Pre-clinical development and clinical validation of a whole blood pharmacodynamic marker assay for the BET bromodomain inhibitor ZEN-3694 in metastatic castration-resistant prostate cancer (mCRPC) patients

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Abstract

Detection of drug activity in patients is essential to confirm its mechanism of action, as well as to ensure proper target engagement at the selected dose to elicit optimal clinical activity. Pharmacodynamic (PD) markers are often developed to detect pharmacological responses and optimize drug dosing. Whole blood is an easily attainable and minimally invasive source of biological material to measure clinical activity of drugs. We designed, developed, and validated a whole blood PD marker assay to detect the activity of ZEN-3694, an orally available inhibitor of the bromodomain and extra-terminal (BET) domain family of proteins currently in phase I clinical trials in mCRPC (NCT02705469 and NCT02711956). Potential BET-specific PD markers were first identified via comparative microarray analysis using a PI3K inhibitor, a BET inhibitor, and a dual PI3K/BET inhibitor in an MV4-11 acute myeloid leukemia (AML) cell line. Further microarray analysis of subsequent in house data and published data of BET inhibitors from different chemical scaffolds in hematologic cell lines allowed us to develop a short list of ~20 candidate genes. Further testing was done by measuring the modulation of these PD markers by various Zenith BET inhibitors from different chemical scaffolds in a number of human cell lines derived from hematological cancers and solid tumors, as well as cryopreserved human peripheral blood mononuclear cells (PBMCs). *In vivo* validation was also done in whole blood obtained from xenograft mice, and cynomolgus monkeys that were dosed orally with ZEN-3694, as well as *ex-vivo* treated human blood derived from normal donors or patients diagnosed with either AML or diffuse large B cell lymphoma. There was also robust target engagement in tumors of mouse AML xenografts, making them suitable tumor PD markers. A quantitative real-time PCR assay was developed for human whole blood matrix with parameters defined based on the multiplex efficiency (85-115%), coefficient of correlation of the standard curve (R^2>0.98), and dynamic copy number range (10-10^6). Assay validation testing demonstrated an inter-assay variability (operator/day/machine) of < 5% and strong dilution parallelism. A final list of 6 genes (MYC, BCL-2, CCR1, IL1RN, GPR183, and HIST2H2BE) met the requirements. Clinical validation of the PD marker assay was done by analyzing whole blood from 11 patients enrolled in the dose-escalation arm of the Zenith mCRPC phase I clinical trial. CCR1 and IL1RN showed robust, exposure-dependent, target modulation at all ZEN-3694 exposures tested, whereas target modulation of MYC, BCL-2, GPR183, and HISTH2BE was only detected at higher ZEN-3694 exposures. The CCR1 and IL1RN data was also confirmed independently of the qPCR assay by testing the patients' samples using Nanostring technology. Another candidate PD marker, HEXIM1, was evaluated in some of the clinical samples, and showed a modest modulation only at higher doses of ZEN-3694, similar to MYC, GPR183, and HISTH2BE. These results demonstrate that the activity of ZEN-3694 is consistent with a BET bromodomain inhibitor in mCRPC patient's whole blood, and that whole blood can be used as a surrogate tissue for measuring the target modulation of ZEN-3694 in the clinic, and guide dose optimization for further development.

Overview

- A whole blood assay was developed to measure target engagement of ZEN-3694 in patients
- 6 PD markers were selected that show modulation in multiple biological systems
- Detected robust dose-dependent target modulation for CCR1 and IL1RN in all patients tested
- CCR1 and IL1RN results were validated independently by Nanostring analysis
- ZEN-3694 shows activity that is consistent with a BET bromodomain inhibitor
- This assay could be used to measure activity of other BET inhibitors and may be a useful tool for guiding dose optimization in clinical studies

Background

Overall objective:
- Develop a qPCR assay to measure target modulation of ZEN-3694 in whole blood

Method:
- Identify genes that are modulated by ZEN-3694 in various cell lines and animal models

Requirements:
- Genes need to be robustly modulated over a large concentration range of ZEN-3694
- Genes need to be reproducibly modulated in different model systems
- Results have to be easily reproduced by different operators and detection systems

Goals:
- Confirm that whole blood can be used to easily measure target modulation by ZEN-3694
- Determine if target modulation correlates with the pharmacokinetics of ZEN-3694 in mCRPC patients
- Verify that the activity of ZEN-3694 in patients' blood is consistent with a BET bromodomain inhibitor

Development Scheme

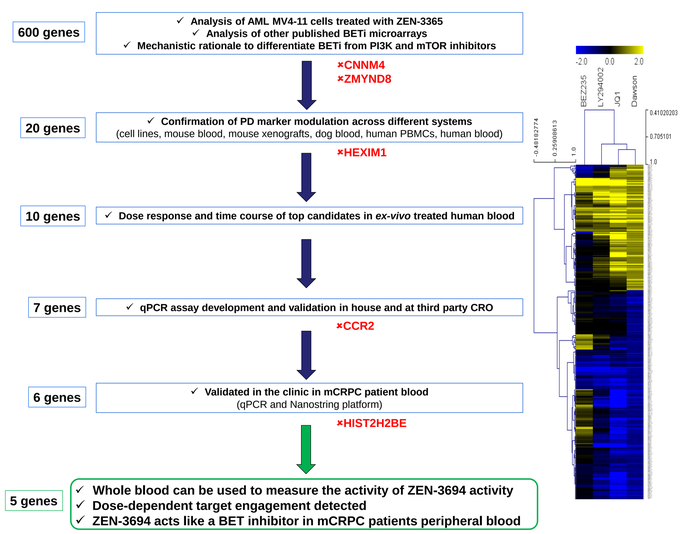


Figure 1.Development of whole blood PD marker assay for ZEN-3694. **LEFT:** Overview of development scheme, including the number of genes that were kept at each selection step. ZEN-3365 was initially used to develop the assay, and was later replaced by the investigational drug ZEN-3694. Specific candidates eliminated during the selection process are indicated in red. **RIGHT:** Two dimensional clustering analysis of BETi (JQ1), dual PI3K/mTORi (BEZ235), dual BETi/PI3K (LY294002), and BETi (IBET-151, Dawson et al, 2011 [1]), to differentiate the BETi signature from PI3K and mTOR inhibitors. Other published microarrays in multiple myeloma and Burkitt's lymphoma were used as comparators as well [2-3].

PD marker assay development and validation

PD marker modulation in different human cell types

Matrix/Cell line (Disease)	BET inhibitor (Concentration)	MYC ⇩	BCL2 ⇩	CCR1 ⇩	IL1RN ⇩	GPR183 ⇩	H2H2BE ⇧
		Mean Fold Change Compared to DMSO Control at 2-4hrs					
Whole blood	ZEN-3365(0.6uM)	**0.51**	**0.47**	**0.43**	**0.34**	**0.46**	**1.85**
	ZEN-3365 (1.7uM)	**0.36**	**0.33**	**0.18**	**0.15**	**0.37**	**2.73**
PBMC	ZEN-3365(0.75uM)	**0.44**	**0.38**	**0.05**	**0.06**	**0.19**	**2.87**
AML PBMC	ZEN-3365(0.33uM)	**0.33**	**0.47**	**0.07**	**0.06**	**0.12**	**6.14**
	ZEN-3365(1uM)	**0.26**	**0.38**	**0.10**	**0.04**	**0.04**	**8.46**
MV4-11 (AML)	ZEN-3365 (5uM)	**0.16**	**0.31**	**0.07**	**0.20**	**0.01**	**3.5**
	JQ1 (1uM)	**0.29**	**0.37**	**0.11**	**0.41**	**0.01**	**5.54**
Kasumi-1 (AML)	ZEN-3118 (5uM)	**0.06**	**0.54**	**NT**	**NT**	**0.06**	**NT**
	JQ1 (0.5uM)	**0.10**	**0.47**	**NT**	**NT**	**0.06**	**NT**
MM.1S (MM)	ZEN-3118 (5uM)	**0.03**	**0.73**	**NT**	**NT**	-	**NT**
	JQ1 (0.5uM)	**0.04**	**0.66**	**NT**	**NT**	-	**NT**
HT29 (CRC)	ZEN-3365 (3uM)	**0.46**	-	-	**0.74**	-	**0.63**
HCT116 (CRC)	ZEN-3365 (3uM)	**0.50**	**0.76**	-	**1.88**	**0.34**	**1.83**
SW480 (CRC)	ZEN-3365 (3uM)	**0.51**	**0.77**	**0.21**	-	**0.22**	**2.21**
RKO (CRC)	ZEN-3365 (3uM)	**0.20**	**0.84**	-	-	-	**2.52**
A375 (MEL)	ZEN-3365 (3uM)	**1.00**	**0.54**	-	-	-	**1.68**
A549 (NSCLC)	ZEN-3365 (3uM)	**2.07**	**0.38**	-	-	-	**1.00**
SK-N-AS (NB)	ZEN-3365 (3uM)	**0.53**	**0.38**	-	**0.55**	**0.06**	**2.38**
ZR75-1 (BC)	ZEN-3365 (3uM)	**0.63**	**0.31**	-	-	-	**1.99**
FaDu (H&N)	ZEN-3365 (3uM)	**0.57**	-	-	**0.62**	-	**3.29**

Table legends: TOP LEFT: PD markers were tested in several human cell lines and primary cells to validate the set of six PD markers. Values highlighted in blue represent PD markers that were modulated as in PBMCs with different BET bromodomain inhibitors. Values not highlighted in blue represent PD markers that were modulated differently than in PBMCs. **TOP RIGHT:** Analysis of whole blood from various species for PD marker modulation. In some cases, whole blood analysis was done in mouse during a xenograft study. In all cases, the PD markers tested were modulated as expected. **RIGHT:** PD markers were also evaluated as markers of target engagement in xenograft tumors.

NT= Not tested; (-)= PD marker not expressed
AML= acute myeloid leukemia; **BC**= breast cancer; **CRC**= coloractal cancer; **H&N**= head & neck cancer; **MM**= multiple myeloma; **MEL**= melanoma; **NB**= neuroblastoma; **NSCLC**= non-small cell lung cancer; **PBMC**= peripheral blood mononuclear cells

PD marker modulation in whole blood from different species

Species (Study)	BET inhibitor	Dose	Day of Analysis (2-4hrs post-dose)	MYC ⇩	BCL2 ⇩	CCR1 ⇩	IL1RN ⇩	GPR183 ⇩	H2H2BE ⇧
				Mean Fold Change Compared to Vehicle Control					
Monkey	ZEN-3694	4 mg/kg	Day 14	**0.70**	**NT**	**0.31**	**NT**	**0.77**	**NT**
		8 mg/kg		**0.63**	**NT**	**0.27**	**NT**	**0.60**	**NT**
		15 mg/kg		**0.39**	**NT**	**0.14**	**NT**	**0.34**	**NT**
Dog	ZEN-3365	3 mg/kg	Day 1	**0.70**	**0.60**	**0.50**	**0.20**	**NT**	**NT**
Female Athymic mice	ZEN-3365	30 mg/kg	Day 1	**0.47**	**0.61**	**0.05**	**0.1**	**0.43**	**1.85**
Mouse (AML Xenograft Efficacy Study)	ZEN-3365	45 mg/kg	Day 19	**0.27**	**0.24**	**0.01**	**0.17**	**0.24**	**6.50**
		65 mg/kg		**0.22**	**0.18**	**0.01**	**0.06**	**0.05**	**11.76**
	OTX-015	65 mg/kg		**0.12**	**0.24**	**0.03**	**0.11**	**0.10**	**1.66**
		50 mg/kg		**0.12**	**0.15**	**0.01**	**0.04**	**0.09**	**1.60**
	ZEN-3365	50 mg/kg	Day 21	**0.46**	**0.43**	**0.18**	**0.35**	**0.40**	**1.67**
	ZEN-3694	50 mg/kg		**0.36**	**0.31**	**0.22**	**0.45**	**0.50**	**1.08**
Mouse (CRC Xenograft Efficacy Study)	ZEN-3365	30 mg/kg	Day 19	**0.21**	**0.36**	**0.05**	**0.25**	**0.40**	**2.15**
		65 mg/kg		**0.31**	**0.16**	**0.02**	**0.16**	**0.23**	**12.82**
Mouse (NSCLC Xenograft Efficacy study)	ZEN-3365	30 mg/kg	Day 19	**0.18**	**0.38**	**0.02**	**0.08**	**0.17**	**4.38**
		65 mg/kg		**0.46**	**0.37**	**0.02**	**0.11**	**0.19**	**16.20**

PD marker modulation in tumor xenografts

Samples (Study)	BET inhibitor	Dose	Day of Analysis (2-4hrs post-dose)	MYC ⇩	BCL2 ⇩	CCR1 ⇩	IL1RN ⇩	GPR183 ⇩	H2H2BE ⇧
				Mean Fold Change as Compared to Vehicle Group					
MV4-11 AML Xenograft	ZEN-3365	30 mg/kg	Day 1	**0.07**	**0.48**	**0.19**	**0.14**	**0.22**	**5.99**
	ZEN-3694	50 mg/kg		**0.24**	**NT**	**0.07**	**0.29**	**0.06**	**2.78**
MV4-11 AML Xenograft	ZEN-3365	65 mg/kg	Day 19	**0.15**	**0.71**	**0.08**	**0.37**	**0.01**	**7.45**
	OTX-015	65 mg/kg		**0.32**	**0.94**	**0.13**	**0.37**	**0.04**	**5.07**
	ZEN-3365	50 mg/kg	Day 21	**0.13**	**0.25**	**0.09**	**0.11**	**0.01**	**5.72**
	ZEN-3694	50 mg/kg		**0.23**	**0.36**	**0.17**	**0.23**	**0.05**	**5.48**

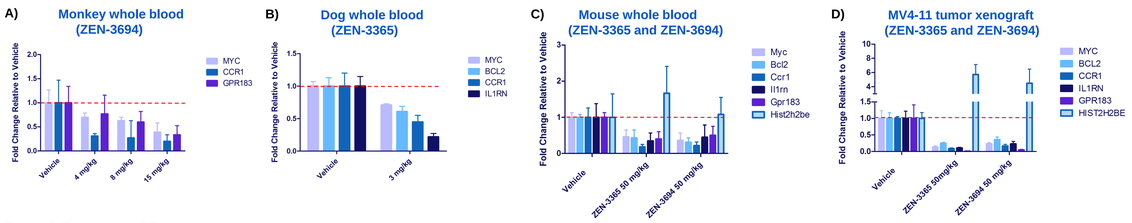


Figure 2. Examples of PD marker modulation detected in whole blood and tumor xenografts. **A)** Whole blood from monkeys treated with a single dose of ZEN-3694 (4, 8, and 15 mg/kg). **B)** Whole blood from dogs treated with a single dose of ZEN-3365 (3 mg/kg). **C)** Whole blood from mice treated with either ZEN-3365 or ZEN-3694 (50 mg/kg) at Day 21 of the study. **D)** In-tumor analysis of PD marker modulation in MV4-11 xenografts in mice treated with either ZEN-3365 or ZEN-3694 at 50 mg/kg. Blood and tumors were harvested 2-4 hours post-dosing.

PD marker PCR assay validation: reproducibility and repeatability parameters, intra- and inter-assay variability

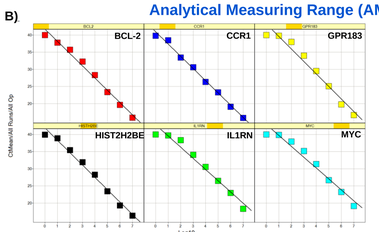
A)

qPCR assay parameters for PD markers
- **Multiplex qPCR Efficiency (with endogenous control):** 85-110%
- **R^2 value:** >0.98
- **Linearity, Analytical Measuring Range (AMR):** 10-1x10^7 copies
- **Inter assay variability (3 runs between 2 users and 3 instruments):** CV< 15% except LOQ, CV<20%
- **Intra assay variability (quadruplicates):** CV <5% based on Ct values
- **Accuracy:** direct comparison of results by conducting a T-test across 3 independent runs p-value ≤ 0.05
- **Lower Limit of Quantitation (LLOQ):** 10 copies (CCR1) to 1000 copies (MYC, IL1RN, GPR183)

Controls for whole blood assay for clinical trial
- **Standard curve:** plasmid with cDNA for each marker, multiplex PCR (copy #)
- **Reproducibility control:** assay done in quadruplicate (CV<5%)
- **Positive control #1:** whole blood treated ex-vivo with ZEN-3694
- **Positive control #2:** universal human reference RNA (UHR), high and low concentrations (CV<5%)
- **Negative control #1:** whole blood treated ex-vivo with vehicle (DMSO)
- **Negative control #2:** no template reaction (Ct ≥40)
- **Negative control #3:** no reverse transcription reaction (Ct ≥40)

B)

Analytical Measuring Range (AMR)



AMR results by Gene	
Gene	AMR (copies)
BCL2	1.00E+00-1.00E+07
CCR1	1.00E+00-1.00E+07
GPR183	1.00E+00-1.00E+07
HISTH2BE	1.00E+00-1.00E+07
IL1RN	1.00E+00-1.00E+07
MYC	1.00E+00-1.00E+07

C)

Dilution Linearity

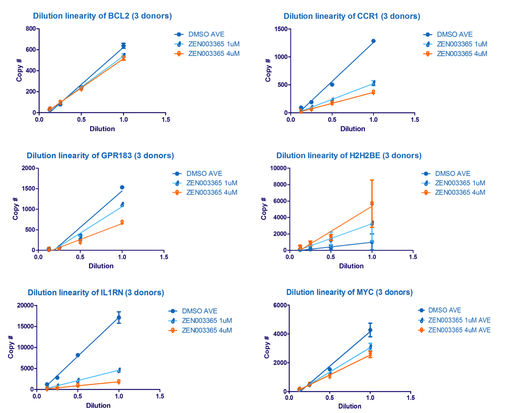


Figure 3 PD marker PCR assay validation. **A)** Summary of the qPCR assay parameters and controls used. **B)** Overall standard curve titration and analytical measuring range (AMR) results for each gene across all operators and all machines. **C)** Dilution linearity for each PD marker done in *ex-vivo* treated blood from 3 healthy donors using two different concentrations of ZEN-3365 (1uM and 4uM).

PD marker modulation by ZEN-3694 in *ex-vivo* treated human whole blood

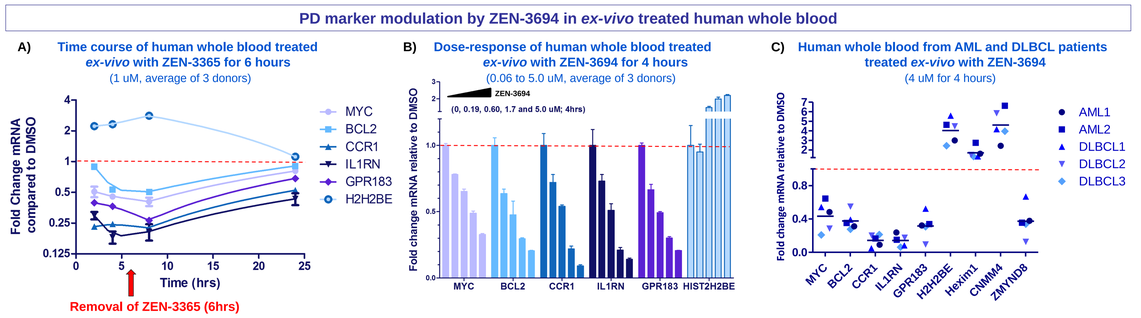


Figure 4. PD marker modulation by ZEN-3694 in *ex-vivo* treated human whole blood. **A)** Incubation and washout time course of whole blood from three healthy volunteers treated *ex-vivo* with 1uM of ZEN-3365 for 6 hours. Fold change in PD marker mRNA was measured at 2, 4, 8, and 24 hrs. The 4 hour time-point was selected as the best readout for target engagement of PD markers. **B)** Human whole blood from three healthy donors was treated *ex-vivo* with four different concentrations of ZEN-3694 for 4 hours. Gene expression changes were quantified by real time PCR, and the average of the three donors is shown with their standard deviation. **C)** PD marker modulation in whole blood from AML and DLBCL patients showing good inter-individual correlation

ZEN-3694 Phase I Trial in mCRPC

Target engagement in mCRPC patients treated with ZEN-3694 at 4hrs post-dose

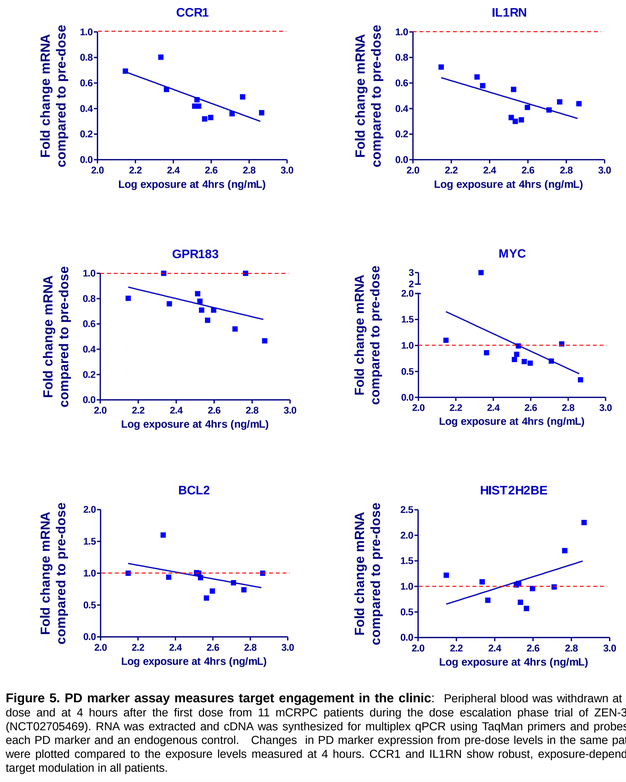


Figure 5. PD marker assay measures target engagement in the clinic: Peripheral blood was withdrawn at pre-dose and at 4 hours after the first dose from 11 mCRPC patients during the dose escalation phase trial of ZEN-3694 (NCT02705469). RNA was extracted and cDNA was synthesized for multiplex qPCR using TaqMan primers and probes for each PD marker and an endogenous control. Changes in PD marker expression from pre-dose levels in the same patient were plotted compared to the exposure levels measured at 4 hours. CCR1 and IL1RN show robust, exposure-dependent, target modulation in all patients.

Nanostring validation of qPCR results

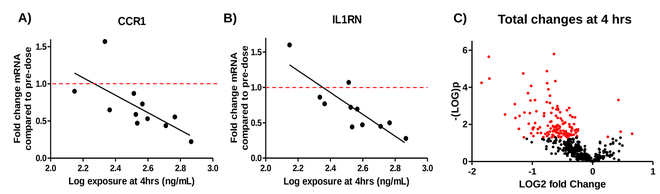


Figure 6. Nanostring digital color-coded barcode dual probe hybridization assay validates qPCR assay: RNA samples isolated from peripheral blood of mCRPC patients treated with ZEN-3694 were assayed via Nanostring and demonstrated good concordance with the qPCR data for both CCR1 **(A),** and IL1RN **(B)** at 4 hours after the first dose. **C)** Volcano plot meta-analysis of mRNA expression from peripheral blood of 8 mCRPC patients treated with ZEN-3694 shows significant modulation of several genes (in red, p<0.05) using the nCounter® PanCancer Immune Profiling Panel.

Summary

- **A qPCR assay was developed to measure the activity and target engagement of ZEN-3694 in patient's whole blood through PD marker modulation**
- **The extent of PD marker modulation correlates with drug exposure**
- **CCR1 and IL1RN are the most robust PD markers showing target engagement at the lowest exposure**
- **qPCR results were independently validated using the Nanostring nCounter® gene expression assay**
- **ZEN-3694 shows activity consistent with a BET bromodomain inhibitor**
- **This assay could be a useful tool for guiding dose optimization in clinical studies, and potentially be used to measure target engagement of different BET inhibitors in the clinic**

References

1. Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature. 2011;478(7370):529-33.
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3. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell. 2011;146(6):904-17.