Validation of the interaction between a candidate compound and the intended drug target by a phenotypic rescue approach

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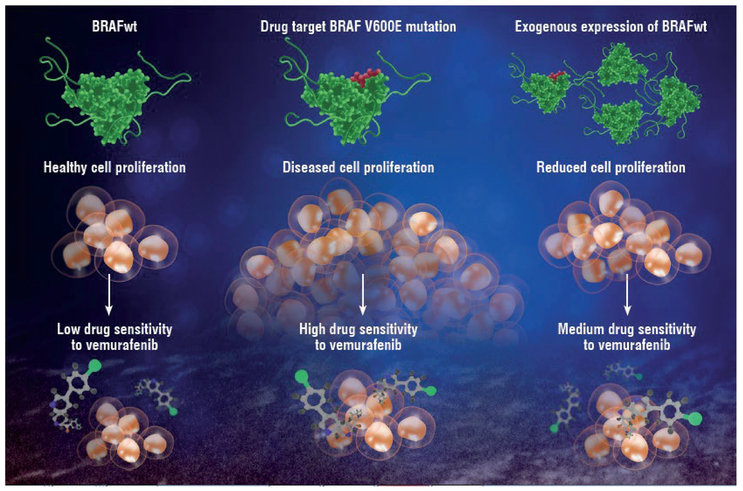
1 INTRODUCTION

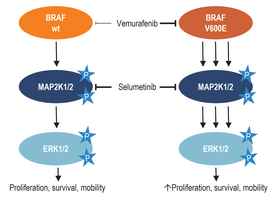
New targets for cancer treatment frequently emerge in literature, but the thorough target validation required to consider these targets for a drug discovery program is often lacking. In pharmacological or genetic perturbation studies using complex biological assays, undesired off-target effects cannot be easily distinguished from the intended mode of action at the desired target. This is especially evident in cancer drug development where it is important to discriminate on-target effects on cell viability from off-target effects resulting in non-specific loss of cellular fitness. Neglecting the possibility of being deceived by off-target effects can have tremendous scientific and financial impact on a drug discovery program. Ideally confidence in a preclinical drug target and a modulating compound is boosted in an early stage by more extensive analysis and validation of the actual drug-target relationship. Rescue of a disease-relevant phenotype by genetic restoration of a target mutation is a gold standard approach in drug discovery by which target validation can be achieved.

We aim to follow this approach targeting the BRAF V600E mutation in A2058 melanoma cell line as well as the MAP2K1 Q56P mutation in non-small cell lung cancer cell line NCI-H1437. Target validation for both BRAF and MAP2K1 will be addressed by assessing viability, phenotypic changes and sensitivity to compound modulation upon doxycycline-inducible overexpression of the wildtype (wt) variant by lentivirus transduction and CRISPR/Cas9 repair of the target mutation.

Distinguishing on-target versus off-target effects with rescue experiments



2 EXOGENOUS EXPRESSION OF WT VARIANT



Schematic overview of the mitogen-activated protein kinase (MAPK) signaling pathway and therapeutic intervention. BRAF is a serine/threonine kinase activating the MAP kinase/ERK-signaling pathway. BRAF V600E is the most common mutation reported in exon15 of BRAF and results in abnormal MAPK signaling. Vemurafenib (PLX4032) acts as a RAF inhibitor with a strong blockade activity against the V600E BRAF mutation. Selumetinib (AZD6244) is a highly selective and reversible inhibitor of MAP2K1/2.

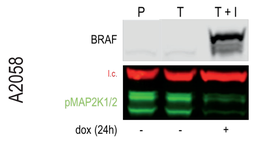


Fig 1. Reduced pMAP2K1/2 levels upon BRAFwt overexpression. High levels of BRAFwt were detected (α-BRAF, ab33899) 24h after dox induction in transduced A2058 cells (upper panel). Overexpression of BRAFwt decreased pMAP2K1/2 signal (α-pMEK1/2, CST#9154; lower panel).

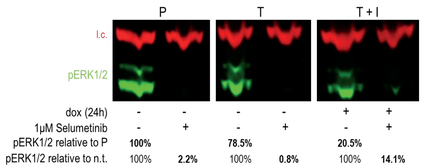


Fig 2. Inhibition of ERK1/2 phosphorylation. Treatment with Selumetinib resulted in a strong reduction of pERK1/2 levels (α-pERK1/2, CST#4370) in parental and transduced A2058. Dox-induced BRAFwt overexpression alone also lowered pERK1/2 levels and resulted in less sensitivity to Selumetinib treatment.

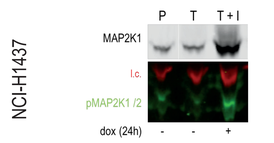


Fig 3. Increased pMAP2K1/2 levels upon MAP2K1wt overexpression. High levels of MAP2K1wt were detected (α-MEK1 ab32091) 24h after dox induction in transduced NCI-H1437 cells (upper panel). Overexpression of MAP2K1wt increased pMAP2K1/2 signal (α-pMEK1/2, CST#9154, lower panel).

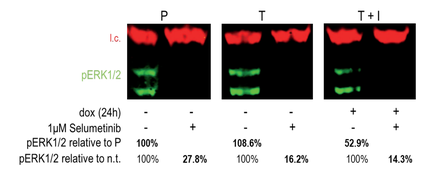
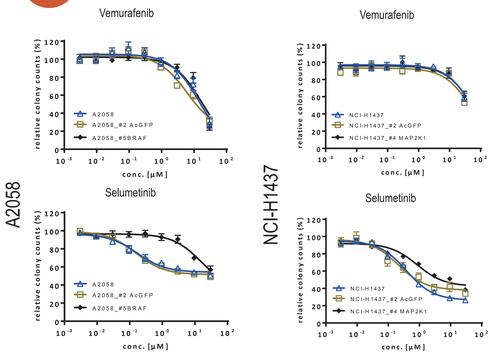


Fig 4. Inhibition of ERK1/2 phosphorylation. Treatment with Selumetinib resulted in a strong reduction of pERK1/2 levels (α-pERK1/2, CST#4370) in parental and transduced NCI_H1437. Dox-induced MAP2K1wt overexpression alone also lowered pERK1/2 levels. Selumetinib sensitivity remained unchanged when MAP2K1wt was overexpressed.

3 COMPOUND SENSITIVITY



IC_{50} (µM)	Vemurafenib	Selumetinib
A2058	14.7	0.21
A2058_#2 AcGFP	9.89	0.16
A2058_#5 BRAF	17.6	8.78

A2058_#2 AcGFP: lentiviral transduction control, A2058_#5 BRAF: BRAFwt overexpressing

IC_{50} (µM)	Vemurafenib	Selumetinib
NCI-H1437	27.9	0.39
NCI-H1437_#2 AcGFP	27.6	0.31
NCI-H1437_#4 MAP2K1	>30	7.09

NCI-H1437_#2 AcGFP: lentiviral transduction control, NCI-H1437_#4MAP2K1: MAP2K1 overexpressing

Fig 5. Reduced Selumetinib sensitivity in BRAFwt and MAP2K1 overexpressing cell lines. Test compounds were added 24h after dox induction. Cell viability was analyzed after 5 days by using the CellTiter-Blue® Cell Viability Assay (Promega). Vemurafenib sensitivity of A2058 remained unchanged after BRAFwt overexpression, however, Selumetinib sensitivity was reduced (left panel). A2058 cell viability was reduced by 25% upon BRAFwt overexpression (data not shown). Selumetinib sensitivity was lowered by MAP2K1wt overexpression in NCI-H1437. Vemurafenib sensitivity was not changed (right panel). NCI-H1437 cell viability was reduced by 34% upon MAP2K1 overexpression (data not shown).

4 REVERTING ENDOGENOUS BRAF V600E TO WT

Fig 6. BRAF c.1799T>A mutation gene editing strategy by CRISPR/Cas9. A2058 is heterozygous for the c.1799T>A mutation in BRAF. CRISPR/Cas9 technology was used to induce a DBS at BRAF exon 15 to trigger HDR to revert SNP to wt (T>A). Different guide RNAs (recruiting Cas9 endonuclease to the target) and HDR templates were designed.

gRNA	I	II	III
% NHEJ	61%	21 %	98.5%
% HDR	5%	10%	7%

Table 1. Comparison of the efficiency of DBS repair through the NHEJ and HDR pathways. Alt-R® CRISPR-Cas9/gRNA RNP complexes (IDT) were delivered in A2058 by electroporation. The efficiency (%) of HDR and NHEJ events was determined through the decomposition of the sequencing trace data using ICE-Knock In (KI) online software (Synthego). ICE-KI analysis indicated 5-10% HDR (restoring the wt variant of BRAF) in the heterogeneous population after gene editing.

P: parental, T: transduced; T+I: transduced and induced, l.c.: loading control, n.t.: non-treated, dox: doxycycline

HDR: homology-driven repair, NHEJ: non-homologous end joining; DBS: double stranded break; RNP: ribonucleoprotein

5 CONCLUSION & PERSPECTIVES

The activation status of the MAPK/ERK signaling cascade in tumor cell lines harboring mutations in either BRAF or MAP2K1 can be reduced by overexpressing the wild-type variants of these kinases, indicating that these mutations indeed play a role in tumor pathogenesis. The change in drug sensitivity to Selumetinib in MAP2K1wt overexpressing NCI-H1437 demonstrates the on-target effect of the drug. The lack of effect of BRAFwt overexpression on Vemurafenib sensitivity in A2058 might be due to the more resistant phenotype of A2058 compared to other V600E carrying tumor cell lines or due to the presence of confounder mutations in this cell line. For future phenotype rescue studies CRISPR/Cas9 gene editing is preferred over exogenous expression, because the effect of reversion to wild-type is studied in the context of physiological levels of the target. This study demonstrates the added value of a phenotype rescue study for newly identified drug targets and their alleged interaction with a drug. Such early in-depth validation of the relationship between a compound and the drug target is vital to mitigate the risk of failure at later steps of drug development.