Utilisation of the InCELL Hunter™ Target Engagement Cell-Based Assay for Profiling of ULK1 Inhibitors

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Introduction

Drug efficacy and cellular target engagement studies have traditionally been monitored by evaluation of downstream cellular responses, such as detection of substrate phosphorylation by upstream kinases for example. Alternative biochemical techniques, based on measuring the thermal stability of a recombinant target protein in response to binding of accessory proteins or small molecules by assessing changes in the thermal shift (T_m) have been widely used in a variety of formats. More recently, this approach has been applied to the study of molecular stabilisation of target proteins expressed endogenously or exogenously in cells, animals or human samples using the Cellular Thermal Shift Assay (CETSA). These approaches rely on melt curve analysis following interaction with drug or compound and subsequent detection of the target protein by immunoblotting. The levels of target protein detected at different temperatures can be compared between control and treated samples as a measure of target engagement.

Commercially-available assays such as the InCELL Hunter™ Target Engagement Assay (TEA; DiscoveRx® Corporation), based on Enzyme Fragment Complementation (EFC) between two inactive β-galactosidase fragments, an enhanced ProLabel® (ePL) and an Enzyme Acceptor (EA) allow the study of protein stability in live intact cells without the need to perform thermal shift studies. Here, we describe the application of this assay for the profiling of a panel of small molecule inhibitors to a key kinase in the autophagy pathway ULK1 (Unc-51 like autophagy activating kinase 1). Data output were compared to other assays measuring indirect target engagement through assessment of a downstream ULK1 substrate, Atg13 using a phospho-specific antibody and a phenotypic autophagy LC3 tandem reporter assay.

Methods

Target Engagement Assay. Human ULK1 kinase domain (16-278; O75385) fused at the C-terminal to the enhanced ProLabel® (ePL) β-galactosidase fragment (pTEA-ePL-C vector, DiscoveRx® Corporation) was transiently expressed for 24 hours in HEK293 cells (4μg/1x10^6 cells using FuGene HD™, Promega). Transfected cells were dissociated with Accutase™ and dispensed in 384-well white-walled plates (2x10^5 cells/well) and incubated with compounds for 3 hours prior to enzyme fragment complementation with the β-galactosidase Enzyme Acceptor (EA) luminescent detection reagent.

LC3 Tandem Reporter Assay. Stably transfected mCherry-GFP-LC3 U2OS cells were treated with an mTOR inhibitor (1μM AZD8055) for 2 hours to induce autophagy. Fixed cells were imaged on an InCell Analyser 2000 and GFP-positive (autophagosome) and mCherry-positive (autolysosome) LC3 puncta were quantified. The ability of compounds to modulate mTOR-dependent autophagy was assessed following 2 hours of treatment in the presence of AZD8055.

Immunoblotting. Compounds were profiled for inhibition of Atg13 phosphorylation, a well described ULK1 substrate, induced by mTOR inhibition (1μM AZD8055, 90 minutes) in HEK293 cells using phospho-specific antibodies to ULK1 (S757, Cell Signalling Technology), Atg13 (S318, Abnova) and LC3 (LC3 A/B, Cell Signalling Technology).

Figure 1. Overview of the DiscoveRx Target Engagement Assay (TEA)

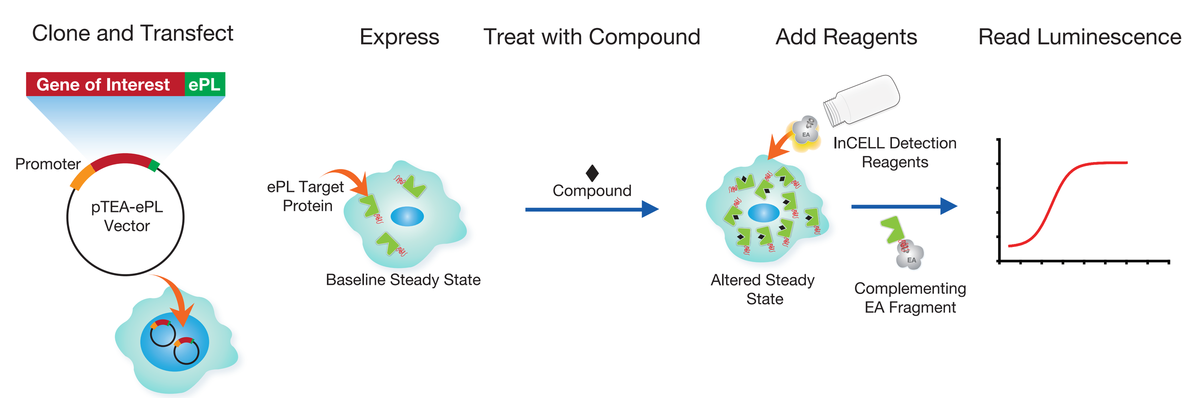


Figure 1. Schematic workflow for the TEA assay. The target gene is cloned in to the pTEA-ePL vector and transiently expressed in cells; following compound treatment, enzyme fragment complementation with the EA enzyme acceptor produces a luminescent signal which is directly proportional to the amount of transfected ePL-tagged protein remaining in the cell.

Figure 2. Cloning and Expression of ePL-Tagged ULK1 Kinase Domain

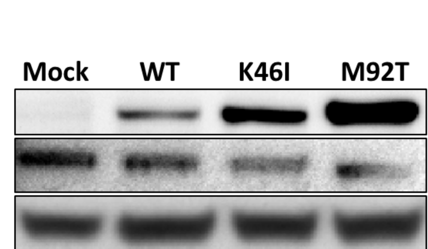
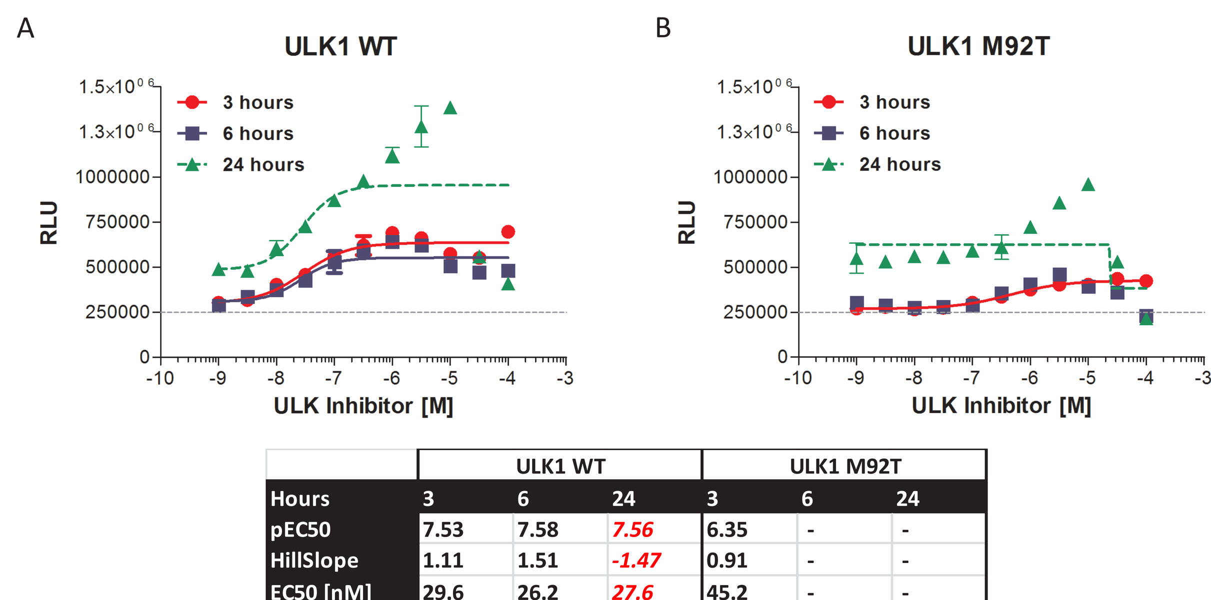


Figure 2. Expression of transiently expressed ePL-tagged ULK1 kinase domain in HEK293 cells by immunoblotting. The wild type (WT) kinase domain (16-278), as well as a kinase-inactive (K46I) and a gatekeeper mutant version (M92T) were transiently transfected in to HEK293 cells and levels of protein expression detected by immunoblotting with an anti-ePL antibody (3F07, DiscoveRx® Corporation) showing that all recombinant versions of ULK1 where expressed. Subsequent studies focussed on comparing the WT ULK1 with the gatekeeper M92T mutant. The levels of endogenous ULK1 are shown for comparison using an antibody that does not recognise the ULK1 kinase domain (D8H5, Cell Signalling Technology).

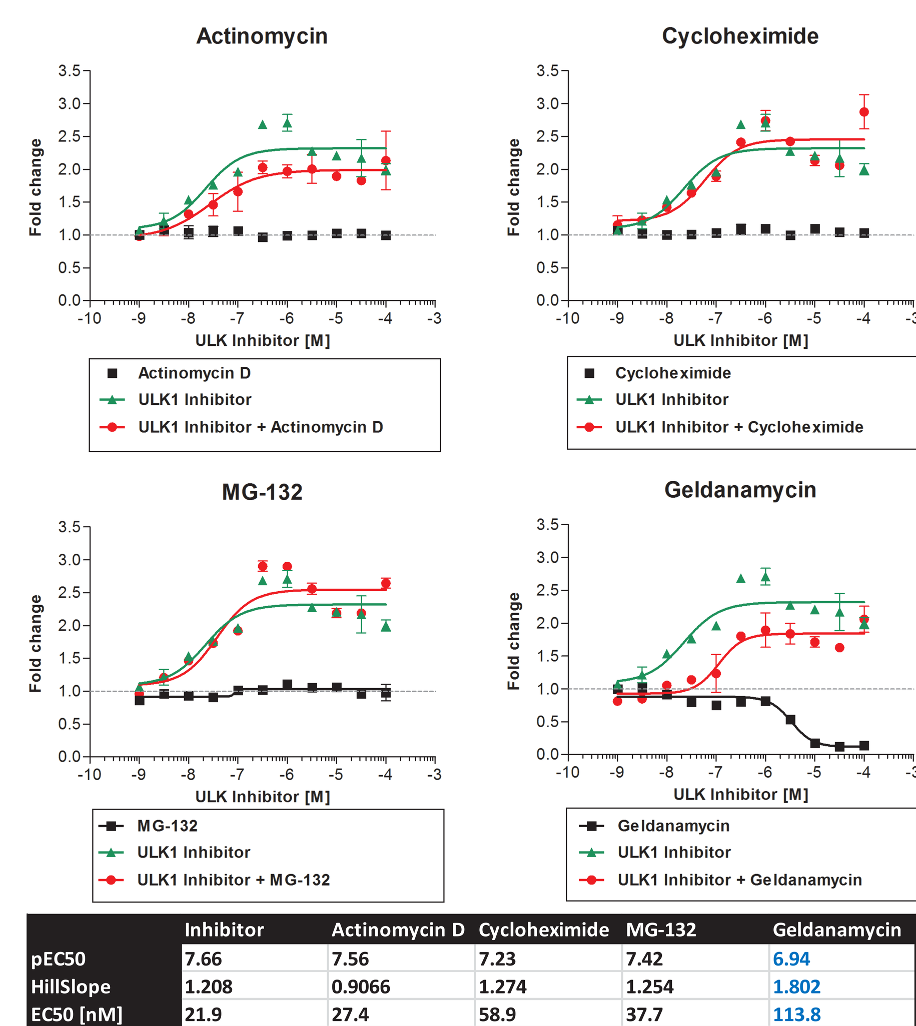
Figure 3. Time Course Analysis of TEA Signal



	ULK1 WT			ULK1 M92T		
Hours	3	6	24	3	6	24
pEC50	7.53	7.58	*7.56*	6.35	-	-
HillSlope	1.11	1.51	*-1.47*	0.91	-	-
EC50 [nM]	29.6	26.2	*27.6*	45.2	-	-

Figure 3. Time-dependent changes in the stability of wild type (WT) ULK1 (panel A) and the M92T mutant (panel B) using a tool ULK1 inhibitor. Data presented as relative luminescent units (RLU) for cells treated for 3, 6 and 24 hours with an 11 point half-log concentration response curve of the tool ULK1 inhibitor. Stabilisation of WT ULK1 was optimal at 3-6 hours and this effect was attenuated in the M92T mutant, as summarised in the accompanying table. The 3 hour time point was used for all subsequent studies and compound profiling. Accurate curve fitting and derivation of EC_{50} values was not possible for the 24 hour time point in both ULK1 WT and M92T. Data presented as mean ± SD.

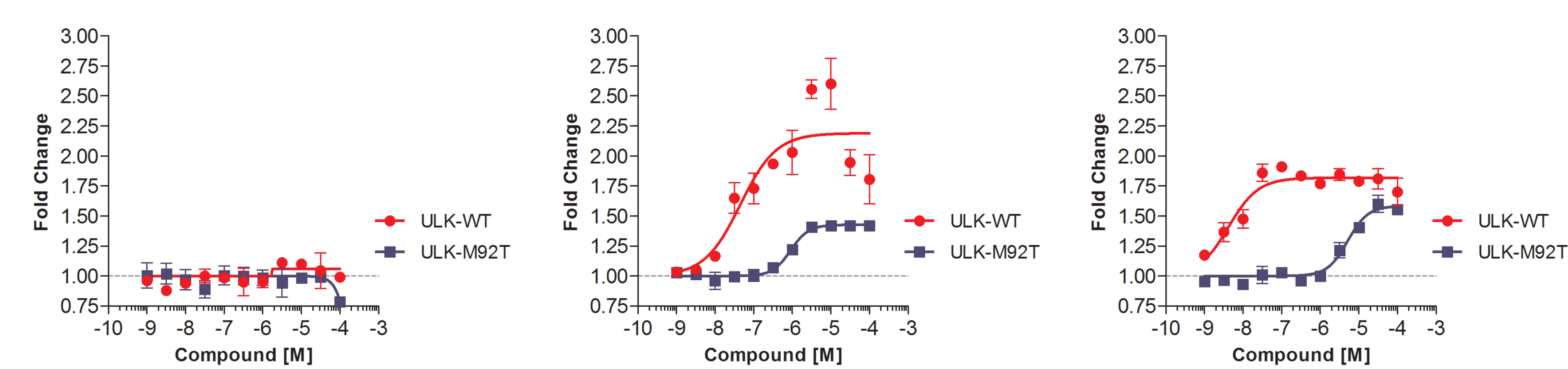
Figure 4. Specificity and Stability of TEA Signal



	Inhibitor	Actinomycin D	Cycloheximide	MG-132	Geldanamycin
pEC50	7.66	7.56	7.23	7.42	6.94
HillSlope	1.208	0.9066	1.274	1.254	1.802
EC50 [nM]	21.9	27.4	58.9	37.7	113.8

Figure 4. Summary of the effects of transcriptional (actinomycin D, 50μM), translational (cycloheximide, 100μM), proteosomal (MG-132, 30μM) and HSP90 (geldanamycin, 1μM) inhibition on stabilisation of WT ULK1 by a tool ULK1 inhibitor (assessed at 3 hours). Actinomycin D, cycloheximide and MG-132 alone had no effect on the basal steady state of ULK1 (black lines) and did not show any significant modulatory effects on the increased stabilisation by the ULK1 inhibitor when tested (red lines). In contrast, geldanamycin caused a concentration-dependent inhibition of basal ULK1 steady state and additionally produced a rightward shift (approximately 0.7 log) in the concentration response curve of the tool ULK1 inhibitor (pEC_{50} ULK1 inhibitor 7.66 vs pEC_{50} ULK1 inhibitor plus 1μM geldanamycin 6.94). These data suggest that the increased stabilisation of ULK1 observed is specific and not due to other cellular effects, but that ULK1, at least under these conditions may act as a client protein for HSP90. Data presented as mean ± SD and expressed as fold change in ULK1 stability.

Figure 5. Profiling of ULK1 Inhibitors



No Engagement	ULK1 WT	ULK1 M92T
pEC50	-	-
HillSlope	-	-
EC50 [nM]	-	-

Engagement	ULK1 WT	ULK1 M92T
pEC50	7.35	6.05
HillSlope	0.934	1.854
EC50 [nM]	44.9	900

Potent Engagement	ULK1 WT	ULK1 M92T
pEC50	8.36	5.41
HillSlope	1.101	2.145
EC50 [nM]	0.4	3850

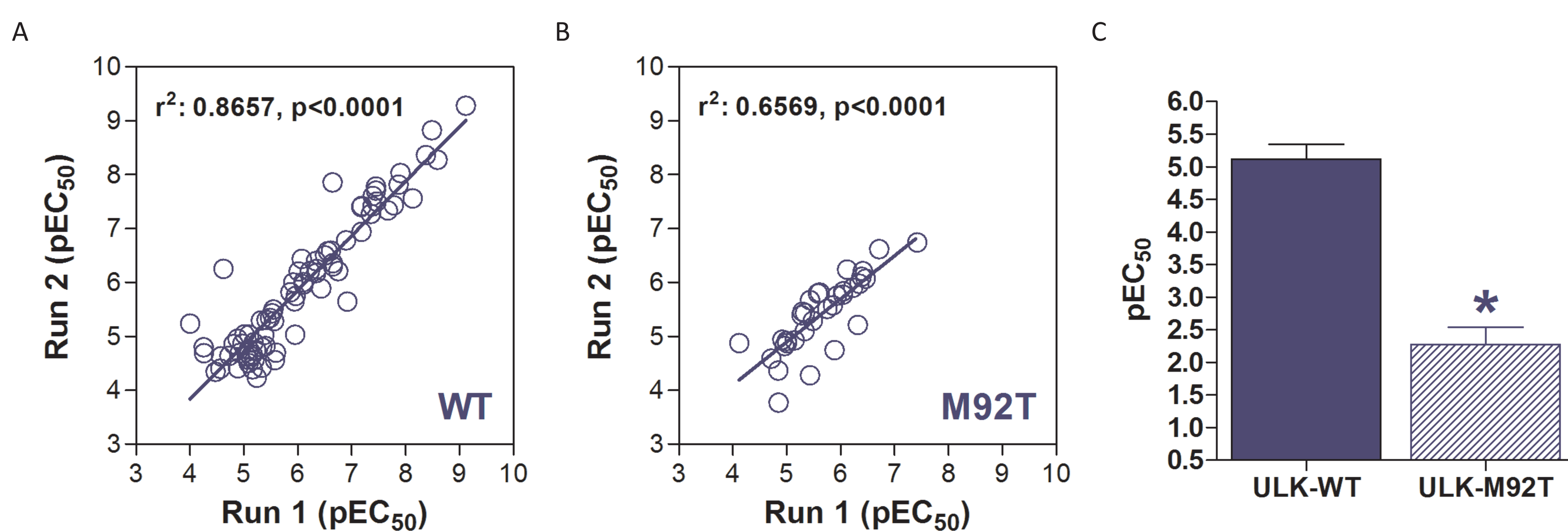


Figure 5. Summary of ULK1 compound profiling in the TEA assay from the hit-to-lead medicinal chemistry programme. The upper panels show 3 examples of the types of profiles that can be obtained with ULK1 inhibitors in the TEA assay, ranging from no engagement (left panel), engagement (centre panel) and potent engagement (right panel); where increased stabilisation by compounds is observed, these effects are significantly attenuated in the M92T gatekeeper mutant compared to the WT ULK1. A total of 101 compounds were profiled for their ability to stabilise both WT ULK1 and the M92T mutant in 11 point half-log concentration response curves. Linear regression analysis where accurate EC_{50} values could be obtained, showed statistically significant inter-assay correlations against both WT (panel A, n=79) and the M92T mutant (panel B, n=33). Stabilisation of the M92T mutant was in all cases significantly lower than stabilisation of the WT ULK1 with respect to pEC_{50} (panel C, paired T-test, p<0.0001), suggesting that the scaffolds used for design of these kinase inhibitors could be ATP-competitive in nature.

Figure 6. TEA Assay, ULK1 Target Engagement and Autophagy

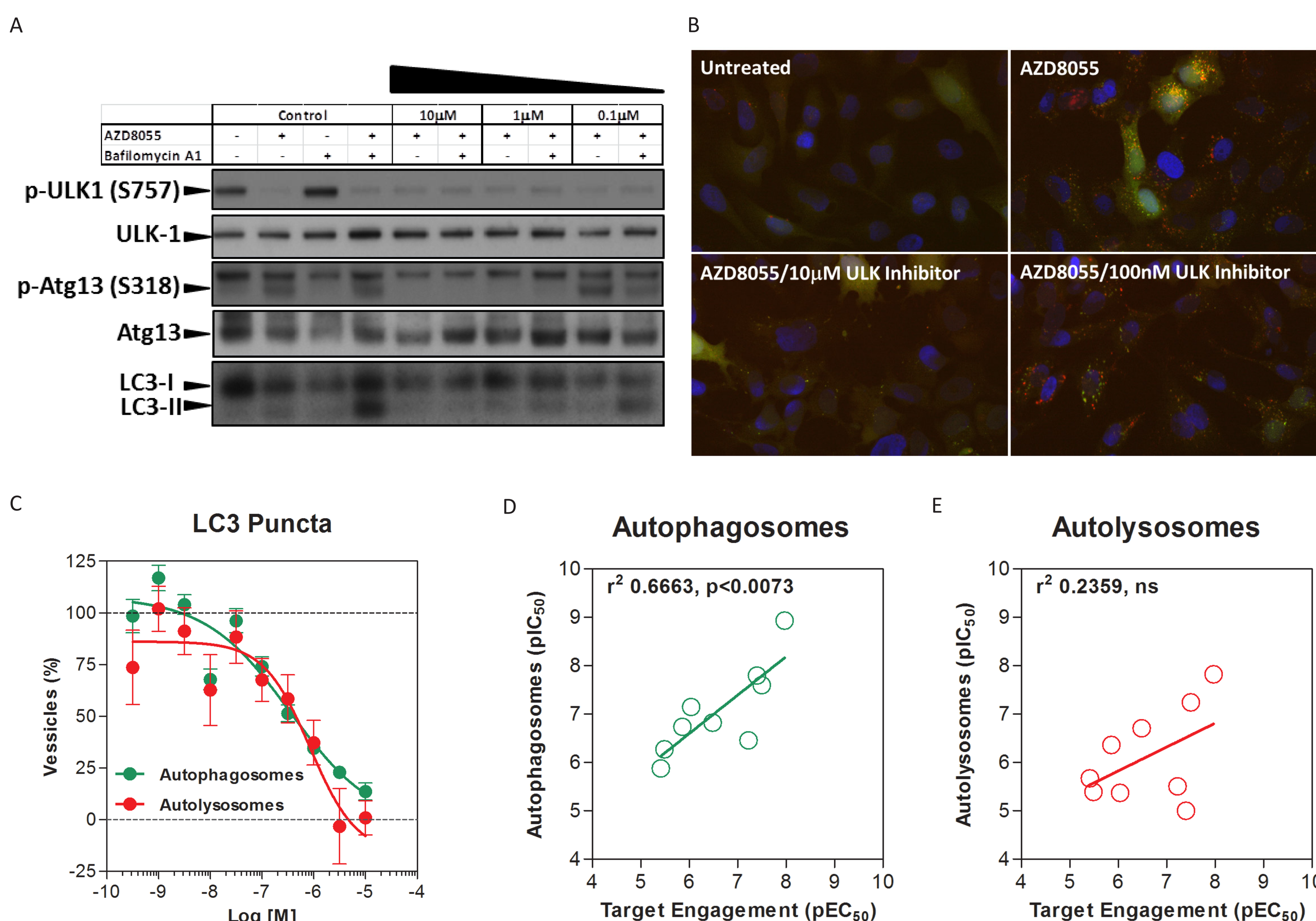


Figure 6. Relationship between ULK1 target engagement, by analysis of Atg13 phosphorylation and a phenotypic autophagy assay. Panel A shows mTOR-dependent autophagy induced by the mTOR inhibitor AZD8055 (1μM, 90 minutes), resulting in loss of ULK1 phosphorylation (S757) and increased phosphorylation of the ULK1 substrate Atg13 (S318); treatment with the tool ULK1 inhibitor caused a concentration-dependent decrease in the phosphorylation of Atg13 and reduced LC3 flux as assessed by lipidated LC3-II (LC3A/B antibody, Cell Signalling Technology). Using an LC3 tandem reporter assay, mTOR-dependent autophagy based on the formation of LC3 puncta in autophagosomes (GFP, green) and autolysosomes (mCherry, red) also shows inhibition of autophagosome and autolysosome formation by the tool ULK1 inhibitor at 10μM (panel B), as quantified by the number of green and red puncta (panel C, mean ± SEM, n=2 independent repeats). A small panel of ULK1 inhibitors were profiled in the tandem reporter assay and inhibition of both autophagosome (panel D) and autolysosome (panel E) formation, correlated significantly with pEC_{50} data generated in the TEA assay (9 compounds in total, n=2 independent repeats for both tandem reporter and TEA assays). Interestingly, this phenomenon was only observed for autophagosome formation and not autolysosomes, however these findings are based on a limited sample set and should be interpreted with caution.

Conclusion

The TEA assay represents a simple, rapid, homogenous and quantitative approach for evaluation of intracellular protein stabilisation. Using ULK1 as an example, we pharmacologically profiled >100 compounds in a medium throughput fashion. Profiling in TEA, allows analysis in live, intact cells providing simultaneous assessment of compound potency, cell-permeability and target engagement.