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Mechanisms of therapeutic synergy between pattern recognition response agonists and CDK4 inhibitors

Victoria Roulstone*, Joan Kyula*, Richard Elliott, Christopher J. Lord, Nik Matthews, Vicki Jennings, Harriet Whittock, David Mansfield, Jyoti Choudhary, James Wright, Lu Yu, Alan Melcher, Richard Vile, Hardev Pandha, Grey Wilkinson, Matt Coffey, Martin McLaughlin*, Kevin Harrington*. Targeted Therapy Team, The Institute of Cancer Research, London, UK.

Palbociclib enhances the anti-cancer efficacy of the oncolytic virus Rt3D



Reovirus type-3 Dearing (Rt3D) is an oncolytic wild-type, double-stranded RNA virus studied in multiple clinical trials. We performed a highthroughput drug screen to employ a non-biased approach to determine any synergistic drugvirus interactions. A range of different cancer drugs (~80) was screened against Rt3D in the BRAFV600E-mutant A375 cell line (a). Palbociclib enhanced Rt3D cell death and was a top hit in the screen. Palbociclib induces a G1/S cell cycle arrest through inhibition of CDK4/6 upstream of retinoblastoma protein (Rb). These effects are shown in A375 fucci cells (**b**).

Validation of Rt3D in combination with palbociclib



Palbociclib potentiates interferon signalling in A375

In addition to the ER stress and demethylation signatures described, proteomics (a) and RNA sequencing (b) data indicated a pronounced virus response and interferon signature due to combination treatment.



IFNβ was up-regulated with Rt3D-Palbociclib combination therapy (c). RNA sensors (RIG-I/MDA-5/TLR3) were also elevated with palbociclib (not shown). It is possible that enhanced interferon signaling was driven by increased levels of the dsRNA Rt3D genome. However, RT-qPCR showed that palbociclib does not alter viral RNA levels (d), nor does it increase viral replication (e). We investigated other possibilities for the increased IFN response.

Rt3D plus palbociclib therapy enhance ERV expression

Cell kill was enhanced between Rt3D (multiplicity of infection dose, MOI 0.1) plus palbociclib (1 µM) in A375 melanoma cells (a). Rt3D plus palbociclib reduced A375 tumour burden in CD1 nude mice invivo (b). Cell cycle stage revealed a G1 arrest with palbociclib (as expected), and an increase in the subG1 population with combination treatment (c). Western analysis shows the appearance of cleaved caspase 3 and PARP due to Rt3D that was enhanced by palbociclib, indicating that combination therapy increases death through an apoptotic process (d).

Palbociclib aggravates ER stress.

To understand the mechanistic interaction, we analysed the proteome in A375 BRAF-mutant melanoma treated with Rt3D plus palbociclib.



Rt3D has been previously shown to induce ER stress. Proteomic analysis indicated altered expression of proteins with gene ontology (GO) terms relating to ER stress in the context of palbociclib-Rt3D combination (a). siRNA against unfolded protein response (UPR) components revealed PERK, ATF6, XBP1 and CCAAT-enhancer-binding protein homologous protein (CHOP) as important mediators of cell death (b). CHOP has been reported to induce death by ER stress. Upregulation of CHOP was confirmed by qPCR (c).



Palbociclib also sensitised cells to other forms of ER stress. Enhanced cell kill was shown with palbociclib combination with thapsigargin (TG), a UPR gold-standard activator, supporting the role of palbociclib as an ER stress sensitizer (d).

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Loss of DNMTs through CDK4/6 inhibition, can release dsRNA endogenous retroviral elements (ERVs) stimulating interferon responses (Goel et al.). In a panel of 26 ERVs, Rt3D induced expression of 5 STAT-dependent ERVs (STAT dependence was shown by loss of expression upon treatment with the JAK/STAT inhibitor, ruxolitinib). Expression of 3/5 ERVs was boosted with palbociclib, including the ERV MLT1C49 (a). Knockdown of RNA sensors showed that RIG-I was critical to the cytotoxicity of combination treatment (b).



ER stress enhances RNA sensor-driven interferon responses

At this point we suspected a link between the unfolded protein response (UPR) and cytoplasmic dsRNA sensing. Complicating this, each single agent impacted multiple cellular processes.



Using tool compounds specific to ER stress / UPR activation (thapsigargin, TG) and RNA sensor activation (poly I:C), co-treatment enhanced cell kill relative to the single agents (a). In response to escalating doses of poly I:C, the transcriptional expression of IFN α/β plateaued. However, the addition of TG accommodated the increased doses of RNA load (b). TG-induced ER stress releases the brakes on RNA sensor transcription, and, consequently, IFN expression. In line with observations from Rt3D and palbociclib, these data mimicked Rt3D-palbociclib-induced IFN expression, confirmed by RT-qPCR (c). To demonstrate the link between the UPR and IFN response, inhibition of the IRE1 α branch of the UPR reduced Rt3D-palbociclib-induced IFN β release (d).

Combination therapy enhances innate immune activity

We hypothesized that enhanced dsRNA sensing and ER stress may also enhance the immunogenicity of therapy.



Rt3D-palbociclib increases HLA-derived proteins (a), and surface exposure of calreticulin (CRT, green) (b). CRT is pro-phagocytic and is associated with immunogenic cell death. Labelled PBMC-derived human macrophages were co-cultured with tumour cells stained with pHrodo, a dye that fluoresces red in acidic conditions such as engulfment. Microscope pictures show macrophages (CD11b FITC+, white arrows), and engulfed tumour cells within macrophages (blue arrows) (c). Phagocytosis was significantly enhanced by combination treatment, measured by dual-positive staining (d).

Palbociclib causes extensive DNA modification



Proteomic analysis revealed that palbociclib markedly altered proteins under the GO term DNA modification (a). This includes inhibition of DNA methyl transferase 1 (DNMT1) and EZH2, regardless of the presence of Rt3D. To test direct DNMT inhibition combined with Rt3D, we used the DNMT inhibitor decitabine. Decitabine sensitized melanoma cells to Rt3D-induced cell kill (b). DNMT inhibitors have been shown to ER upregulate chaperones, demethylation indicating by palbociclib as a possible cause of the ER stress profile observed in combination with Rt3D.



HLA-DQB1 RAB10 RAB35 RAB34 RELB CTSS CTSL CD74 HLA-DMA RAB4A CTSH ^ × × × × 0 0'

Summary

HLA-DQA

Oncolytic viruses are an attractive treatment option because they are self-amplifying, kill through multiple mechanisms and have good potential to promote anti-tumour immune responses.

• Combinatorial therapy of Rt3D plus palbociclib was identified by a high-throughput drug screen.

• Palbociclib altered cell cycle dynamics and DNA methylation, while Rt3D activated interferon signaling via RIG-I. In addition, both agents appeared to have non-overlapping ER stress profiles.

• The combination, however, enhanced ER stress, potentiated ISG-induction, ERV liberation and phagocytosis in vitro.

Our pre-clinical data show a strong rationale for the combination of Rt3D with novel pharmacological inhibitors, such as the CDK4/6 inhibitor palbociclib.

Contact

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The Institute of Cancer Research Division of Radiotherapy & Imaging Targeted Therapy Team 237 Fulham Road, London



The Royal Marsden

Victoria.Roulstone@icr.ac.uk