Inhibition of Porcupine reduces Wnt-dependent colony formation and/or proliferation in both autocrine and paracrine cell models

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Introduction

Aberrant activation of the pro-survival Wnt signalling pathway is a key determinant in numerous cancers (Nusse and Varmus, 2012) and therefore constituents of this pathway are potentially promising therapeutic targets.

The membrane-bound o-acyltransferase porcupine (PORCN) is required for post-translational modification of all Wnts and is vital for Wnt secretion (Herr and Basler, 2012). Due to the complex and varied oncogenic roles of the known Wnt ligands, prevention of active ligand secretion provides an effective mechanism for inhibiting canonical and non-canonical Wnt signalling in both paracrine and autocrine systems.

We therefore sought to determine whether a PORCN inhibitor could effectively reduce Wnt-dependent proliferation in both paracrine and autocrine *in vitro* models.

REDX05562 is a potent, novel inhibitor of the Wnt pathway

REDX05562 is an early exemplar from a novel chemical series of Wnt pathway inhibitors currently under optimisation.

An assay was designed to test the potency of compounds (Fig. 1).

- L-cells stably secreting active murine Wnt3a (ATCC) were seeded in 96-well plates.
- After 24 hrs, media was removed and replaced with assay media containing compound or DMSO control (final DMSO concentration = 0.1%) and incubated for a further 24 hrs.
- The media was removed and transferred to cells stably expressing luciferase under control of Wnt pathway response elements (Enzo Life Sciences; seeded 24 hrs prior to media transfer).
- After 24 hrs, Wnt pathway activation was quantified with ONE-Glo Luciferase assay system (Promega) and luminescence was measured with an EnVision plate reader (PerkinElmer).

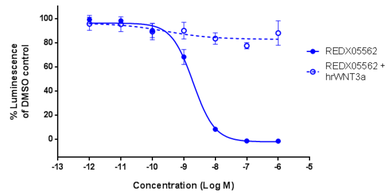


Fig. 1: REDX05562 potently inhibited Wnt signalling in a concentration dependent manner (REDX05562 pIC_{50} 8.9 +/-0.16, N=5). Addition of recombinant murine Wnt3A (35ng/mL) in combination with REDX05562 treatment restored Wnt signalling.

REDX05562 potently inhibited Wnt-dependent signalling. Addition of recombinant Wnt3a restored Wnt signalling, confirming that the observed compound-mediated inhibition is occurring upstream of the Wnt receptor, Frizzled.

REDX05562 inhibits AXIN2 expression

Having shown potent inhibition of Wnt signalling in our cell-based gene reporter assay we next determined whether REDX05562 exhibited Wnt pathway inhibition in a human pancreatic cancer cell model (CAPAN-2). Pathway engagement by REDX05562 was determined by the expression of AXIN2, an established marker of Wnt pathway activation (Jho *et al*., 2002).

- CAPAN-2 cells were grown to 70% confluence.
- Cells were treated with compound or DMSO control (final DMSO concentration = 0.1%) for 24 hrs before being harvested.
- Relative changes in AXIN2 mRNA with respect to 2 housekeeping genes (β-Actin and GAPDH) were tested by qPCR and processed using CFX manager software (BioRad).

At 10nM REDX05562 appeared to decrease AXIN2 mRNA expression more than 25-fold (Fig. 2). This inhibition of AXIN2 could be reversed by addition of recombinant human Wnt3a (250ng/mL), consistent with compound-mediated inhibition occurring upstream of Frizzled.

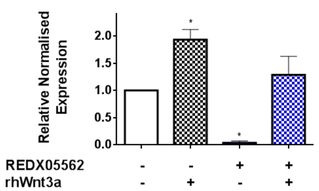


Fig. 2: Treatment of 2D HPAF-II cells with 10nM of REDX05562 reduced AXIN2 mRNA expression 25-fold compared to DMSO (N=2, relative expression 0.04 and 1 respectively, p=0.02). Addition of 250ng/mL recombinant human Wnt3a 6 hrs prior to harvesting, restored AXIN2 expression.

PORCN inhibition restricts proliferation of CAPAN-2 cells

Figure 2 shows that REDX05562 potently inhibits Wnt signalling in CAPAN-2 cells. We therefore sought to determine the effect of PORCN inhibitors on the proliferation of *in vitro* models of pancreatic cancer cell lines (CAPAN-2 and HPAF-II).

- CAPAN-2 or HPAF-II cells were seeded in 96-well plates (2000 or 3000 cells/well respectively).
- After 24 hrs, media was removed and replaced with growth media containing compound or DMSO control (final DMSO concentration = 0.1%).
- After 5 (HPAF-II) or 7 (CAPAN-2) days, media was removed and cells fixed with ice cold methanol and stained with Hoechst33342. Nuclei were counted using an Operetta HCS System (10x objective; PerkinElmer) and analysed using the Columbus image analysis system (PerkinElmer; Fig. 3A).

REDX05562 inhibited CAPAN-2 (Fig. 3B) and HPAF-II (Fig. 3C) proliferation in a concentration dependent manner.

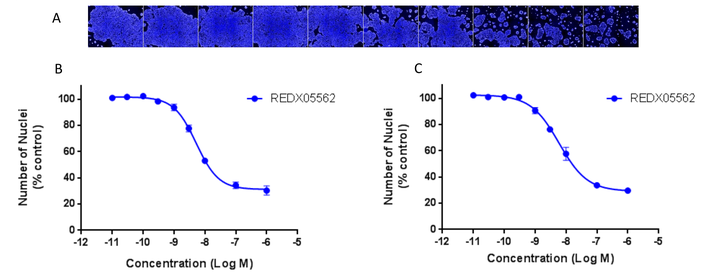


Fig. 3: A, An exemplar series of fluorescent images with each panel representing 1 well of a 96 well plate. Each panel represents CAPAN-2 cells treated with DMSO (far left) or REDX05562 at a concentration of 10pM to 1nM (left to right) and stained with Hoechst33342. **B**, CAPAN-2 proliferation data (REDX05562 pIC_{50} 8.4 +/-0.19, N=5). **C**, HPAF-II proliferation data (REDX05562 pIC_{50} 8.2 +/-0.26, N=6).

REDX05562 restricts cancer stem cell activity

To determine whether PORCN inhibition could also effect the growth of anoikis resistant cells, a sphere-forming efficiency (SFE) assay was performed. Briefly, HPAF-II cells were seeded in ultra-low attachment plates (Corning) at clonal density. After 5 days the number of established colonies were counted and expressed as a percentage of the original cell number. In this assay, only cells that exhibit anoikis resistance survive to form colonies. These cells are reported as having "stem-like" properties that are comparable to primary cancer stem cells (Lamb *et al*., 2013). Incubation of these cells with 10nM REDX05562 significantly inhibited colony formation (Fig. 4A) and appeared to also reduce colony size (Fig. 4B and C).

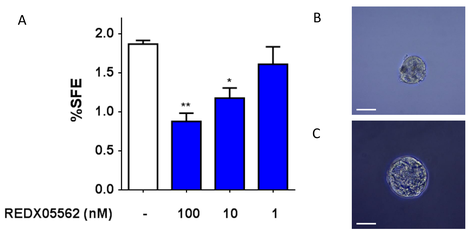


Fig. 4: A, HPAF-II cells treated with REDX05562 showed significantly decreased sphere forming efficiency from 1.87 +/- 0.04% to 0.88 +/- 0.11% (100nM, p=0.0076, N=2) and to 1.18 +/- 0.13% (10nM p=0.027, N=2). **B-C,** Example light microscope images (10x objective; Nikon) of colonies from cells treated with 10nM REDX05562 (B) or with DMSO (C). White bar = 40μm.

PORCN inhibitors do not directly effect MCF-7 proliferation

In contrast to HPAF-II cells, treatment of MCF-7 cells with REDX05562 did not inhibit SFE (Fig. 5A). SFE was significantly improved, however, by addition of 100ng/mL recombinant human Wnt3a (Fig. 5B), suggesting that Wnt3a can bestow anoikis resistance in this model via a paracrine route.

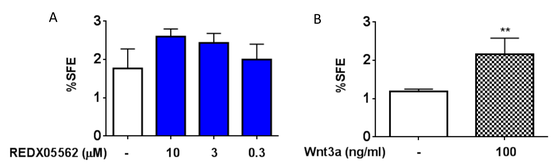
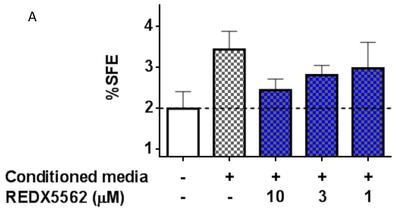


Fig. 5: A, MCF-7 cells treated with high concentrations of REDX05562 showed no significant change in %SFE (N=3). **B**, MCF-7 cells showed significant increase in %SFE when treated with Wnt3a at 100ng/ml (N=3, p=0.0037).

PORCN inhibition in a paracrine model

With no direct effect of PORCN inhibition on MCF-7 SFE we sought to determine whether breast cancer cells were stimulated by Wnt ligands released from stromal cells. To this end, primary human cancer-associated fibroblasts (CAFs) were isolated from human breast cancer samples (which were generously donated by the Liverpool Tissue Bank as part of an ethically approved study). Incubation of MCF-7 cells with conditioned media from primary CAFs induced a significant increase in MCF-7 SFE (Fig. 6A). When CAFs were incubated with REDX05562, the subsequent conditioned media resulted in significantly reduced SFE in the MCF-7 mammosphere assay. PORCN inhibition had no significant effect on primary human CAF proliferation (data not shown). This suggests that there may be a paracrine Wnt signalling mechanism between stroma and tumour in breast cancer.



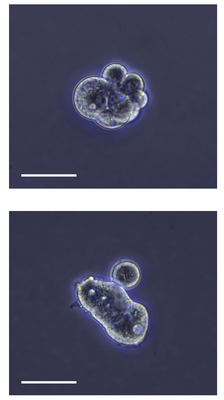


Fig. 6: A, MCF-7 cells treated with media conditioned for 7 days on primary human CAFs showed significant increase in %SFE (N=3, p=0.045). This effect could be inhibited when cells were incubated in the presence of REDX05562 at 10 and 3μM. Accounting for basal SFE, this inhibition was shown to be significant (10μM p=0.002, 3μM p=0.02, N=2). **B-C,** Representative images of mammospheres (20x objective; Nikon) formed in conditioned media with DMSO (B) or with 10μM REDX05562 (C). White scale bar = 40μm. Unlike the spheroids observed in the HPAF assay (Fig. 4 B-C) there was no apparent difference in mammosphere size.

The different responses of CAF and MCF-7 cells to PORCN inhibition is supported by the observation that PORCN mRNA expression is markedly higher in CAFs than in MCF-7 cells (Fig. 7).

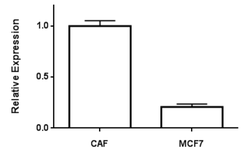


Fig. 7: qPCR analysis shows that untreated MCF-7 cells exhibited 5-fold less mRNA expression of porcupine compared to untreated primary human CAFs. GAPDH and β-Actin were used as housekeeping genes.

Conclusions

- REDX05562 potently inhibit Wnt-mediated signalling in both a gene reporter assay and an *in vitro* model of pancreatic cancer proliferation.
- Conditioned media from patient derived CAFs increased anoikis resistance in MCF-7 cells. REDX05562 treatment of CAFs significantly inhibited colony formation in this paracrine model. This novel methodology could be used with matched patient samples to further investigate the role of paracrine Wnt pathway signalling in various human cancers.
- Consistent with the early position of PORCN within the Wnt pathway signalling cascade, inhibition with REDX05562 reduced the impact of Wnt ligand-mediated signalling in both paracrine and autocrine models.
- Taken together these data emphasise the broad therapeutic potential of PORCN inhibition in Wnt-dependent cancers.

References

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