Niclosamide and Palbociclib act synergistically to reduce cholangiocarcinoma cell viability *in vitro* and inhibit tumour growth in a mouse model.

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Introduction

- Despite the emergence of new treatment modalities including targeted therapies that benefit some patients with tumours carrying specific mutations, the prognosis for patients with cholangiocarcinoma (CCA) remains poor.
- Novel therapeutic approaches that can benefit the majority of patients whose tumour cells do not carry targetable mutations are urgently needed.
- To identify mutation-agnostic treatment approaches we screened a library of well-tolerated off-patent drugs against CCA cells and tested their effects on both CCA cells and normal biliary epithelial cells.

Screening a drug library revealed Niclosamide as a drug of interest

We screened a drug repurposing library of off-patent drugs and Niclosamide was the most effective hit in the library (Fig.1A). Niclosamide decreased the viability of multiple CCA cell lines (Fig.1B-C) but had less effect on normal human biliary epithelial cells (BECs) (Fig.1E-F).

The EC50 value is the drug dose required to achieve a 50% reduction in viability.

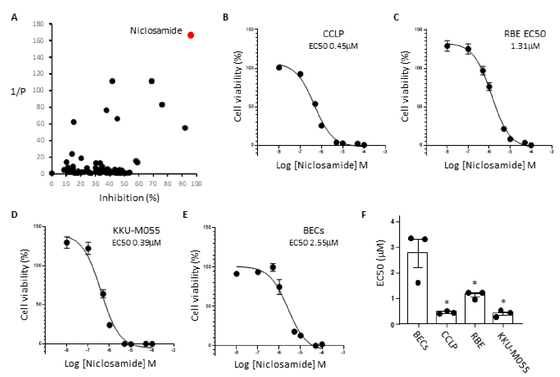

Figure 1 Drug screening and validation of Niclosamide using CCA cell lines and primary biliary epithelial cells.
(A) CCLP CCA cells were plated at $1x10^4$ cells per well in a 96 well plate and treated with a library of drugs each at its clinically relevant peak serum concentration for 96 hours. Cell viability was then determined using an MTT assay and normalized to cells treated with the corresponding vehicle control for each drug. N=3 biological repeats with a paired, two-tailed T-test followed by Benjamini-Hochberg multiple corrections. The inhibition of viability (%) is plotted against the reciprocal of the P value for each drug. (B-E) Niclosamide dose response curves for CCA cell lines (CCLP, RBE, and KKU-M055) and primary normal biliary epithelial cells (BECs) plated as in (A) and treated with Niclosamide (10nM-100µM) or vehicle control for 72 hours. Cell viability was measured using an MTT assay and normalised to vehicle control. N=3 biological repeats each performed in triplicate. (F) The graph shows the relative EC50 values for each cell type calculated using GraphPad Prism. Error bars represent SEM (*=p<0.05 unpaired t-test). When error bars cannot be seen they are smaller than the symbols.

Niclosamide treatment decreased the expression multiple proteins known to control CCA cell proliferation including the PRH oncoprotein

Treating CCLP cells with Niclosamide resulted in decreased expression of PRH (Fig.2A), a protein that we have previously shown to act as an oncoprotein in CCA cells (Kitchen et al Cancer Res. 2020). Niclosamide treatment also decreased the expression of the growth control proteins β-catenin and Cyclin D1 (Fig.2A).

Niclosamide has been reported to increase protein ubiquitination levels in some non-CCA cancer cell lines (Cheng et al Plos One 2020). In three CCA cell lines Niclosamide treatment increased global protein ubiquitination levels (Fig.2B). Interestingly the increase in protein ubiquitination appeared to correlate with the EC50 for Niclosamide in each cell line (Fig.2C).

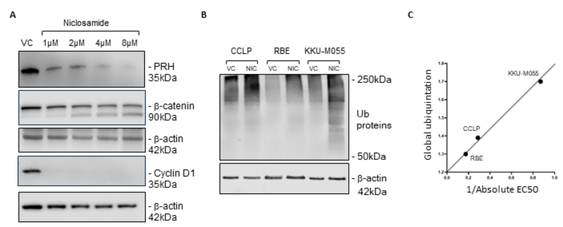

Figure 2 Niclosamide decreased the levels of multiple proteins that determine CCA cell viability and increased global protein ubiquitination levels.
(A) CCLP cells were treated with vehicle control (VC) or Niclosamide at the concentrations given for 24 hours before harvesting for western blot analysis for PRH, β-catenin and Cyclin D1 with β-actin as a loading control. (B) CCLP, RBE and KKU-M055 cells were treated with VC or 8µM Niclosamide (NIC) for 24 hours before harvesting for western blot analysis using a ubiquitin antibody to measure global protein ubiquitination levels with β-actin as a loading control. (C) Global protein ubiquitination was quantified by normalising the ubiquitinated proteins to β-actin and relativising to VC and plotted against the absolute EC50 values observed in each cell line.

Niclosamide induced proteasome-dependent degradation of PRH but PRH alone is not responsible for the effects of this drug

We have shown that PRH interacts with the proteasome (Bess et al Biochem J 2003) and is proteasomally processed in other cancer cell types (Noy et al NAR 2012). CCLP cells were treated with vehicle control or Niclosamide in the absence and presence of the proteasome inhibitor MG132 and PRH protein levels were determined using western blotting. MG132 treatment increased PRH levels in the absence of Niclosamide and abrogated the down-regulation of PRH protein levels seen in the presence of Niclosamide (Fig.3A and Fig.3B). This shows that Niclosamide induces proteasome-dependent degradation of PRH. However, when we knocked out the gene encoding PRH using CRISPR technology (Fig.3C) there was no difference in the response to Niclosamide treatment (Fig.3D).

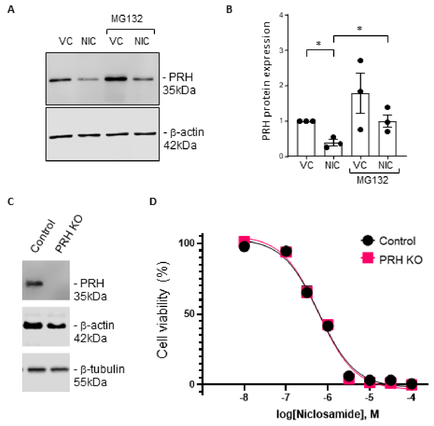

Figure 3 PRH knockout does not reduce sensitivity to Niclosamide.
(A) CCLP cells were treated with VC or 8µM Niclosamide (NIC) in the absence and presence of 5µM MG132 and PRH protein levels were determined using western blotting. (B) The experiment show in (A) was performed to N=3 and PRH levels relative to VC were determined by densitometry. Error bars represent SEM and *=p<0.05 unpaired t-test. (C) PRH protein expression in CCLP cells in which the gene encoding PRH is knocked out (PRH KO) and control CCLP cells (control) was examined by western blotting. (D) Niclosamide dose response curves were generated for PRH KO and control cells. The cells were plated at $1x10^4$ cells per well in triplicate in a 96 well plate and treated with Niclosamide (10nM-100µM) or vehicle control for 72 hours. Cell viability was then measured using an MTT assay and values normalised to vehicle control. N=4 biological repeats. Error bars represent SEM and are smaller than the symbols.

Treament with the CDK4/6 inhibitor Palbociclib reduced the viability of CCLP cells but had less effect on normal cells

Dysregulation of genes encoding cell cycle components and regulatory proteins is frequent in CCA and we have previously demonstrated that cell cycle inhibition using the CDK4/6 inhibitor Palbociclib reduces the viability of CCLP cells (Kitchen et al Cancer Res. 2020). We therefore examined the effect of Palbociclib treatment on CCA cell lines and primary BECs. Palbociclib treatment had a significant inhibitory effect on the viability of CCLP cells, RBE cells and KKU-M055 cells (Fig.4A-C). More interestingly Palbociclib treatment had very little effect on BECs under the same conditions (Fig.4D and Fig.4E).

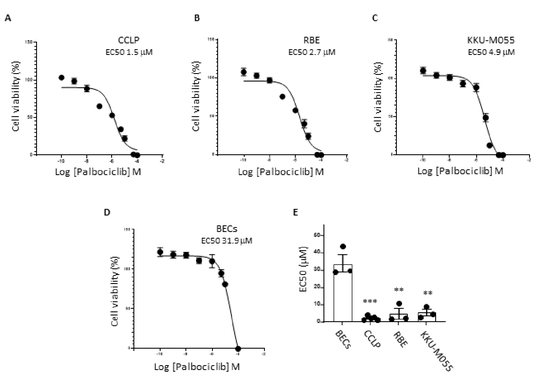

Figure 4 Palbociclib decreased the viability of CCA cells but had less effect on primary biliary epithelial cells.
CCA cell lines - CCLP (A), RBE (B), KKU-M055 (C) - or primary biliary epithelial cells (D) were plated at $1x10^4$ cells per well in a 96 well plate and treated with increasing concentrations of Palbociclib for 96 hours. Cell viability was then measured using an MTT assays and normalised to vehicle control. Three biological repeats (five for CCLP cells) each performed in triplicate. Error bars represent SEM. When error bars cannot be seen they are smaller than the symbols. (E) The graph shows the relative EC50 values calculated using GraphPad Prism. Error bars represent SEM (**=p<0.01 ***=p<0.001 unpaired t-test).

Niclosamide and Palbociclib acted synergistically

Niclosamide is well known as a mitochondrial uncoupler and it is also known to impact a variety of cell signalling pathways (Tao et al Nat. Med 2014). We therefore considered whether Niclosamide might act synergistically with CDK4/6 inhibitors such as Palbociclib and achieve a more potent cell killing effect in combination. Increasing doses of either drug alone or the combination of both drugs were tested in CCLP cells (Fig.5A). Both drugs alone reduced the viability of CCLP cells and the drugs in combination were more effective than single treatments. Combination index (CI) values for the two drugs in combination were all <1 indicating synergistic behaviour (Fig.5B). As expected Palbociclib reduced the levels of phosphorylated RB (pRB) in the treated cells (Fig.5C). More interesting, Niclosamide treatment also reduced pRB levels in these cells (Fig.5C) and the combination of Palbociclib and Niclosamide further reduced pRB levels, again suggesting a synergistic effect.

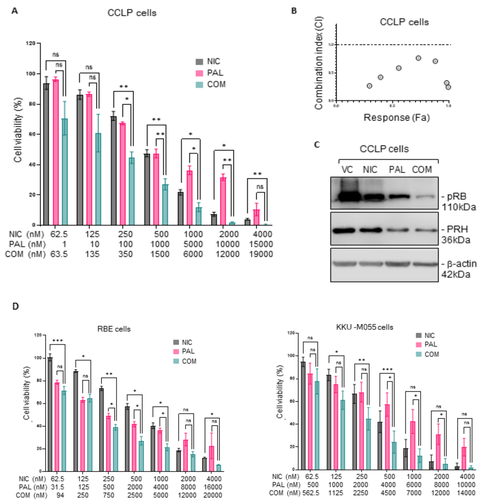

Figure 5 Niclosamide and Palbociclib acted synergistically to reduce CCA cell viability.
(A) CCLP cells were plated at 1x104 cells per well in a 96-well plate before treatment with Niclosamide (NIC), Palbociclib (PAL), or both drugs in combination (COM) at the concentrations shown and for 72 hours. Cell viability was then measured using an MTT assay. N=3 biological experiments each performed in triplicate. Error bars represent SEM (*= p<0.05, **= p<0.01) two-tailed paired t-test. (B) From the data shown in (A) combination index (CI) values were calculated and plotted against the corresponding response (Fa) values in a CI plot. CI<1 indicates synergism. (C) CCLP cells were treated with 0.5mM Niclosamide (NIC), 1mM Palbociclib (PAL), or both drugs in combination (COM) for 24 hours then proteins were extracted for western blotting for phosphorylated RB (pRB), PRH, and β-actin. (D) The experiment shown in (A) was repeated using RBE cells and KKU-M055 cells.

Niclosamide and Palbociclib reduced the growth of CCA cells as spheroids.

We cultured CCLP and BEC 3D spheroids for 5 days and then treated them with each drug alone and in combination for a further 3 days. Treatment with Niclosamide alone or Palbociclib alone resulted in a reduction in the size of CCLP spheroids and the number of viable CCLP cells but had no effect on the size of BEC spheroids or the number of viable BEC cells (Fig.6A and Fig.6B, respectively). The combination of Niclosamide and Palbociclb had a more potent effect on CCLP spheroid size and cell viability than individual treatment but had no effect on the size of BEC spheroids and little or no effect on the viability of BEC cells.

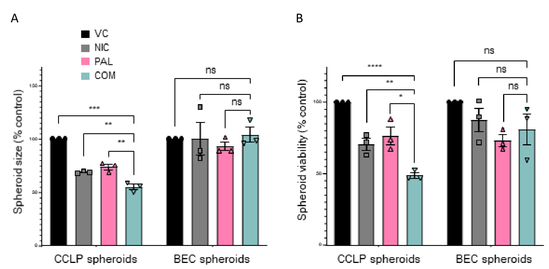

Figure 6 Niclosamide and Palbociclib synergistically reduced CCA cell viability.
(A) CCLP cells and BECs were grown as spheroids for five days before treatment with 0.5mM Niclosamide, 1mM Palbociclib, or both drugs in combination for a further 72 hours. Images were taken using a Nikon brightfield microscope and spheroid size calculated using ImageJ. N=3 biological repeats. Error bars represent SEM (*= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001) two-tailed paired t-test (B) Cell viability was measured in the spheroids from (D) using a Presto blue assay.

Niclosamide and Palbociclib as a combination treatment induced cell cycle arrest and apoptosis in CCA cells

We next treated CCLP cells with Niclosamide and Palbociclib either individually or in combination for 72 hours and assayed cell cycle progression and cell death using flow cytometry. Treatment with Palbociclib and Niclosamide under these conditions resulted in greater G1 arrest than either treatment alone (Fig.7A). Similarly, the combination of drugs resulted in greater apoptotic cell death than either single treatment (Fig.7B). Very similar results were obtained using RBE and KKU-M055 cells.

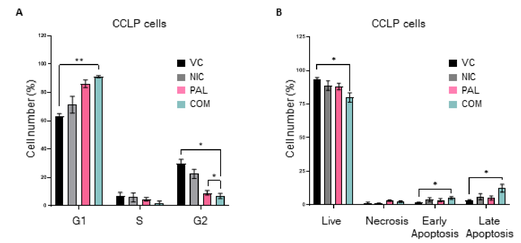

Figure 7 Combining Niclosamide and Pabloclib resulted in decreased cells in G2 and increased cells in apoptosis.
(A) CCLP cells were treated with vehicle control (VC), 0.5µM Niclosamide (NIC), 1µM Palbociclib (PAL) or both drugs (COM) for 72 hours before harvesting for cell cycle analysis using flow cytometry. The graph shows the proportion of the cell population in each stage of the cell cycle. N=3 biological experiments. Error bars represent SEM (*= p<0.05, **= p<0.01) two-tailed paired t-test. (B) CCLP cells were treated with Niclosamide (NIC), Palbociclib (PAL) or both drugs (COM) as in (A) then the number of live cells, and cells in necrosis and cells in early and late apoptosis was determined using flow cytometry. Error bars represent SEM (*= p<0.05, **= p<0.01) two-tailed paired t-test.

Niclosamide and Pabloclib acted synergistically to reduce CCA tumour growth in a mouse model.

CCLP tumours cells were grown in immunodeficient mice until they reached 3mm in diameter. The mice were then treated with vehicle alone, Niclosamide alone, Palbociclib alone, or Palbociclib plus Niclosamide, for 30 days. All of the treatments decreased tumour volume (Fig.8A) but when the tumour volumes were analysed using the Bliss Independence model for drug synergy, the observed tumour volume for the combination was significantly lower than the expected tumour volume through additive effects and thus the two drugs are considered synergistic (Fig.8B). Moreover, when the average tumour weights for the four groups of mice were compared only the combination treatment resulted in a statistically significant difference from vehicle (Fig.8C).

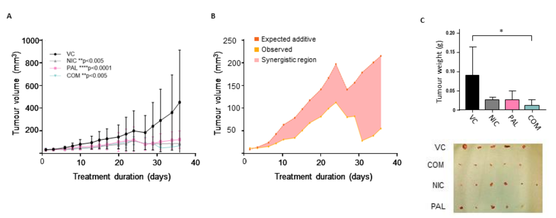

Figure 8 Niclosamide and Pabloclib acted synergistically to reduce CCA tumour growth in a mouse xenograft model.
(A) CCLP cells were injected subcutaneously into 4 groups of nude mice and allowed to grow until the tumours reached 3mm in diameter. Vehicle control (VC), Niclosamide (NIC) (20mg/kg), Palbociclib (PAL) (10mg/kg), or both drugs (COM) was then administered three times weekly intraperitoneally and tumour volume was measured using calipers over 30 days. ****p<0.0001 **p<0.005 two-way ANOVA. (B) The plot shows a comparison between the observed tumour volumes from the combination treatment and the expected additive tumour volumes over time. The expected tumour volume (under the assumption of an additive effect) was calculated by using the Bliss independence model. The red shaded region indicates synergistic effects where the observed tumour volume is significantly lower than the expected additive effect. (C) Top - Comparison of tumour weights across all treatment groups *p<0.05 two-way ANOVA. Bottom - the excised tumours.

Conclusions

- Niclosamide treatment reduced the viability of multiple cholangiocarcinoma cell lines but had a lesser effect on normal primary biliary epithelial cells.
- Niclosamide treatment reduced the growth of cholangiocarcinoma cells as tumour spheroids *in vitro* and reduced the growth of cholangiocarcinoma cells as tumours in a xenograft mouse model.
- Palbociclib also reduced the viability of cholangiocarcinoma cell lines compared to normal biliary epithelial cells and synergised with Niclosamide to reduce cholangiocarcinoma cell viability *in vitro* and reduce tumour growth in a mouse xenograft model.
- These preclinical results suggest that Niclosamide and/or Niclosamide in combination with an inhibitor of CDK4/6 are worthy of clinical evaluation as potential treatments for this disease.