

The histone deacetylase inhibitor PXD101 synergises with 5-fluorouracil to inhibit tumour cell growth *in vitro* and *in vivo*.

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Summary

Gene expression is controlled in part by deacetylation of specific lysine residues in histone tails and this process controls repression of many genes involved in cell cycle progression. Histone deacetylase inhibitors (HDACi) are currently being developed as anti-tumour agents and have been shown to inhibit effectively the growth of hyper-proliferating cancer cells. It has also been demonstrated that combinations of HDACi with well-established chemotherapeutics can synergise with their anti-tumour effects. We have investigated in detail the effects of PXD101 (HDACi in phase I clinical trials) in combination with the thymidylate synthesis inhibitor 5-fluorouracil (5-FU), on tumour cell proliferation and apoptosis both *in vitro* and *in vivo*.

Incubation of the HCT116 cell line (an established model of colon cancer) with PXD101 alone for 24 hours down-regulated thymidylate synthase expression (Fig 1). High level thymidylate synthase expression in colon cancer is a marker of poor prognosis. The HCT116 cell line was studied using WST-1 proliferation assays. Synergistic inhibition of proliferation was obtained with combination index (CI) values of 0.7 or below over a wide range of concentrations, as calculated by CalcuSyn™ (1). Several different incubation schedules were tested and the greatest degree of synergy was obtained when cells were incubated in PXD101 for 24 hours, followed by a further 48 hours in 5-FU alone (Fig 2). Furthermore, synergism was confirmed in HCT116 cells in clonogenic assays when PXD101 and 5-FU were combined (Fig 3). 5-FU combined with PXD101 also increased DNA fragmentation (demonstrated using FACS TUNEL assay) and increased PARP cleavage in HCT116 cells (Figs 4 and 5), compared to each compound alone.

In vivo studies using the mouse P388 *i.p.* tumour model showed an increase in median survival of 3 days (log rank analysis $p < 0.002$) compared to each drug alone, when PXD101 and 5-FU were combined (Fig 6). HCT116 xenograft models also showed a beneficial outcome when PXD101 and 5-FU were combined. Tumour volume was significantly reduced in HCT116 xenografts with combinations compared to each drug alone (Fig 7). PXD101 is nearing the end of Phase I oncology clinical trials and subsequent Phase II trials will assess the benefits of combinations of this HDACi with existing chemotherapeutics, including 5-FU.

Thymidylate Synthase Down-regulation

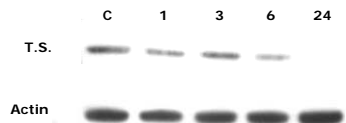


Figure 1. Western blot demonstrating down regulation of thymidylate synthase levels in HCT116 cells. Cells were incubated +/- 0.9 μM PXD101 for 1, 3, 6 and 24 hours. C = untreated.

In vitro effects on proliferation

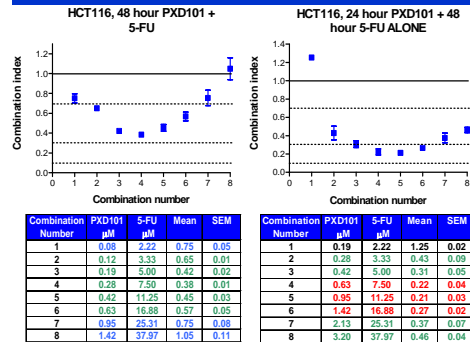


Figure 2. PXD101/5-FU combinations analyzed using CalcuSyn™. Each combination number refers to a different dose of each drug used in combination. CI values in black represent antagonism, blue additive effects, green synergy and red strong synergy.

In vitro effects on colony formation

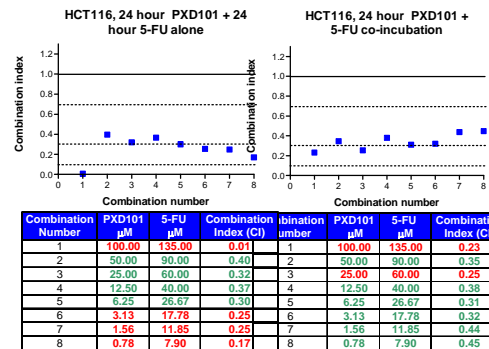


Figure 3. Effect of PXD101 and 5-FU combinations on HCT116 cell colony formation. HCT116 cells were cultured +/- PXD101 and 5-FU for the indicated times and seeded onto 35 mm dishes in 3% agar containing a sheep erythrocyte feeder layer. Agar plates were cultured for 14 -21 days at 37°C and colonies counted. CI values in green represent synergy and red strong synergy.

TUNEL Analysis

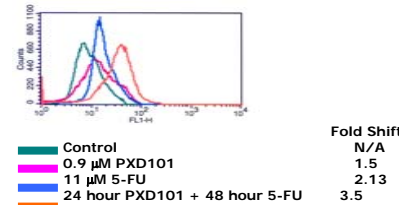


Figure 4. Terminal dUTP Nick End Labelling (TUNEL) analysis of HCT116 cells treated for 24 hours +/- PXD101 followed by 48 hours +/- 5-FU alone.

PARP Cleavage

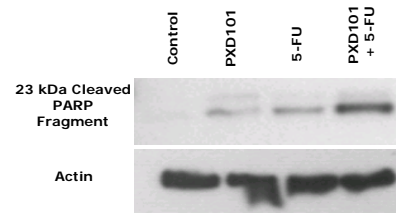


Figure 5. Western blot demonstrating cleavage of Poly ADP-Ribosyl Polymerase (PARP) in HCT116 cells. Cells were incubated +/- 0.7 μM PXD101 and 17 μM 5-FU for 48 hours.

Prolonged survival in mice with intraperitoneal P388 cells

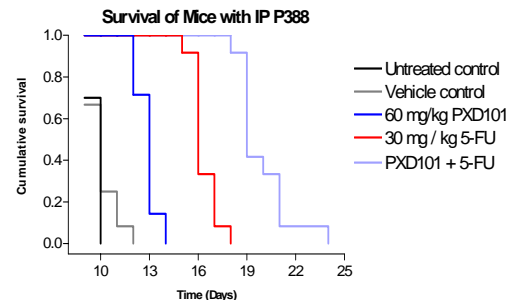


Figure 6. Effect of PXD101 and 5-FU treatment on the mouse intraperitoneal P388 leukaemia survival model. Mice were inoculated with 1x10⁶ P388 cells and treated 3 days later +/- PXD101 and/or 5-FU *i.p.* daily for 5 days. Combined treatment significantly prolonged survival compared to treatment with either PXD101 or 5-FU alone; $P < 0.002$ using Log Rank statistic analysis.

Tumour growth inhibition in an HCT116 xenograft

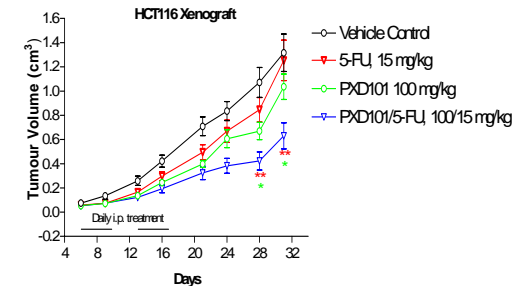


Figure 7. Effect of PXD101 and 5-FU treatment on tumour growth in a HCT116 subcutaneous xenograft established in NCR:nu/nu mice. Mice were treated on days 6-10 and 13-17. Tumour volume was monitored until day 31. Statistical analysis was performed using Mann-Whitney U test; * $P < 0.05$, ** $P < 0.01$.

Major Conclusions

> Combinations of PXD101 and 5-FU synergistically inhibit HCT116 cell proliferation and colony formation *in vitro*.

> Pre-treatment of HCT116 cells with PXD101 demonstrated stronger synergistic effects. This may be explained by the down-regulation of thymidylate synthase by PXD101.

> The combination of PXD101 and 5-FU produced an increase in PARP cleavage and DNA fragmentation in HCT116 cells *in vitro*, compared to each compound alone. This is indicative of increased cell death by apoptosis.

> Treatment of P388 implanted mice with PXD101 and 5-FU significantly prolonged survival compared to treatment with each drug alone.

> Significant tumour growth inhibition of an HCT116 xenograft was observed when mice were treated with PXD101 followed by 5-FU.

Reference

1. Chou, T.-C., and Talalay, P. Quantitative analysis of dose effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv. Enz. Regul.* 22:27-55, 1984