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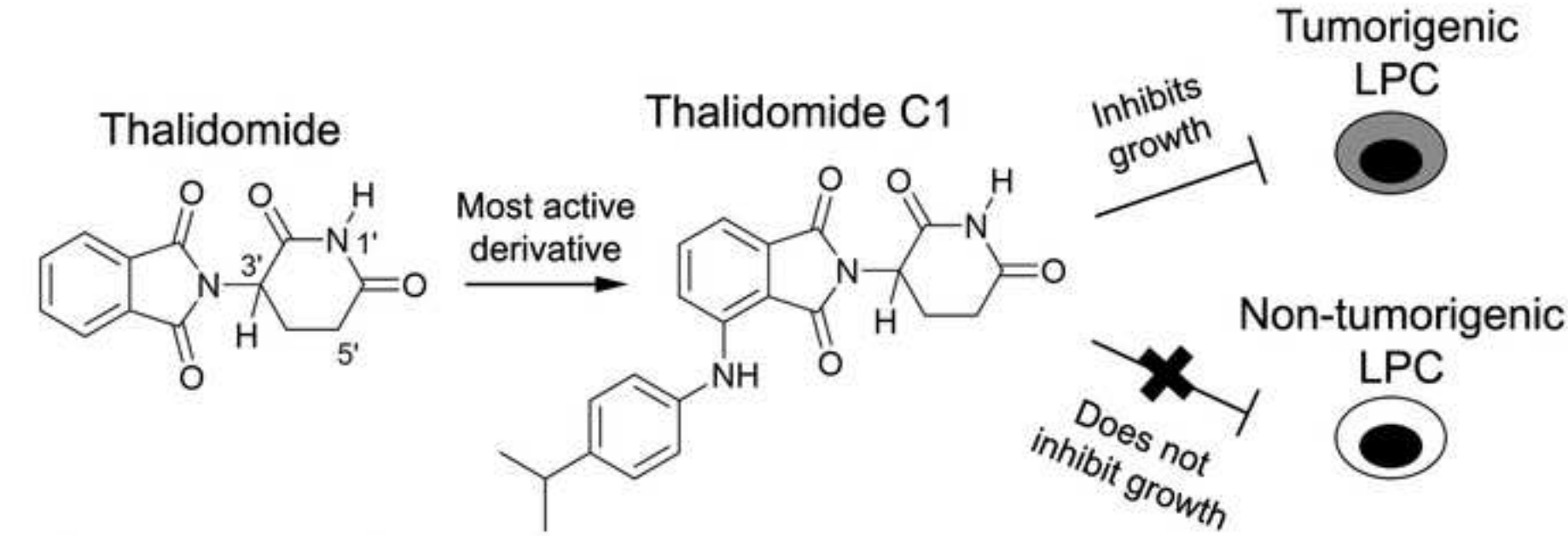

Identification of a thalidomide derivative that selectively targets tumorigenic liver progenitor cells and comparing its effects with lenalidomide and sorafenib.

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Abstract

Background & Aims: The availability of non-tumorigenic and tumorigenic liver progenitor cell lines affords a method to screen putative anti-liver cancer agents to identify those that are selectively effective. To prove this principle we tested thalidomide and a range of its derivatives and compared them to lenalidomide and sorafenib, to assess their growth-inhibitory effects.

Methods: Cell growth, the mitotic and apoptotic index of cell cultures were measured using the Cellavista instrument (SynenTec) using commercially available reagents.

Results: Neither lenalidomide nor thalidomide (100 µM) affected tumorigenic progenitor cells but killed their non-tumorigenic counterparts. Sorafenib arrested growth in both cell types. All but two derivatives of thalidomide were ineffective; of the two effective derivatives, one (thalidomide C1) specifically affected the tumorigenic cell line (10 µM). Mitotic and apoptotic analyses revealed that thalidomide C1 induced apoptotic cell death and not mitotic arrest.

Conclusions: This study shows that screens incorporating non-tumorigenic and tumorigenic liver cell lines are a sound approach to identify agents that are effective and selective. A high throughput instrument such as the Cellavista affords constant and consistent objective measurements with a large number of replicates that are reliable. These experiments show that neither lenalidomide nor thalidomide are potentially useful for anti-liver cancer therapy as they kill non-tumorigenic liver cells and not their tumorigenic counterparts. Sorafenib in contrast, is highly effective, but not selective. One tested thalidomide derivative has potential since it induced growth arrest; and importantly, it selectively induced apoptotic cell death only in tumorigenic liver progenitor cells.

Keywords: liver cancer, progenitor cells, thalidomide, analogues, lenalidomide, sorafenib, apoptosis, mitosis

List of abbreviations: Liver progenitor cells (LPCs), hepatocellular carcinoma (HCC), Federal Drug Administration (FDA), structure activity relationship (SAR), bipotential mouse oval cell (BMOL), non-tumorigenic (NT), tumorigenic (T), Williams' E medium (WEM), Hank's Balanced Salt Solution (HBSS)

Introduction

Thalidomide and its analogues elicit a wide range of cellular effects including growth inhibition and promotion of apoptosis [1] that suggest this class of compounds may be useful for treating cancers [2]. Indeed, one of the most publicised uses of thalidomide is that of a dual agent; targeting angiogenesis [3] and the tumour, in treating multiple myeloma [4-6]. Clinical trials have established that a range of thalidomide related compounds are particularly effective in treating hematological cancers as they are able to block the action of cytokines such as tumour necrosis factor (TNF) and the interleukins that provide the growth stimulus for maintenance of myelomas [7].

The biological effects of thalidomide and its derivatives suggest they may be especially useful for treating liver cancer. Firstly, inflammation is proposed to cause liver cancer [8] and many liver cancers display elevated levels of inflammatory cytokine expression [9]. Secondly, there is extensive evidence to support the view that some hepatocellular carcinomas (HCC) may be derived from liver progenitor cells (LPCs) [10] and growth of these cells is stimulated by TNF [11, 12] and TNF-related cytokines such as tumour necrosis factor-like weak inducer of apoptosis (TWEAK) [13] as well as IL-6 [14]. These cytokines mediate their effects through nuclear factor kappa B (NFκB) signalling that is targeted by thalidomide and its derivatives [15]. We have generated a panel of liver progenitor cell (LPC) lines [16] some of which undergo transformation following extensive passaging. Further characterisation revealed these tumorigenic cell lines show increased expression of cell-cycle related genes, e.g. Yes associated protein (YAP) and anti-apoptotic genes e.g. cellular inhibitor of apoptosis (cIAP) [17]. Therefore, these cells are particularly amenable to test the toxicity of thalidomide and its analogues. Our possession of unique non-tumorigenic and tumorigenic LPC lines also affords the opportunity to screen these agents for analogues that selectively target tumorigenic cells [18].

Liver cancers are notoriously resistant to therapy for they do not respond well to chemotherapeutic agents or to radiotherapy [19]. Various reasons have been advanced to explain their resistance including their ability to detoxify chemical toxins and to efflux noxious agents. Presently, resection is the only avenue for treating HCC, and even then, recurrence is common. Although it is the fifth most common cancer, HCC ranks third as the most common cause of cancer-related deaths worldwide [20].

The tyrosine kinase inhibitor sorafenib, was shown to be effective against HCC, and it has received Federal Drug Administration (FDA) approval for treating patients with liver cancer. While there are numerous studies concerned with sorafenib's effects on hepatoma cell lines, its action on LPCs, and in particular tumorigenic LPCs is unknown. Since we have both the non-tumorigenic and their tumorigenic LPC counterparts, it is also possible to concomitantly evaluate the selectivity of these agents.

This study was undertaken to establish proof-of-principle that tumorigenic LPCs are useful to screen agents that have potential for treating HCC; and that incorporating non-tumorigenic cells will help identify agents that selectively target the tumour. It also evaluates the effects of lenalidomide, an FDA approved thalidomide derivative used to treat myeloma and selected derivatives we synthesised based on their ability to inhibit TNF expression [21]. These initial structure activity relationship (SAR) studies rationally identified a group of compounds that effectively inhibited TNF expression. This study identifies an isopropyl aniline C4 derivative of thalidomide that restricts the growth of tumorigenic LPCs relative and not non-tumorigenic LPCs. We then compared it to sorafenib an FDA-approved drug for treating HCC patients. Sorafenib affected both non-tumorigenic as well as tumorigenic LPCs, and it inhibited mitosis as well as induced apoptotic cell death. Finally, we show that this C4 derivative of thalidomide exerts its growth inhibitory effects by killing cells through apoptosis and not by inhibiting mitosis. In contrast, sorafenib exerts its growth inhibitory effect by inhibiting mitosis and inducing apoptotic cell death in both non-tumorigenic and tumorigenic cells.

Materials and methods

Cell lines

The original BMOL (for Bipotential Mouse Oval Liver) cell line was established and characterised by our laboratory [16]. Its LPC status was confirmed by its ability of differentiate into both cholangiocytes and hepatocytes in culture [16]. At low passage ($p<15$) it is deemed to be non-tumorigenic (NT) by virtue of its inability to grow in soft agar and to produce tumours upon subcutaneous transplantation into nude mice and is designated BMOL-NT. In contrast, following extensive passage ($p>30$) it is able to grow in soft agar and readily produces tumours; hence tumorigenic (T) in nude mice and is designated BMOL-T.

Cell maintenance

BMOL-NT and BMOL-T cells were maintained in 25cm^2 flasks (FAL353108, Corning®) with 4 mL of Williams' E medium supplemented with 1% v/v Fungizone (15290-018, Life Technologies), 48.4 µg/mL Penicillin (5161, Calbiochem), 675 µg/mL Streptomycin (11860-038, Life Technologies), 2 mM Glutamine (G8540, Sigma-Aldrich), 5% Fetal Bovine Serum (16000-044, Life Technologies), 20 ng/mL EGF (FAL354001, Corning®), 30 ng/mL IGF-II (OU001, GroPep Bioreagents) and 0.25 U/mL Humulin R (Eli Lilly and Company). The medium was replaced every 2 or 3 days over the weekend with an additional 1 mL of medium. Once cultures reached 80% confluence, cells were passaged 1 in 10 into new flasks. Cells were detached by rinsing with 1 mL of Hank's Balanced Salt Solution (HBSS) (H2387, Sigma-Aldrich), followed by rinsing with 1 mL of 0.05% Trypsin (15400-054, Life Technologies) in HBSS and a 5 min incubation at 37°C. After the cells had rounded and detached, they were then collected in 1 mL of medium, resuspended and passaged as required.

Cellavista proliferation assay

Cells (1,500 for BMOL-NT and 1000 for BMOL-T) were plated in a 96 well plate (167008, Thermo Scientific) in 100 µL of WEM per well. The following day after cells had attached, 100 µL of WEM was added and the plate was scanned with the Cellavista instrument (300007, SynenTec) twice daily for 5 days or until the cells reached 80% confluency. Parameters for the image acquisition were set according to the manufacturer's instructions to obtain an average background intensity of

approximately 120 in the brightfield channel using the "Cell Confluence" application (Fig 3a, b). The efficiency of cell detection was checked to ensure cells were detected appropriately (Fig 3c, d). The log of cell confluency (% well coverage) is plotted against time to generate a growth curve. The doubling time was determined from the linear portion of the growth curve corresponding to the log phase of cell growth.

Treatment of cells with sorafenib or thalidomide C1

Cells were plated as described above. They were then treated with each compound by addition of a 2x solution of each compound in 100 µL of WEM to achieve a final concentration of 1x in each well containing a final volume of 200 µL.

Cellavista mitotic cell assay

Following three days of treatment with the compound of interest, cells were treated with the Phospho-Histone H3 Imaging Kit (06569161001, Roche) according to the manufacturer's instructions. Briefly cells were fixed in 4% paraformaldehyde, 0.1% Triton X-100 in 1x phosphate buffered saline (PBS) for 10 minutes, washed with 200 µL 1x PBS and incubated for 5 minutes. Cells were then labelled with 50 µL of Antibody Solution for 60 minutes at room temperature and nuclei stained by the addition of 50 µL of Nuclei Dye (Roche) for 10 minutes. Finally cells were washed twice with 200 µL 1x PBS and analysed with the Cellavista instrument. Cells treated with 1 µg/mL Gibco® KaryoMAX Colcemid (15210-040, Life Technologies) overnight served as positive controls and to adjust detection settings for the Cellavista (Fig 4a, b) enabling identification of mitotic cells. The mitotic index was expressed as the percentage of positive cells detected in each well.

Cellavista apoptotic cell assay

Following three days of treatment with the compound of interest, cells were treated with the DNA Fragmentation Imaging Kit (06432344001, Roche) according to the manufacturer instructions. Briefly cells were fixed in 4% paraformaldehyde, 0.1% Triton X-100 in 1x PBS for 10 minutes and washed with 200 µL 1x PBS. Cells were then labelled with 45 µL of Reaction Solution for 60 minutes at 37 °C and nuclei stained by the addition of 150 µL of Nuclei Dye (Roche) for 5 minutes. Finally wells were washed once with 200 µL 1x PBS and analysed with the Cellavista instrument. Cells treated with 8

µg/mL Cisplatin for 24 h served as positive controls and to optimise the Cellavista (Fig 4c, d) for identifying apoptotic cells. The apoptotic index was expressed as a percentage of the cells containing fragmented DNA in each well.

Western blot analysis

BMOL T cells were exposed to thalidomide C1 as indicated above and lysates were prepared in DISC lysis buffer as described previously [22]. Its effect on NFκB and ERK signalling pathways were assessed by Western Blot using the following antibodies; anti-ERK (#4696), anti-Phos-ERK (#9101), anti-p100/p52 (#4882), anti-Phospho-p65 (#3033, Cell Signaling Technology) and anti-β-actin (A1978, Sigma-Aldrich). Briefly the lysates were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (#10600018, GE Healthcare) for blotting. Membranes were incubated with the primary antibodies overnight followed by HRP conjugated secondary antibodies for 1hour. All antibodies were diluted in in 5% skim milk powder in Tris Buffered Saline with 0.1% Tween (TBS-T). The chemiluminescent signal was developed using the Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500, Merck Millipore) and captured using a BioRad ChemiDoc™ MP System.

Quantification of thalidomide C1 effects on NFκB and ERK signalling

Captured images of the western blots were exported to a TIFF image and quantified using ImageJ. NF□B signalling was assessed by changes to the level of phospho-p65 (canonical pathway) and the cleavage of NF□B p100 to p52 (non-canonical pathway). ERK signalling was assessed by the conversion of ERK to phospho-ERK. The data are presented as ratios of the active:inactive forms of the respective proteins.

Chemical synthesis

Lenalidomide (Revlimid®) was purchased from Celgene Ltd. Thalidomide was prepared through literature procedures [23-26]. Compounds **2**, **3**, **4** and **6** were all prepared through the methods described previously [25, 26]. Full spectroscopic characterisation of these compounds are also described in these reports[25, 26]. Additionally ^{1}H NMR of 1-6 can be found in the Supplementary Data.

(*R,S*)-3-(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)propiolaldehyde (5): Dess-Martin periodinane (180 mg, 0.42 mmol, 1.2 eq.) was added to a stirred solution of C4-propargyl alcohol-thalidomide [25] (109 mg, 0.35 mmol, 1.0 eq.) in dichloromethane (4.5 mL) and stirred for 1 h. A sodium bicarbonate (saturated in water) /sodium thiosulfate (1 M in water) solution (1:1, 3 mL) was then added and stirred for a further 1 h. The resulting mixture was extracted with dichloromethane (3 x 20 mL) and ethyl acetate (2 x 10 mL). The combined organic layers were dried over $MgSO_4$, filtered and the solvents were removed under reduced pressure. The organic mixture was then absorbed onto silica and purified via flash column chromatography (ethyl acetate/hexane 1:1 → 1:0) to afford the title aldehyde product **5** as a yellow/orange solid (58 mg, 54%). Recrystallisation from ethyl actetate afforded a pale yellow powder. **m.p.** = 237-240°C. $\mathbf{R_f}$ = 0.29 (1:1 ethyl acetate/hexane). **^{1}H NMR** (600 MHz, d_6-DMSO): δ = 11.15 (s, 1H, NH), 9.53 (s, 1H, CHO), 8.12 (d, *J* = 7.5 Hz, 1H, 5-H/6-H), 8.10 (d, *J* = 7.5 Hz, 1H, 5-H/6-H), 7.98 (‘t’, *J* = 7.5 Hz, 1H, 6-H), 5.18 (dd, *J* = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 2.84-2.92 (m, 1H, 4’H/5’H), 2.52-2.66 (m, 2H, 4’-H_2/5’-H_2), 2.02-2.11 (m, 1H, 4’H/5’H). **^{13}C NMR** (100 MHz, d_6-DMSO) δ = 178.6 (CHO), 172.7 (C=O), 169.7 (C=O), 165.9 (C=O), 165.2 (C=O), 139.0 (Ar-C), 135.2 (Ar-CH), 132.3 (Ar-C), 131.9 (Ar-C), 125.7 (Ar-CH), 114.9 (Ar-C), 92.0 (C-3’’), 87.3 (C-2’’), 30.9 (C-5’), 49.1 (C-3’), 21.9 (C-4’). **IR** (neat) υ = 3076 (N-H), 2197 (C≡C), 1701 (C=O), 1655 (C=O) 1381, 1258, 1201, 1114, 987, 744, 567 cm^{-1}. **MS** (ESI) *m/z* (%) = 333.0 $[M+Na]^+$ (100), 282.2 (7), 210.0 (10). **$C_{16}H_{10}N_2O_5$** (310.26) calc.:333.0487 $[M+Na]^+$ found: 333.0488 (TOF-HRMS).

Results

1.1 Thalidomide and its derivatives exhibit a range of effects on LPCs

The structures of thalidomide, lenalidomide and six derivatives of thalidomide synthesised in-house designated **1** to **6**, are depicted in Fig 1. All were tested to ascertain their effects on growth of BMOL-NT and BMOL-T cells. Compounds **1**-**4** were chosen from a series of aminated thalidomide analogues, previously tested that inhibited TNF expression [18, 21, 23, 24]. In this regard these compounds were between 6-11 times more effective than thalidomide and 3-5 times more effective than lenalidomide. The proparyl aldehyde derivative **6** was identified in earlier studies as an inducer of cell death in Jurkat cell lines, while the derivative **5** showed similar effects in a later study [23]. At concentrations up to 100 µM, neither thalidomide nor lenalidomide affected the growth rate of BMOL-T cells. However, at 100 µM thalidomide and lenalidomide induced cell death of BMOL-NT cells. Aldehyde-alkyne containing compounds **5**, and **6** tested at concentrations up to 100 µM had no effect on either BMOL-NT or BMOL-T cells, unlike their ability to kill Jurkat cells [21]. Aniline thalidomide derivatives **3** and **4** inhibited the growth of both BMOL-NT and BMOL-T cells equally at a concentration of 100 µM. In contrast, analogues **1** and **2** were both effective growth inhibitors at lower concentrations ~ 10 µM and furthermore showed selectivity towards the tumorigenic cell line, BMOL-T. Since the aniline isopropyl derivative **1** showed greater selectivity it was chosen for further study. Henceforth it will be designated as thalidomide C1.

1.2 Thalidomide C1 displays selectivity towards tumorigenic LPCs

Thalidomide C1 was then tested at concentrations between 2 and 10 µM. At all concentrations tested it had no effect on the growth of BMOL-NT cells. However, at 8 and 10 µM thalidomide C1 significantly increased the doubling time of BMOL-T cells by 11% and 27% respectively (Fig 2).

2.1 Sorafenib substantially inhibits the growth of both BMOL-NT and BMOL-T cells

When tested on the BMOL cell lines at the effective concentrations for thalidomide C1 (8 and 10 µM), sorafenib showed substantial growth inhibitory effects that were greater than those of the thalidomide analogue (Table 1). At 8 and 10 µM, sorafenib increased the doubling time of BMOL-NT cells by 100% and 134% respectively and that of BMOL-T cells by 144% and 193% respectively. Thus sorafenib is more effective on the tumorigenic line (T) than the non-tumorigenic LPC line (NT).

2.2 Thalidomide C1, but not sorafenib, selectively inhibits growth of BMOL-T cells

A comparison of thalidomide C1 and sorafenib effects on growth of BMOL-NT and BMOL-T cells showed thalidomide C1 had no significant effect on BMOL-NT cells, whereas sorafenib efficiently increased the doubling time of BMOL-NT cells by 85, 85 and 132% (average – 101%) in three independent experiments (Table 2). In contrast, thalidomide C1 increased the doubling time of the tumorigenic BMOL-T cells by 39, 15 and 25% (average – 26%), while sorafenib was more effective at 272, 245 and 187% (average – 235%) in three experiments (Table 2). Therefore, although sorafenib is more effective, thalidomide C1 shows selectivity against the tumorigenic BMOL-T cells when tested at the same concentration (10 µM). Representative images of control (Fig. 3a and c) and sorafenib treated (Fig 3b and d) cultures captured by Cellavista for growth analyses show growth inhibition of 70%.

3a. Thalidomide C1 does not suppress mitosis of BMOL-NT cells in contrast to sorafenib

Thalidomide C1 did not significantly suppress mitosis of BMOL-NT cells; whereas sorafenib reduced the mitotic index by 26, 84 and 89 % in three independent experiments (average – 66%) (Table 3). In experiment 2, there was, in fact, a small but not significant increase in the mitotic index in response to thalidomide C**1**. Images captured by the Cellavista Instrument comparing control and treated cells show a reduction of 87% in the mitotic index (Fig 4a and b).

3b. Thalidomide C1 does not suppress mitosis of BMOL-T cells in contrast to sorafenib

Thalidomide C1 did not significantly suppress BMOL-T cell mitosis; in fact in experiment 2, it elicited a small (34%) but significant ($p<05$) increase. In contrast sorafenib reduced the mitotic index by 12, 83 and 91 % (average – 62%) (Table 3).

4a. Thalidomide C1 does not induce apoptosis of BMOL-NT cells in contrast to sorafenib

Thalidomide C1 did not consistently induce apoptosis of BMOL-NT cells; there is no effect in two experiments and 1.5-fold increase in another (Table 4). We have observed this variation with other batches of LPCs and it may be due to different growth status and/or condition of the thawed cells. However, the lack of an effect of thalidomide C1 dominates in multiple experiments. In contrast, sorafenib consistently increased the apoptotic index of BMOL-NT cells by 16, 18.1, and 2.5 fold respectively.

4b. Both thalidomide C1 and sorafenib induce apoptosis of BMOL-T cells

Thalidomide C1 significantly induced apoptosis of BMOL (T) cells by 4- and 1.6- fold in experiment 2 and 3 but not in experiment 1 in three independent experiments (Table 4). Sorafenib, on the other hand, consistently increased the apoptotic index by 17, 86 and 11.4 –fold (average – 38-fold). Images captured by the Cellavista Instrument comparing control and treated cells show an increase of 15.4-fold in the apoptotic index (Fig 4c and d).

5. Thalidomide C1 inhibits NFκB but not ERK signaling in BMOL-T cells.

Cleavage of the inactive (p100) to the active (p52) form of NFκB2 was significantly inhibited (30%, $p<0.05$) by thalidomide C1 (Fig 5b). In contrast phospho-p65 NFκB was consistently increased by exposure to thalidomide C1 (Fig 5c) but this was not significant ($p=0.0512$). ERK activity as assessed by examining the ratio of ERK to phospho-ERK was variable, increasing in two experiments and decreasing in the third (Fig 5d).

Discussion

The availability of tumorigenic LPC lines facilitates the screening of compounds that are potentially useful for treating liver cancer. This is a strategically sound approach as there is considerable evidence that at least some forms of HCC are derived from LPCs [9, 11, 12] and it is also possible that some LPCs may function as liver cancer stem cells [10]. An additional advantage afforded by using LPCs is that both tumorigenic and non-tumorigenic lines are available. In a previous study, we reported the utility of such LPC lines that displayed differential chemoresistance to cisplatin *in vitro* as well as in an orthotopic HCC model [19]. In this study we refined the model further by comparing non-tumorigenic BMOL-NT and tumorigenic BMOL-T derivatives of the same lineage to test the selectivity of putative anti-cancer agents. We investigated the effects of thalidomide and several derivatives to identify a compound that can selectively target tumorigenic LPCs relative to non-tumorigenic LPCs

We find that neither thalidomide, nor a commercially available derivative, lenalidomide, affect the growth of BMOL-T cells when tested at a dose level up to 100 µM. In contrast, both kill BMOL-NT cells. This observation is consistent with previous studies that show cell survival pathways are highly upregulated in HCC [27] and in our tumorigenic LPCs [19]. This prompted our attempts to modify thalidomide and then apply our screen, based on its effect on cell growth to identify a derivative that is selectively active against tumorigenic LPCs.

This approach has led to the identification of an isopropyl aniline derivative of thalidomide, compound C1. Our results show that thalidomide C1 is superior to lenalidomide, for it is effective at lower doses and selective against tumorigenic cells; although we note a marginal, but not significant effect on non-tumorigenic cells is obtained in three separate experiments. We then compared this derivative with sorafenib, an FDA approved therapeutic for HCC. The data shows that Sorafenib is highly effective at the same concentration, in fact more effective than thalidomide C1 or all of the thalidomide derivatives tested. However it should be noted that sorafenib significantly affects both tumorigenic and non-tumorigenic LPCs.

We then focussed on the mechanism of the growth-inhibiting effects of thalidomide C1 and sorafenib using our model. Specifically, we evaluated their effects on mitosis and apoptotic cell death. This

evaluation is important in the context of therapy for it is reasonable to assume that simply arresting cell division compared to cell killing is unlikely to be an effective way to treat cancer. In this regard we found that thalidomide C1 elicited its effect essentially by inducing apoptosis. In contrast, sorafenib exerted its effect both by inhibiting mitosis and inducing apoptosis. This suggests that thalidomide C1 may have fewer side effects as a therapeutic agent than sorafenib. We investigated the signalling pathways that thalidomide may affect and found that it significantly and consistently down-regulated the non-canonical NFκB pathway. In conclusion, this study highlights the value of having non-tumorigenic and tumorigenic LPC lines to test putative live cancer drugs, especially to ascertain the basis of their growth inhibitory property. This approach has identified thalidomide C1 as having promise in treating HCC and potential in combined therapy approaches, while accepting that precautions have to be in place to avoid adverse outcomes from potential teratogenic properties associated with this class of compounds.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Treatment	BMOL-NT		BMOL-T	
	Doubling time (h)	% increase	Doubling time (h)	% increase
0 µM	29	-	27	-
8 µM	58	100**	66	144**
10 µM	68	134**	79	193***

Table 1. Sorafenib substantially inhibits the growth of BMOL-NT and BMOL-T cells.

*Growth rate is presented as the mean of the doubling time in hours. ** indicates a p-value of <0.05 and *** <0.005 respectively.*

BMOL-NT

	Control			Thalidomide C1			Sorafenib		
Exp	1	2	3	1	2	3	1	2	3
Growth	**26**	**21**	**31**	**28**	**21**	**33**	**48**	**48**	**72**
SEM	0.57	0.62	2.32	0.61	1.16	1.76	2.62	4.98	0.00
n	6	8	3	6	8	3	5	8	1
p	-	-	-	NS	NS	NS	***	***	***
Change over control	-	-	-	-	-	-	85%	85%	132%

BMOL-T

	Control			Thalidomide C1			Sorafenib		
Exp	1	2	3	1	2	3	1	2	3
Growth	**18**	**20**	**24**	**25**	**23**	**30**	**67**	**69**	**69**
SEM	0.15	0.44	0.5	0.8	0.74	1.57	3.38	1.76	0
n	6	8	3	6	8	3	6	8	1
p	-	-	-	***	***	***	***	***	***
Change over control	-	-	-	39%	15%	25%	272%	245%	187%

Table 2. Sorafenib and not thalidomide C1 affects the growth of BMOL-NT and both agents affect the growth of BMOL-T cells

*Data for three different cell lots are presented as experiments 1, 2 and 3. Sorafenib and thalidomide C1 are tested at a concentration of 10 µM. Growth rate is presented as the mean of the doubling time in hours. NS = not significant and *** p-value <0.001.*

BMOL-NT

	Control			Thalidomide C1			Sorafenib		
Exp	1	2	3	1	2	3	1	2	3
Mitosis	**3.21**	**3.09**	**3.62**	**3.25**	**3.56**	**3.69**	**0.34**	**0.50**	**0.38**
SEM	0.13	0.26	0.08	0.06	0.39	0.07	0.03	0.06	0.04
n	6	6	6	6	6	6	4	6	6
p	-	-	-	NS	NS	NS	***	***	***
Change over control	-	-	-	-	-	-	-26%	-84%	-89%

BMOL-T

	Control			Thalidomide C1			Sorafenib		
Exp	1	2	3	1	2	3	1	2	3
Mitosis	**3.14**	**3.13**	**4.54**	**3.33**	**4.19**	**4.22**	**0.17**	**0.52**	**0.43**
SEM	0.20	0.22	0.13	0.18	0.16	0.11	0.10	0.10	0.15
n	5	6	6	5	6	6	4	6	6
p	-	-	-	NS	**	NS	***	***	***
Change over control	-	-	-	-	34%	-	-95%	-83%	-91%

Table 3. Sorafenib reduces the mitotic index of both BMOL-NT and BMOL-T cells while thalidomide C1 is not consistently effective on either BMOL-NT or BMOL-T cells.

*Data for three different cell lots are presented as experiments 1, 2 and 3. Sorafenib and thalidomide C1 are tested at a concentration of 10 µM. Mitotic cells were identified by phosphohistone H3 staining and visualized using the Cellavista instrument. The mitotic index is presented as the mean of percentage positive cells. NS = not significant, ** p-value <0.01 and ***<0.001.*

BMOL-NT

	Control			Thalidomide C1			Sorafenib		
Exp	1	2	3	1	2	3	1	2	3
Apoptosis	**0.60**	**1.13**	**1.65**	**0.46**	**3.67**	**4.00**	**9.59**	**20.53**	**4.17**
SEM	0.06	0.31	0.25	0.12	1.00	0.34	1.84	3.36	0.43
n	4	4	4	4	4	4	4	4	4
p	-	-	-	NS	NS	**	***	**	**
Change over control	-	-	-	-	-	+1.5-fold	+16-fold	+18.1-fold	+2.5-fold

BMOL-T

	Control			Thalidomide C1			Sorafenib		
Exp	1	2	3	1	2	3	1	2	3
Apoptosis	**0.79**	**0.51**	**1.35**	**0.78**	**2.03**	**2.20**	**13.05**	**44.08**	**15.43**
SEM	0.17	0.08	0.21	0.10	0.18	0.17	1.60	6.20	1.13
n	4	4	4	4	4	4	4	4	4
p	-	-	-	NS	***	*	***	***	***
Change over control	-	-	-	-	+4-fold	+1.6-fold	+17-fold	+86-fold	+11.4-fold

Table 4. Sorafenib consistently induces apoptosis of BMOL-NT and BMOL-T cells whereas thalidomide C1 induces apoptosis of BMOL-T cells.

*Data for three different cell lots are presented as experiments 1, 2 and 3. Sorafenib and thalidomide C1 are tested at a concentration of 10 µM. Apoptotic cells were identified by staining fragmented DNA visualised using the Cellavista instrument. The apoptotic index is presented as the mean of the percentage cells with fragmented DNA. NS = not significant, * p-value <0.05, **<0.01 and ***<0.001.*

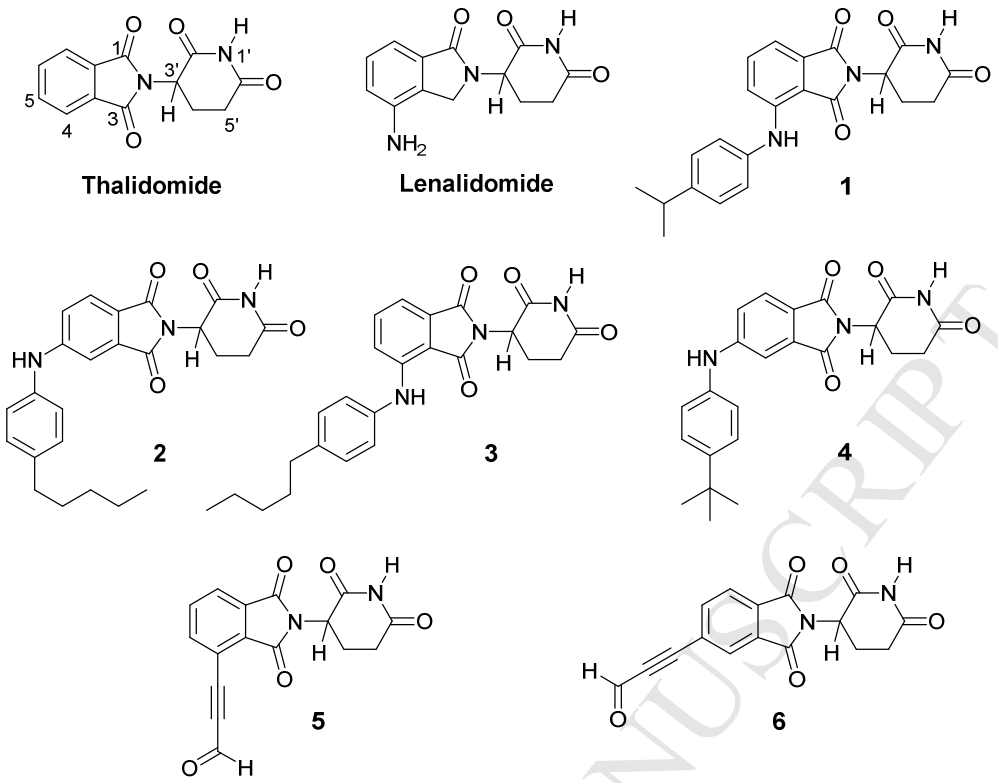

Fig. 1. *The chemical structures of thalidomide, Revlamid and prepared C4 and C5 substituted derivatives of thalidomide. C4 and C5 substituted derivatives of thalidomide are designated 1-6. Compound 1 from here on is named as thalidomide C1*

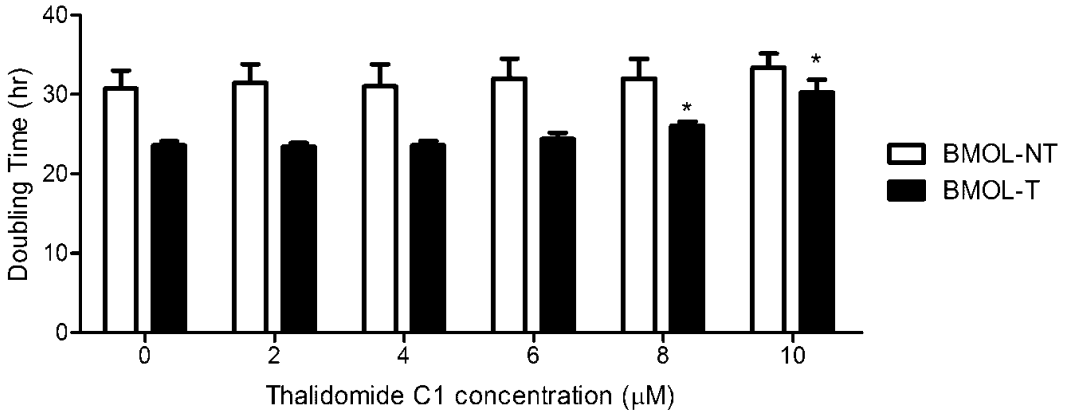

Fig. 2. *Differential effect of thalidomide C1 on BMOL-T cells. BMOL-NT cells maintained a doubling time of approximately 31 hr when cultured with 0-10μM of thalidomide C1. BMOL-T cells had doubling times of approximately 23 hr when cultured with 0-6μM thalidomide C1, but increased to 26 hr with 8μM thalidomide C1 and 30 hr with 10μM of thalidomide C1. * indicates a p-value of <0.05*

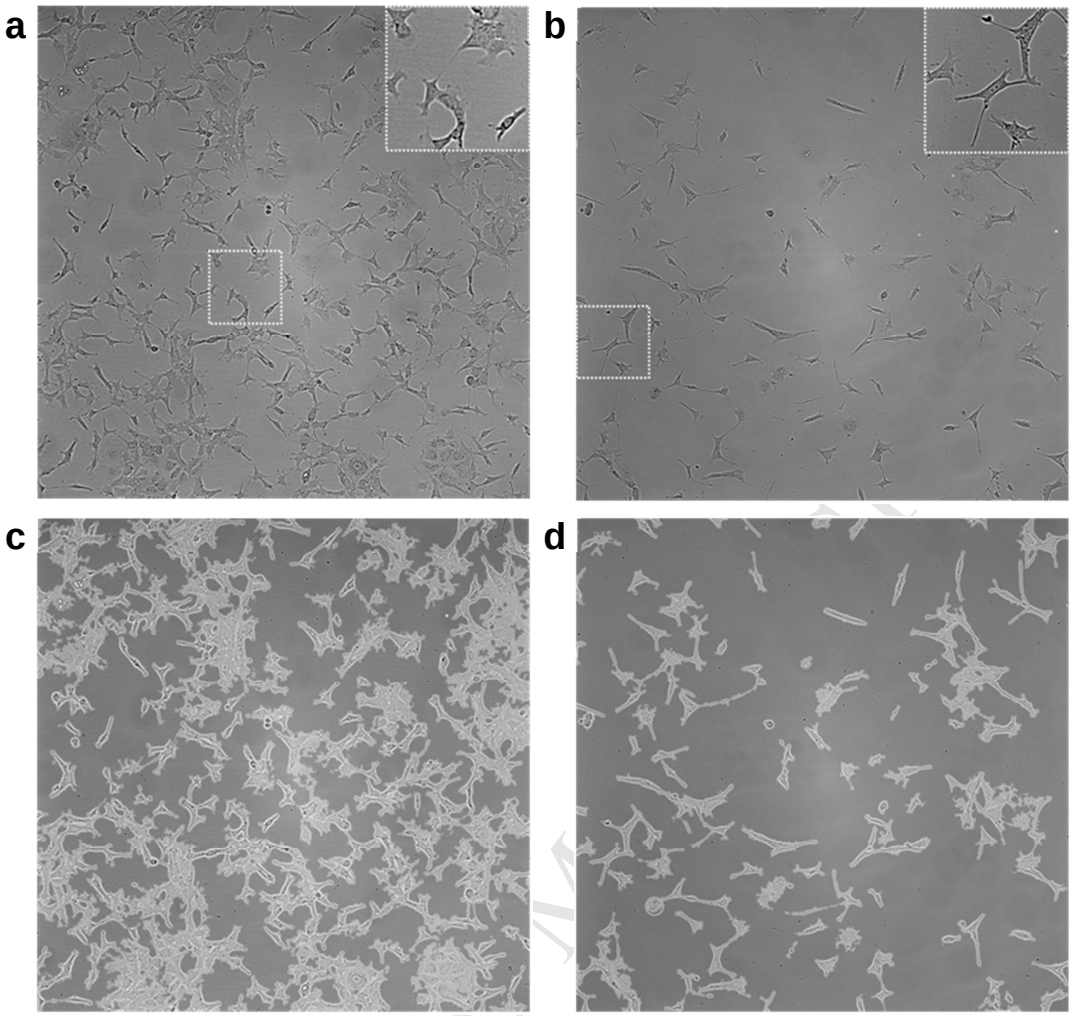

Fig. 3. *Cell detection and area calculation by the Cellavista instrument. Representative images of control BMOL-NT (a) and sorafenib treated BMOL-NT (b) cells imaged at 2 days with a 10x objective on the Cellavista instrument. Cellavista highlights areas (white) where it detects cells (c, d). Cell confluency is determined by the area of the well covered by cells and it is 44.9% for Control BMOL-NT cells (c) and 13.2% for sorafenib treated BMOL-NT cells (d) giving a growth inhibition of 70% at 2 days*

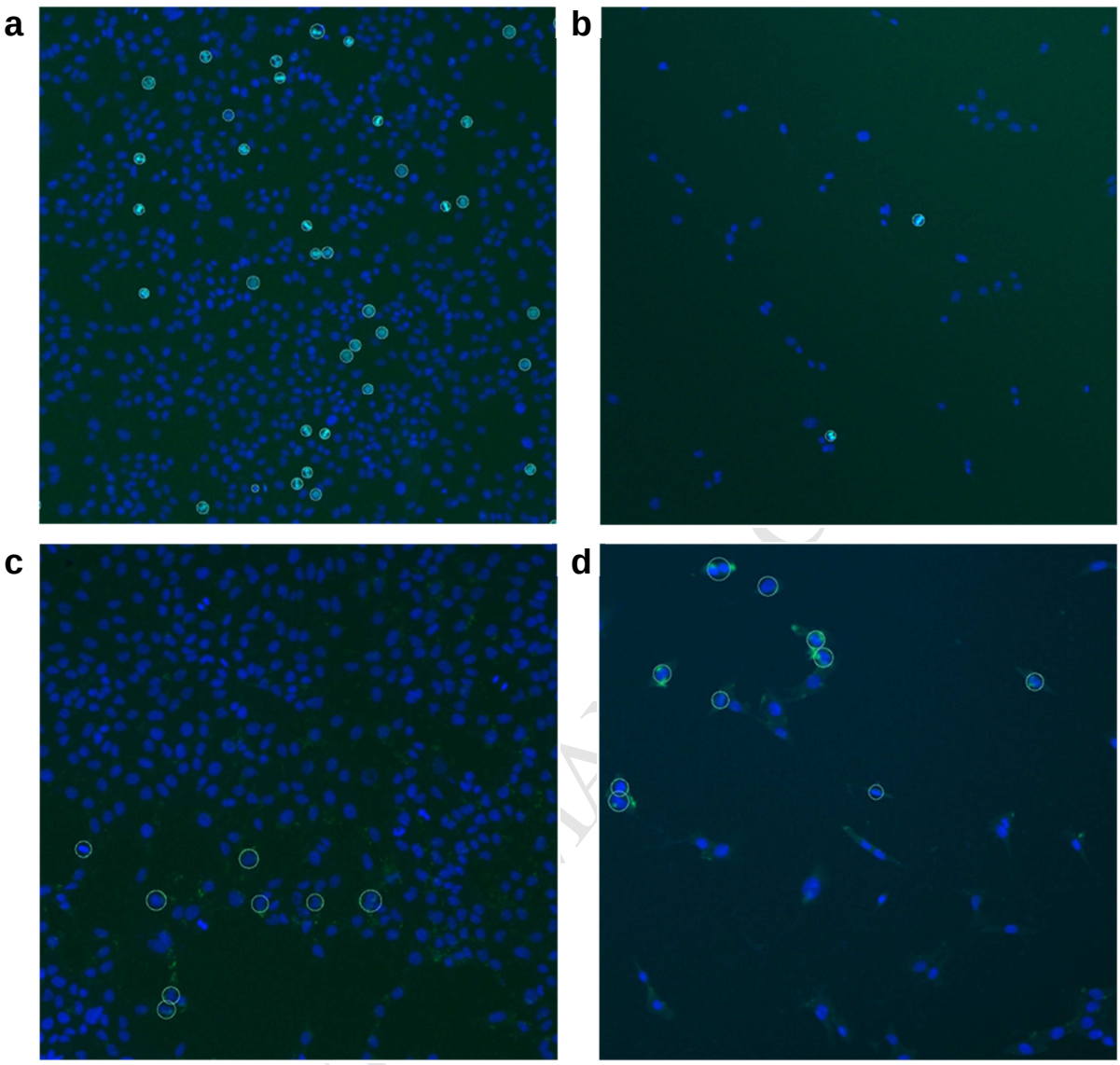

Fig. 4. *Mitotic and Apoptotic BMOL-NT cells visualised with the Cellavista instrument are decreased and increased respectively as a result of sorafenib treatment. Representative images of mitotic (a, b) and apoptotic (c, d) BMOL-NT cells (both green fluorescence) identified by the Cellavista instrument and marked by circles. Cell nuclei are counterstained with Hoechst (blue). Images were captured using the 10x objective. Control BMOL-NT cultures contained 3.79% mitotic cells (a) while sorafenib treated BMOL-NT cultures contained 0.48% mitotic cells (b) giving a mitotic index reduction of 87% as a result of sorafenib treatment after 3 days culture. Control BMOL-NT cells (c) contained 0.91% apoptotic cells while sorafenib treated BMOL-NT cells (d) contained 14% apoptotic cells indicating a 15.4-fold increase of apoptotic cells after 3 days of culture*

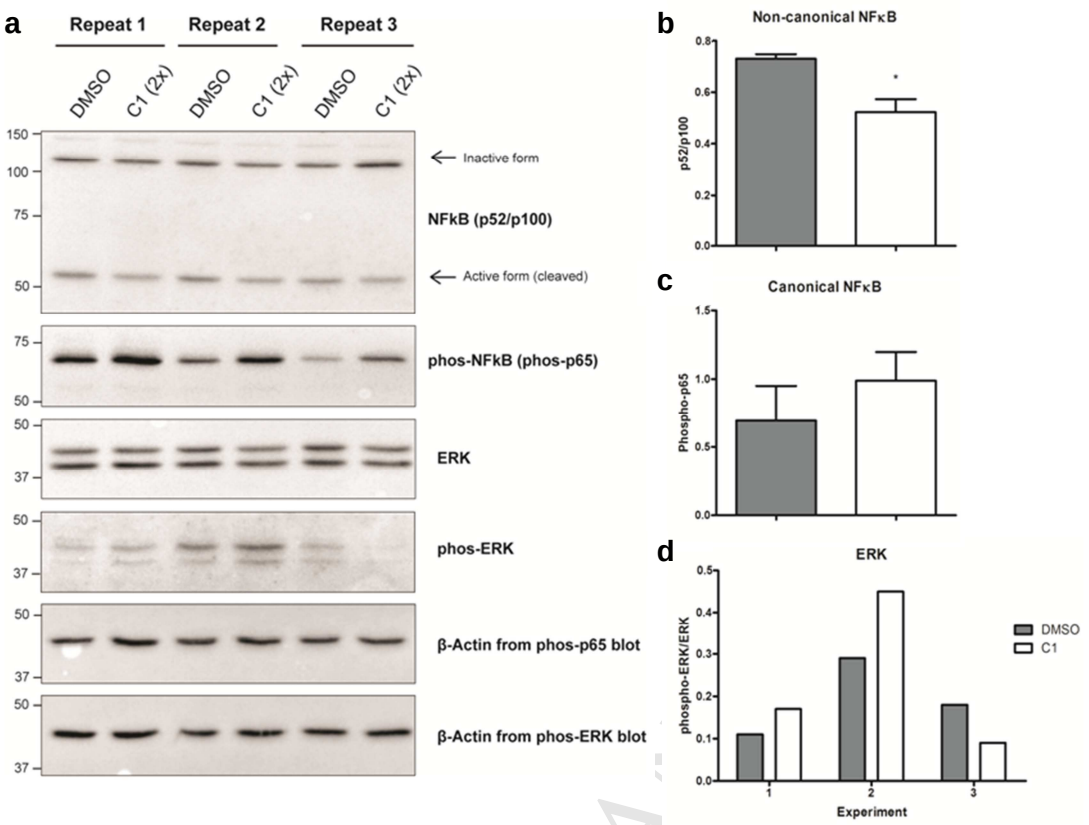

Fig. 5. Thalidomide C1 inhibits non-canonical NFκB signaling in BMOL-T cells. Expression of NFκ□ and ERK was assessed relative to β-actin by their chemiluminescence signal on a Western Blot (a). The ratio of the active (p52) form of NFκB to the inactive (p100) was significantly reduced (30%) by thalidomide C1 (b). In contrast phospho p65 was consistently but not significantly increased by exposure to thalidomide C1 (c). The ratio of phospho ERK to ERK was variable following treatment with thalidomide C1 (d). * indicates p-value<0.05 compared to the control

- Thalidomide C1 selectively inhibits growth of tumorigenic liver progenitor cells
- Growth inhibition was achieve through the induction of apoptosis in treated cells
- Thalidomide C1 treatment did not consistently alter ERK signalling
- However non-canonical NFκB signalling consistently decreased following treatment