Combination of cannabidiol with low-dose naltrexone increases the anticancer action of chemotherapy *in vitro* and *in vivo*

WAI M. LIU[1], NADINE K. HALL[1], HARRY S.Y. LIU[1], FRANCIS L. HOOD[2] and ANGUS G. DALGLEISH[1]

[1]Institute for Infection and Immunity, St George's University of London, London SW17 0RE;
[2]LDN Pharma Ltd., London EC2R 6AY, UK

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Abstract. We previously reported that both cannabidiol (CBD) and low-dose naltrexone (LDN) exhibit complex effects on G-protein coupled receptors, which can impact the expression and function of other members of this superfamily. These receptors feed into and interact with central signalling cascades that determine the ease by which cells engage in apoptosis, and can be used as a way to prime cancer cells to other treatments. The present study was designed to investigate the effect of combining these two agents on cancer cell lines *in vitro* and in a mouse model, and focused on how the sequence of administration may affect the overall action. The results showed both agents had minimal effect on cell numbers when used simultaneously; however, the combination of LDN and CBD, delivered in this specific sequence, significantly reduced the number of cells, and was superior to the regimen where the order of the agents was reversed. For example, there was a 35% reduction in cell numbers when using LDN before CBD compared to a 22% reduction when using CBD before LDN. The two agents also sensitised cells to chemotherapy as significant decreases in cell viability were observed when they were used before chemotherapy. In mouse models, the use of both agents enhanced the effect of gemcitabine, and crucially, their use resulted in no significant toxicity in the mice, which actually gained more weight compared to those without this pre-treatment (+6.5 vs. 0%). Overall, the results highlight the importance of drug sequence when using these drugs. There is also a need to translate these observations into standard chemotherapy regimens, especially for common tumour types where treatment is often not completed due to toxicities.

Correspondence to: Dr Wai M. Liu, Institute for Infection and Immunity, St George's University of London, 2nd Floor, Jenner Wing, Cranmer Terrace, London SW17 0RE, UK
E-mail: wliu@sgul.ac.uk

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Introduction

Naltrexone is an opiate receptor antagonist preventing opiate stimulation; it was licensed in 1984 as a treatment for addiction to opiates as it prevented the euphoria induced by recreational use of morphine and heroin. It was observed, however, that naltrexone action also had an immune-modulatory element, which was therapeutically beneficial to certain patients (1). This effect appeared to be observed only at doses much lower than given to treat addiction, and its profile of action was distinct from that seen with the higher dose (2). Since then, naltrexone used at this lower dose was informally referred to as low-dose naltrexone (LDN), to distinguish it from naltrexone used at the conventional higher dose.

Mechanistically, naltrexone interferes physically with the interaction between opiate and the receptor, and repeated and chronic stimulation/blockade by naltrexone could lead to unintentional changes in the expression and distribution of these receptors as well as others. Specifically, complex and varied dimerisation of the receptor with other G-protein coupled receptors (GPCR) means that its blockade can often impact the distribution and functioning of other members of the receptor superfamily (3). Ultimately, binding to these receptors can feed into and affect the action of central signalling pathways such as the PI3 kinase and MAPK pathways, which together influence cell fate (4). There are many drugs that exert a therapeutic effect through these pathways; this is not a surprise considering conservative estimates put the value of cancers involving or driven by malfunctions in these cascades at 90% (5).

Another drug that our laboratory has actively researched is cannabidiol (CBD), which is a cannabinoid commonly extracted from the cannabis plant. Surprisingly, it shares many features with LDN. It possesses biological activity and has recently been approved for use by the EMA and FDA as a treatment for a rare type of childhood epilepsy (6). Evidence from the earlier 1970s suggests that, in general, phytocannabinoids possess anticancer activity, and it took until the mid-2000s before CBD was specifically mentioned as having anticancer action (7). The principal mechanism of action for CBD is slightly unclear, but it was first thought to involve binding of the canonical cannabinoid receptors; however, binding studies have shown its affinity for the receptor was low (8). A number of possible receptors were suggested as

being important in determining the function, with agonism and antagonism of related GPCRs being mooted as central to the MOA (9). Modulation of these receptors gives CBD the ability to interact with intracellular signalling cascades, and as with LDN, tapping into these allows CBD to fundamentally manipulate key processes such as cell growth and survival (7).

The effects that LDN and CBD have on these signalling pathways in cancer cells often does not lead to an increase in cytotoxicity. Instead, cell proliferation is reduced resulting in smaller numbers of cells. Another effect that changes to these pathways, particularly MAPK, can produce are alterations to the proteins that regulate and determine apoptosis (10). This effect is important as this can lead to a way that LDN and CBD can influence further cell fate. Apoptosis is a process that is strictly regulated by the BCL-2 family of proteins. The balance of these members, which are made up of those that can either oppose or promote apoptosis, is crucial in determining whether a cell commits to apoptosis (11). These proteins therefore act as an apoptosis switch, and when correctly engaged, apoptosis can proceed fully when the cytotoxic signal/stimulus is received (12).

Some cancer cells have aberrant signalling within the BCL-2 family, that results in disruption to apoptotic capability; cancer cells may overexpress the family members that oppose apoptosis, making it more difficult for the cell to initiate apoptosis. The inverse scenario can also exist, namely when the proteins that support apoptosis are mutated or absent (13). Overall, this means that cancer cells are unable to activate apoptosis even though there is a 'kill' signal, and are effectively resistant to certain treatments. Approaches, such as BCL-2 inhibitors and/or mimetics have been developed to correct the errors in the expression levels of these proteins, and in doing so, restore the ability to undergo cell death (14). It is by altering the balance of these apoptosis proteins that LDN and CBD are able to restore a level of cell killing in cancer cells (2,15).

In the present study, a part of our ongoing investigations into the anticancer actions of LDN and CBD is described. Specifically, combination studies were performed to understand how these two related agents can be combined in a way to prime cancer cells to conventional chemotherapy. The best combinations identified by *in vitro* experiments were then forwarded on to further examinations in a murine model.

Materials and methods

Cell culture and drugs. All cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK), and maintained and grown in the culture medium specified by the depositor. For A549 (human lung cancer) and HCT116 (human colorectal cancer) cells this was DMEM (Sigma-Aldrich; Merck KGaA), while for MCF7 (human breast cancer) cells the medium was RPMI-1640 (Sigma-Aldrich; Merck KGaA). Media were supplemented with 5% foetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine (Thermo Fisher Scientific, Inc.). All cells were grown in a humidified atmosphere with 5% CO_2 in air at 37°C, and discarded after 8 weeks. Authentication of the cell lines was performed by the service providers using the AmpFISTR Identifier Plus PCR amplification kit looking for the presence of <10 known loci for each cell line.

Naltrexonehydrochloride(naltrexone;Sigma-Aldrich;Merck KGaA), cannabidiol (CBD; THC Pharm GmbH, Germany); gemcitabine (GEM; Sigma-Aldrich; Merck KGaA) and oxaliplatin (OXP; Sigma-Aldrich; Merck KGaA) were dissolved in DMSO, with the final DMSO concentration in individual tests being <0.05%.

Viability assays. Our previous research has shown that the effects of naltrexone are highly dependent on dose; at lower concentrations <50 nM, the cellular and molecular effects are mainly anticancer in nature, while these are lost at conventionally higher doses that are typically >10 μM (2). To study the effects of each agent on cell growth, exponentially growing cells were seeded onto 6-well plates at a density of $1x10^5$ cells per well and left to adhere overnight. Cells were then treated with the agents. Naltrexone was used at 10 μM (designated NTX) and at a lower 10 nM concentration. This lower concentration was the C_{max} as determined by pharmacokinetic studies in volunteers administered approximately 4.5 mg of naltrexone, which is the low dose of naltrexone (LDN) that is used clinically. CBD was used at 1 μM, which is a concentration seen in the sera of patients given 400 mg i.v. (intravenously). GEM and OXP were used at ~IC_{20} as established in a previous study (16). Cell number and viability were assessed after 48 h by using trypan blue staining to discriminate live from dead cells. Aliquots were also harvested for flow cytometric analysis of the cell cycle using propidium iodide (PI) and RNAse staining as western blot analysis, both as previously described (16).

Combination studies. The effect of combining the drugs was assessed by culturing the cells according to a regimen that was made up of two phases of treatment, with each lasting 48 h. The total duration was therefore 96 h. In between the phases, exhausted medium containing any drugs was removed, and the cells were gently washed and replenished with fresh culture medium containing the next phase of treatment. Methodologically, cells were seeded onto 6-well plates at a density of $1x10^5$ cells per well and left to adhere overnight. After this time, the drugs were added to the cells as part of the first phase of the treatment at concentrations detailed in the 'Viability assays' section, and left for 48 h. The second phase of treatment followed directly after the exhausted medium was removed from the cells, and fresh medium added containing the appropriate concentration of the test drug. Cells were left for a further 48 h before the cell number and viability were assessed by cell counting and processing for western blot analysis.

Immunoblot analysis. Following individual treatments, the cells were then harvested by scraping into lysis buffer (New England Biolabs, UK), and standard western blot protocols were followed as described previously (2). Primary probing was with specific antibodies generated against phosphorylated (p)AKT (cat#: 9271S), AKT (cat#: 9272S), pERK (cat#: 9101S), ERK (cat#: 9102S), cannabinoid receptor (CBR) 1 (cat#: 93851S) and 2 (cat#: PA1-744) (all New England Biolabs), used at a dilution of 1:1,000. GAPDH (1:5,000; New England Biolabs; cat#: 2118S) was the loading control, and secondary

probing was performed using the species-appropriate HRP conjugated antibody (New England Biolabs; cat#: 7074S) at a dilution of 1:1,000. Bands were visualised using the SuperSignal chemiluminescent detection system (Thermo Fisher Scientific, Inc., UK). Densitometry of band intensity was determined using Adobe Photoshop CS3, v10.0 (Maidenhead, UK), and normalised to GAPDH.

In vivo tumour model. The present study assessed the effect that differing treatment-schedules had on the growth of HCT116 cells in athymic nu/nu BALB/c mice. A total of 60 female mice (Charles River Laboratories, Harlow, UK), aged 6-8 weeks, were separated into groups each containing at least 4-mice, and treatments began after one week of acclimatisation. The average starting weight for each animal was ~17.2 g. The schedules involved two phases of treatment that each lasted one week. Exponentially growing HCT116 cells, with viabilities >90% were resuspended in phosphate-buffered saline (PBS) at a concentration of $5x10^7$ cells/ml. Tumour suspension (100 μl) was injected subcutaneously in the dorso-lateral flanks of the mice and allowed to grow until the masses were palpable. This was often after about 7 days, and for ease of recording, this day was designated day 1. Drugs were then administered on days 1 and 4 for the first phase of treatment, and on days 8 and 11 for the second phase. The drugs used were LDN (1.2 μg/mouse), CBD (35 μg/mouse) or GEM (9 μg/mouse), and administered intraperitoneally (100 μl). Mice were housed under standard conditions appropriate for nu/nu mice, in rooms with filtered air. Tumour growth was checked daily, and final tumour volume was determined on day 14 by taking measurements of the tumour in two dimensions [width (W) and length (L)], and using the equation $V=0.5 \times W \times L^2$. Mice were sacrificed on day 14 by using a schedule 1 method according to the UK Home Office and involved cervical dislocation and confirmation by snipping the femoral artery. Tumours were excised for further analysis by western blotting.

Statistical analyses. All statistical analyses were carried out using Graph-Pad Prism 7 (GraphPad Software, Inc.) or Microsoft Excel (v1808). Datasets were tested for normality by the Shapiro-Wilk test, and differences between variable and control groups were determined by the appropriate one-way ANOVA with multiple comparisons at a level to $P<0.01$. Paired tests were then performed to further determine any differences following Bonferroni's testing. Unless where otherwise stated, data where a statistical difference was noted are indicated in the text and/or in figures. All sets consisted of data from at least three separate experiments, and data are presented as the mean and SD.

Results

CBD, LDN and NTX have minimal effects on cell growth and viability. Our earlier studies showed that both LDN and CBD had minimal effects on cell viability (2,17), and the results of the present study recapitulated this. The two agents, however, were capable of significantly altering the expression of intracellular signalling proteins such as AKT and ERK, which underpinned key cell functions such as cell survival and apoptosis. We also showed that affecting these proteins caused a 'priming' effect, rendering cancer cells more susceptible to the cytotoxic effects of certain chemotherapy drugs. A principle component of the present work was to understand the effects of using LDN and CBD together on intracellular signalling in cancer cells, and whether or not they could exert similar priming effects seen when used individually. We therefore combined LDN or naltrexone at a more conventional concentration (10 μM; designated NTX) with CBD, and assessed the effect it may have on cancer cells.

The results showed that single-agent CBD, LDN and NTX had no significant effect on cell viability (Fig. 1A-b, d and f). NTX alone also had no effect on cell numbers; however, CBD alone resulted in small but significant reductions in cell number when compared to the untreated (UN) cells ($P<0.01$) (Fig. 1A-a, c and e). Similarly, LDN alone also significantly reduced cell numbers, but only in HCT116 and MCF7 cells ($P<0.01$) (Fig. 1A-c and e). Combining either LDN or NTX with CBD generally had no significant effect on cell parameters (Fig. 1A). Although in A549 cultures, there appeared to be fewer cells in the combination groups; comparing cell numbers actually observed in these groups against predicted numbers showed no significant differences (Fig. 1A-a). For example, the number of cells predicted to have remained after LDN and CBD were administered together was $47\pm8.3x10^4$ cells, which was not significantly different to $51\pm2.1x10^4$ cells that was actually observed ($P=0.508$). Thus overall, the effect of combining LDN with CBD did not affect the number of cells predicted to remain after the combination treatment. Parenthetically, predicted cell numbers were calculated by subtracting the number of cells reduced by individual treatments with LDN and CBD alone from the number of cells seen in untreated control cultures. There were no significant changes in the distribution of the cells in the different phases of the cell cycle (Fig. 1B).

The sequence of drugs influences overall activity. Guided by our previous studies that showed that the actions of LDN and CBD were influenced by the sequence of administration, we next examined the effect using one drug before the other may have on cell number and viability. We did not have a positive control in this section as LDN and CBD alone have little cytotoxic activity on its own. This is an important consideration when assessing the current dataset. This was tested by comparing cell numbers and viabilities after treatment schedules where one drug was used before the other and vice versa. ANOVA showed statistical differences within some of the groups ($P<0.01$), which were then analysed further using paired t-tests. The results showed that the sequential administration of LDN and CBD irrespective of order (LDN-CBD or CBD-LDN), appeared to be more active than a treatment-regimen involving the concomitant administration of the drugs (CBN + LDN) (Fig. 2A). More importantly, cell number and viability were generally reduced more when LDN was used before CBD (LDN-CBD) compared to the reverse treatment order (Fig. 2A). In some instances, the differences were significant; for example, in HCT116 cells, the cell number was $40\pm3.5x10^4$ after treatment with the sequence CBD-LDN vs. $33\pm2.1x10^4$ using the LDN-CBD sequence ($P=0.014$).

Cell number and viability are modulated in part by intracellular signalling pathways. Thus, whether the two sequences of CBD and LDN exerted differing effects on AKT and ERK was ascertained. These parameters were then compared to untreated

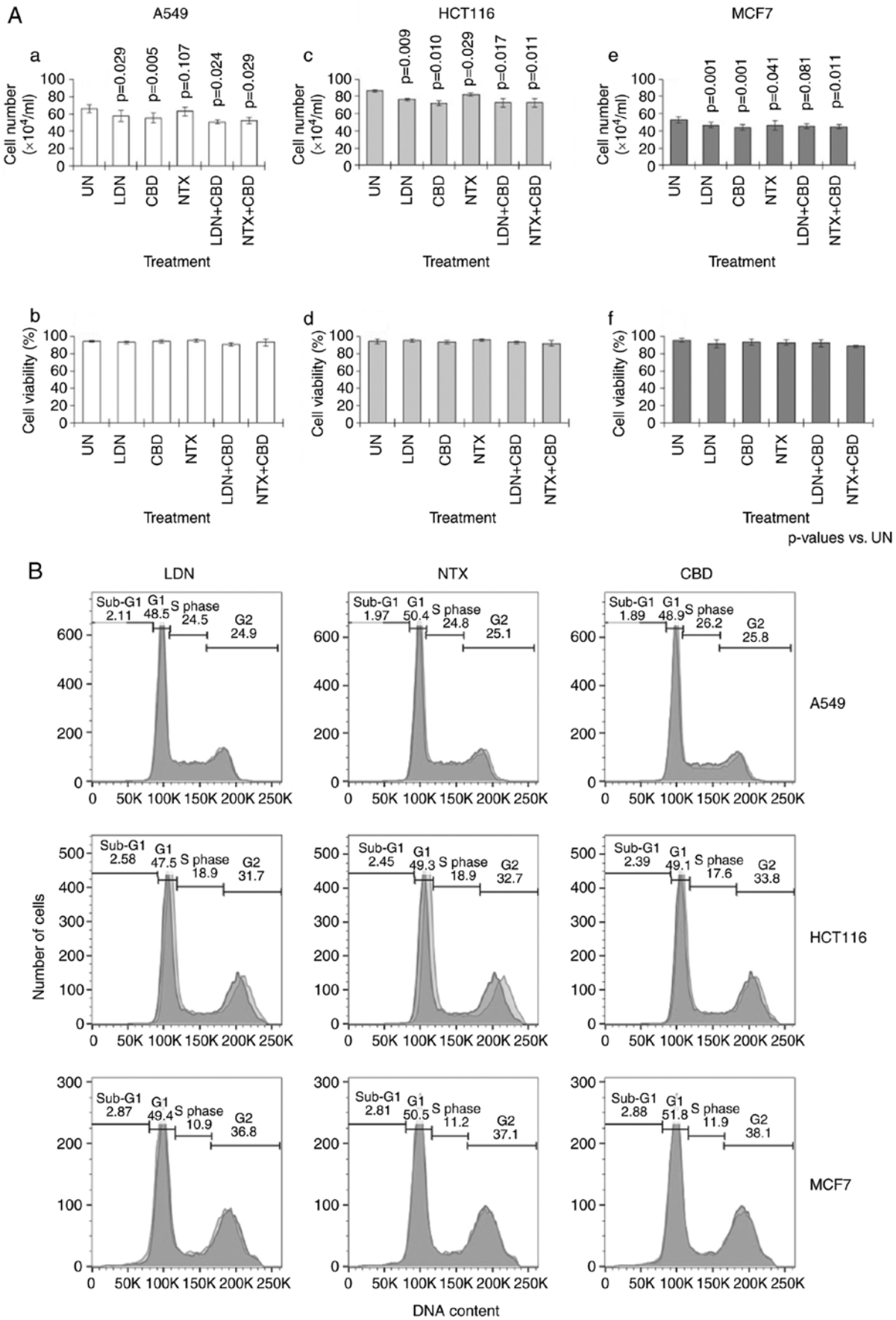

Figure 1. Effect of CBD and LDN on cell growth parameters. The A549, HCT116 and MCF-7 cell lines were cultured with the agents alone or in combination with each other for 2 days before assessing (A) cell number (a, c and e) and viability (b, d and f) and (B) cell cycle distribution. Naltrexone was used at two different concentrations: 10 nM (LDN) or 10 μM (NTX). CBD was used at 1 μM. Data points represent the mean and SD of at least three separate experiments, and P-values are from paired tests following ANOVA, comparing groups against the untreated one. Representative cell cycle histograms are presented. UN, untreated; CBN, cannabidiol.

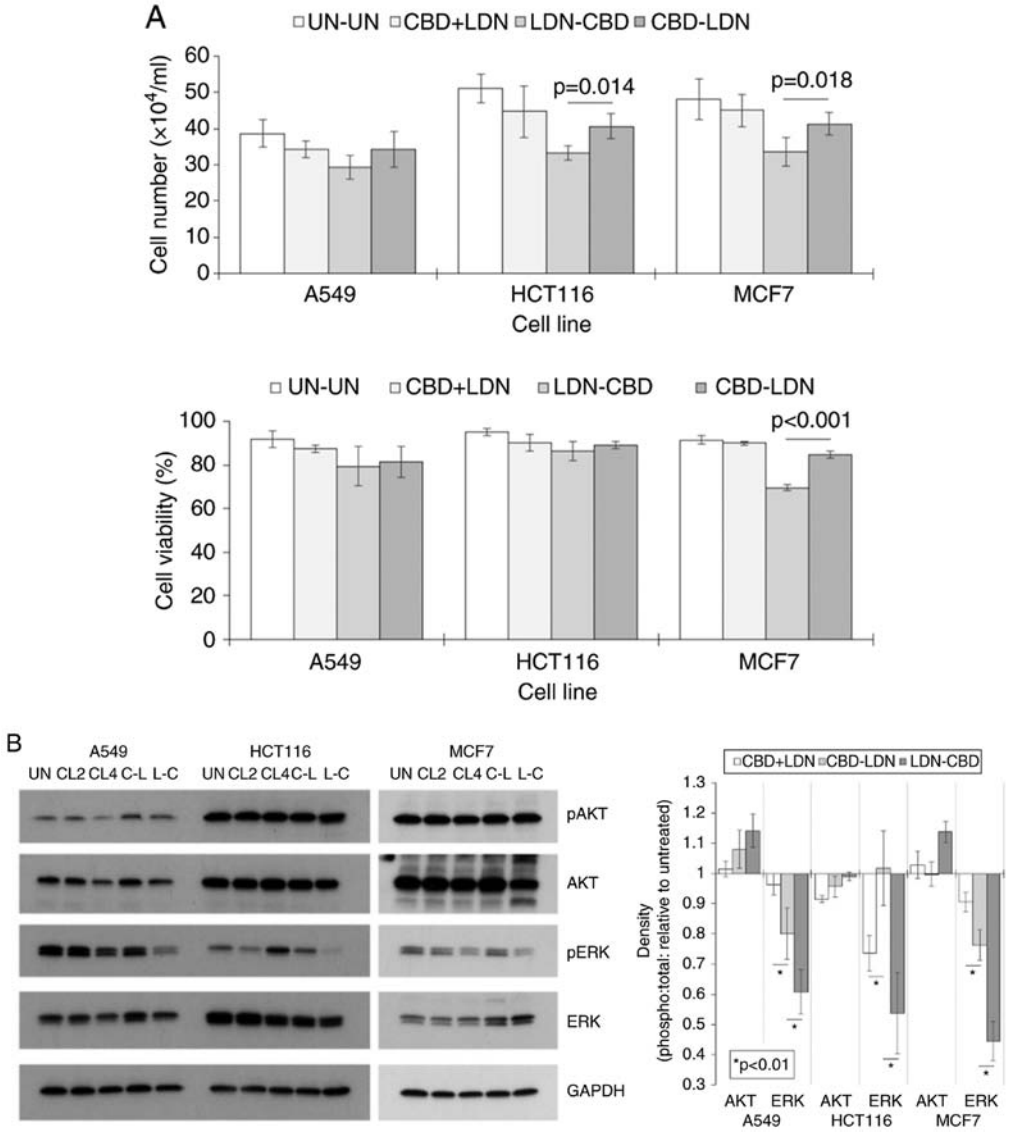

Figure 2. Effect of combining CBD with LDN. A549, HCT116 and MCF-7 cells were cultured with LDN and CBD in three different ways: i) concomitantly for 2 days (CBD + LDN); ii) LDN for 2 days followed by CBD for 2 days (LDN-CBD) iii) CBD for 2 days followed by LDN for 2 days (CBD-LDN). (A) Cell number and percentage of viability were assessed. (B) The effect of treatments on the expression levels of AKT and ERK were assessed by western blotting, with the data analysed and presented as the ratio of phosphorylated to total protein levels. In addition to the concomitant 2-day treatment of CBD with LDN (CL2), the effect of a concomitant treatment lasting 4 days (CL4) was also assessed in this way. *P<0.01. Data columns represent the mean and SD of at least three separate experiments, and representative blots are presented. UN, untreated; CBN, cannabidiol; LDN, low-dose naltrexone.

(UN) controls and to cells treated with CBD and LDN simultaneously for either 2 or 4 days (CL2 or CL4). The results showed that pAKT levels were generally unchanged in cells treated with LDN and CBD in any of the treatment schedules, and that also the sequence by which the drugs were applied had no bearing on this (Fig. 2B). Conversely, pERK levels were generally reduced following treatments. Crucially, the reduction in expression was greater when the sequence LDN followed by CBD was used (P<0.01). For example, in HCT116 cells, the ratio of the densities of the pERK:tERK bands relative to untreated bands were +2% after treatment with CBD-LDN vs. -46% after treatment with LDN-CBD (Fig. 2B).

CBD and LDN can sensitise cells to the effects of chemotherapy. ERK signalling regulates cell proliferation and survival, so consequently, drugs that alter this may sensitise cancer cells to the cytotoxic effects of chemotherapy drugs. Considering the effect that LDN and CBD combinations had on pERK expression, we explored the possibility that pre-treating cancer cells with LDN and CBD could influence their sensitivity to the common drugs GEM and OXP. Cells were pre-treated for 2 days with CBD and LDN at a molar ratio of 100:1, before treatment with chemotherapy used at suboptimal cytotoxic concentrations. The results showed that cell numbers and cell viability were significantly reduced compared to the untreated (UN) controls (Fig. 3A). For example, in HCT116 cells pre-treated with CBD + LDN (C + L), OXP significantly reduced cell number to $41\pm5.0\times10^4$ vs. $94\pm13\times10^4$ cells in the untreated cells (P<0.001), and cell viability to 50±2.7% from 98±3.0% seen in the untreated cells (P<0.001).

There was also an element of a 'priming' effect of C + L, as both number and viability of cells pre-treated with these prior to chemotherapy were significantly lower than cultures when chemotherapy was used without the C + L pre-treatment

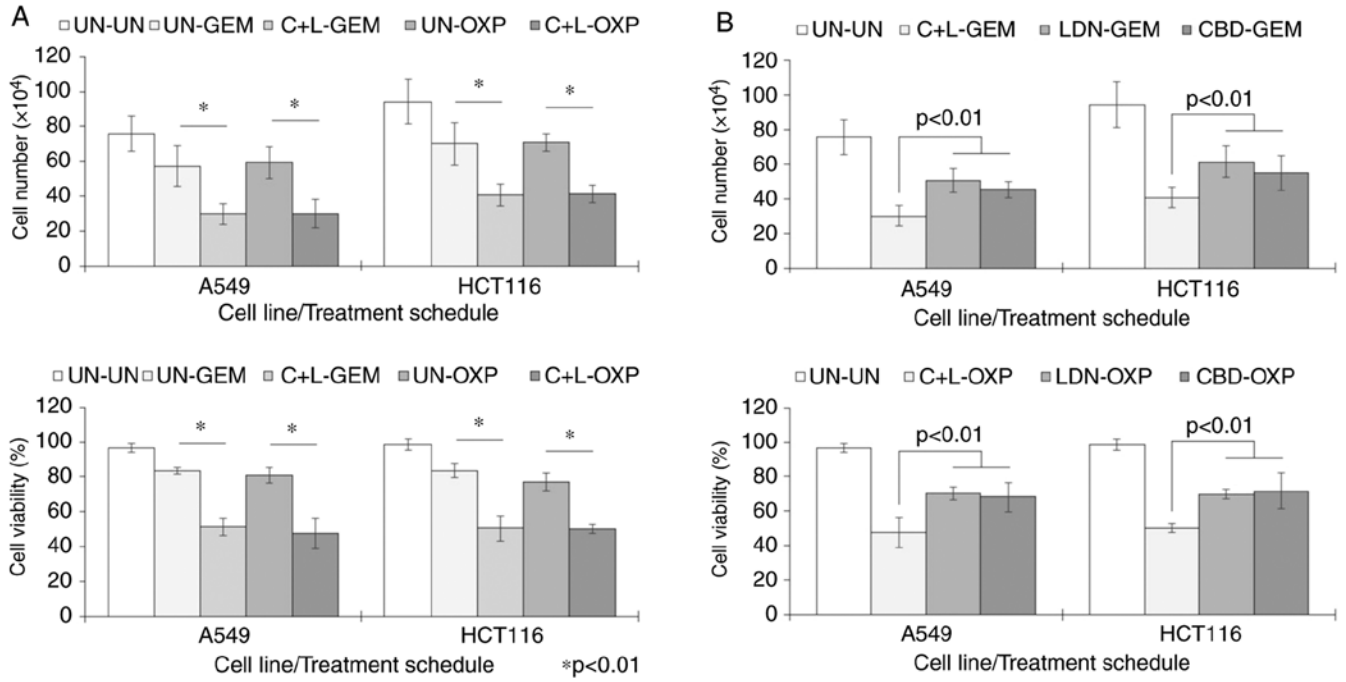

Figure 3. Effect of a triple combination of CBD, LDN and chemotherapy on cell number and viability. A549 and HCT116 cells were cultured with drugs according to a treatment schedule that involved two treatment phases that each lased 2 days. The total number of treatment days was 4 days, at which time, cell number and cell viability were assessed. (A) The effect of using CBD and LDN together (C + L) in the first phase before gemcitabine (GEM) or oxaliplatin (OXP) was compared to cultures where there was no treatment (UN) in the first phase. *P<0.01. (B) The effect of CBD and LDN together before GEM or OXP was compared to regimens where just CBD or LDN alone were used. Data points represent the mean and SD of at least three separate experiments, and P-values are from paired tests following ANOVA. CBN, cannabidiol; LDN, low-dose naltrexone.

(Fig. 3A). For example, cell number and viability of HCT116 cells exposed to OXP without the C+L pre-treatment was 71±5.0x10^4 cells and 77±4.8%, respectively. However, with the C + L pre-treatment, these values were reduced significantly further to 41±5.0x10^4 cells and 50±2.7% (P<0.01). As both CBD and LDN were not cytotoxic on their own, the increase in death suggested cells were primed to the killing effects of OXP.

This priming effect also appeared to be most effective when CBD and LDN were used in combination, as results also showed that priming separately with just CBD or LDN did not enhance the cytotoxic effect of GEM or OXP as much as if both components were used together (P<0.01 in all cases) (Fig. 3B).

GEM is tolerated better when used with CBD and LDN. The efficacy of treatments using CBD and/or LDN were also assessed in a xenograft murine model. The growth of human HCT116 cells implanted into the flanks of nude mice was tracked for about 25 days, and the effects of CBD ± LDN with or without GEM on final tumour volumes were examined. The treatment regimens were composed of two stages of treatment that each lasted 7 days. Those involving just LDN and/or CBD were well tolerated, and there were no marked changes in body weights compared to the untreated groups (data not shown). Mice that were treated with just a single agent, viz. either LDN or CBD only for both stages of the regimen, exhibited tumours that were not significantly different to those observed in the untreated mice (Fig. 4A-a). However, the sequential administration of LDN and CBD, regardless of their particular order generally resulted in tumour volumes that appeared to be smaller than those seen in the control (UN) group; however, this was not statistically significant (Fig. 4A-b).

Next, mice were either given C + L or PBS in the first phase of treatment before being treated with GEM. Tumour volumes were then measured and compared at the end of treatment. The results showed that tumour volumes when compared with the control group were smaller in the groups where GEM was used after PBS (P=0.018) or with C + L (P=0.002) (Fig. 4A-c). There was no difference in tumour volumes between C + L primed and un-primed animals; however, GEM was better tolerated by mice in the group pre-treated with C + L compared to those without. Specifically, the change [median (IQR)] in animal body-weight after treatment with C + L then GEM was 1.5 g/mouse (1.0-1.8) vs. -0.25 g/mouse (-0.55-0.3) in the PBS then GEM group (data not shown).

The order in which C + L and GEM was administered was also examined to see whether this influenced overall efficacy. The results revealed there was no significant difference in the final tumour volumes from animals in the C + L-GEM group vs. the GEM-C + L group (Fig. 4A-d). However, there was a difference in how the treatments were tolerated as the change [median (IQR)] in animal body-weight was 1.3 (1.2-2.3) in the C + L-GEM group vs. -0.60 (-1.1-0) in the GEM-C + L group (data not shown). The effect of these two treatments on standard markers of cell growth and survival were also measured. The results showed the treatment using C + L followed by GEM resulted in a significant increase in the expression of BAD and p21 compared to the untreated group (P<0.01) (Fig. 4B).

Discussion

The present study was a continuation of our earlier ones, which focused on understanding further the anticancer effects

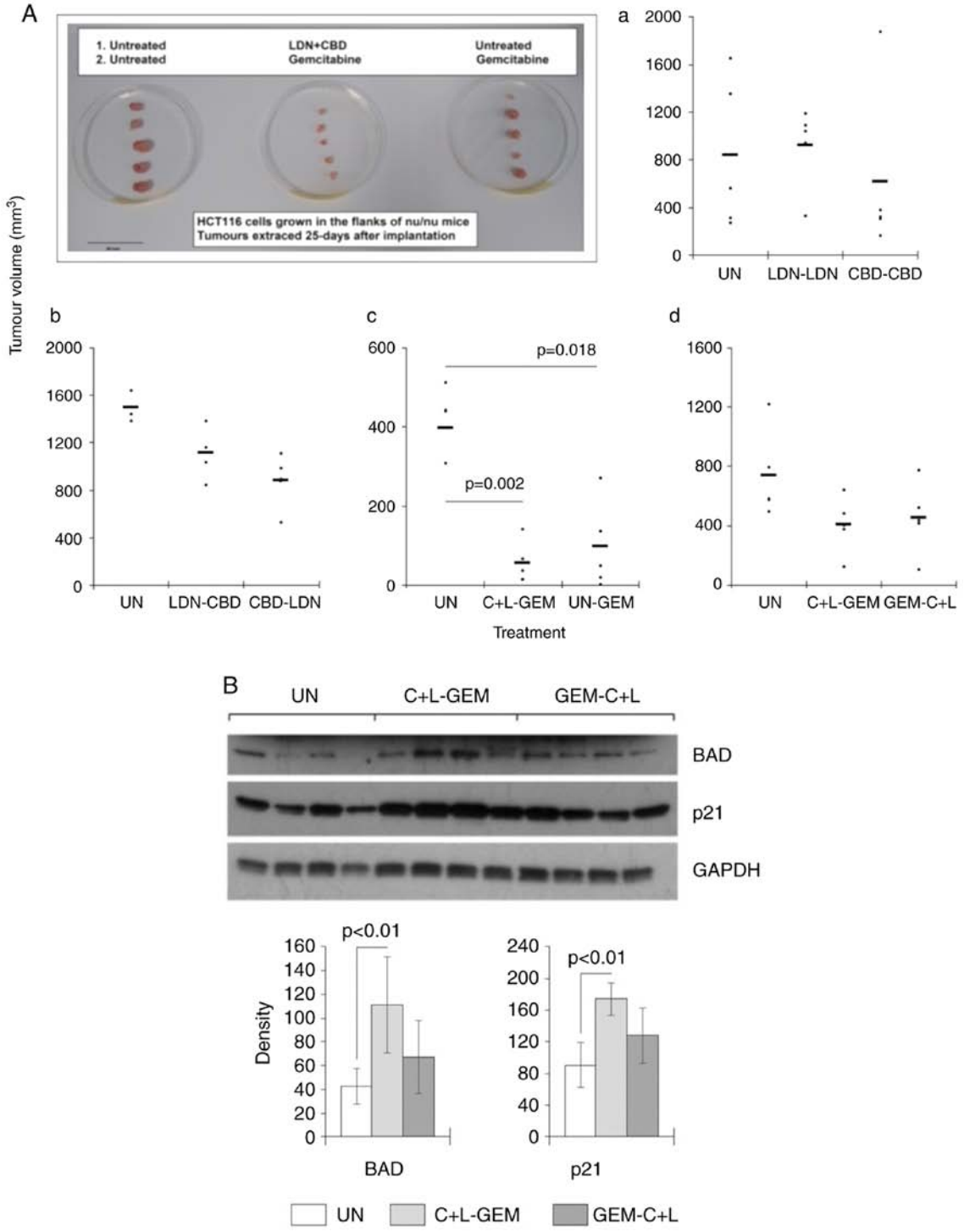

Figure 4. Effect of CBD, LDN and GEM on tumour cells *in vivo*. HCT116 cells were implanted subcutaneously into the flanks of immune-compromised mice, and when palpable, tumours were treated with CBD, LDN and/or GEM according to a treatment schedule consisting of two stages of treatment that each lasted one week. For example, LDN could be used in the first period followed by CBD in the second week (designated LDN-CBD). In some situations, CBD and LDN were used together (C + L). (Aa-d) Tumour sizes were assessed at the end of the treatment (end of week 2). The tumours extracted from the mice in experiment c were photographed and included in the figure. (B) Tumours were disaggregated at the end of week 2, and subjected to western blot analysis for expression of BAD and p21. Black bars represent the mean of each mouse represented by the circles. The blots are of four mice, and the columns represent the mean of all the mice in each group. CBN, cannabidiol; LDN, low-dose naltrexone; GEM, gemcitabine.

of low-dose naltrexone (LDN) and cannabidiol (CBD). Our previous studies showed that both agents when used in isolation were able to enhance the activities of common chemotherapy agents. Mechanistically, this was because both LDN and CBD were able to enhance the level of certain proteins within cell signalling cascades such as BAD, BAX and p21 that influence cell death and survival. Both agents were found to share a similar MOA, which is that they are able to prime cancer cells to the cytotoxic effects of standard cytotoxic agents (2,17,18).

The potential anticancer effects of these two agents have been known for some time and are supported by a range of *in vitro* studies and animal modelling. Many of the key studies

have been examined and collated into review articles (19,20), yet there have been scant clinical trials to confirm the efficacy and benefit; especially as single-use agents. This has not, however, dissuaded the public from using these sorts of drugs in the hope that they will work 'better' than conventional treatments. Indeed, in more recent times, LDN and CBD have actually been used together in the belief they are complementary irrespective of the fact that there are no data suggesting a benefit to using them together as anticancer agents. The only study we could find that reported the use of LDN and CBD was a murine study reporting a benefit to using the two as a way of reducing the motivation of mice to self-administer ethanol (21). The benefit was only observed when the two drugs were used concomitantly as CBD appeared to support/promote the action of LDN. Although the MOA was not defined, the need for signalling cascades common to both agents appeared important. Thus, as an attempt to shed more light in this field, we examined the effect that combining LDN with CBD may have on the growth and survival characteristics of cell lines *in vitro*. We also assessed the effect of these novel combinations on the cytotoxic effects of certain chemotherapy drugs. Finally, we examined the best drug-combinations utilizing murine studies to establish the optimum tumour-reducing treatment schedule.

LDN and CBD display actions and features that are extremely similar to each other. They both interact loosely with receptors that result in intracellular modifications, which lead to the desired response. The receptors involved are not the same for both drugs, but are all part of the G-protein coupled receptor (GPCR) superfamily (22). Activation of these receptors can cause changes to similar downstream signalling cascades, which although overlapping in places, lead to effects specific to each drug individually. Anticancer actions have been loosely attributed to and associated with two routes of action. Firstly, both CBD and LDN can directly interfere with cell signalling systems within cancer cells that can result in an arrest of cell proliferation. In some circumstances, this arrest in cell cycling can lead to apoptosis (23,24). Second, both are capable of modifying the host immune system, which can re-educate and stimulate and direct an immune-based cytotoxic response against cancer cells (1,25). Given these actions, it appears that both CBD and LDN reduce cell numbers by inducing a cytostatic effect rather than killing cells directly.

The cannabinoid receptor and opioid receptor systems are known to interact with each other (26), which suggests that the use of LDN with CBD together could lead to an enhanced effect overall. *In vitro* and murine models have indeed shown that the binding to cannabinoid receptor 1 can influence activity of the δ-opioid receptor (27). The cross-play between receptor systems is not uncommon and can involve a compensatory increase in expression of a receptor to make up for the loss or antagonism of another (28). In fact, our previous study examining the effect of LDN on the gene expression of a range of receptors within this family, including the adrenergic, GABA, glutamate and serotonin, revealed 15% were upregulated and 17% were downregulated following culture with LDN (2). Crucially, as the incoming 'substitute' receptor may interact differently with intracellular signalling, the ultimate effect achieved may actually differ from that originally initiated. This introduces the possibility that a ligand with classical actions can engage other processes distinct from what would be expected, and two drugs that work on receptors may work in unison. Taken together, these introduce the overarching concept that using methods to modulate the activity of one receptor may be exploited as a mechanism by which the activity of another can be altered. We therefore utilised LDN and CBD together to see if their interplay could influence further cancer cell fate.

Using LDN and CBD at the same time had no effect on cell number and cell viability. Using the two together was no better than using the agents separately, and in some situations negated the reduction in cell number seen when the drugs were used separately. This was not a complete surprise as GPCR signalling is not a uniform process but instead one that is spatial and temporal in nature (29). Receptor activation and signalling via secondary messengers are timed and orchestrated, with one event having to occur before another can. We thus examined the effects on cell number and viability when LDN and CBD were used sequentially and showed that the order in which they were used was crucial. Using CBD before LDN was no different to using the two drugs at the same time; however, cell number and viability were significantly reduced when LDN was used before CBD. The levels of pERK expression in cells were also tracked, and were reduced after treatment with CBD and LDN, with the largest reduction seen in the schedule where LDN was used before CBD. There are other readouts such as migration and invasion that may have been affected by these treatments, which is something that should be explored further in the future.

Signalling through pERK plays a central role in determining the fate of cancer cells, as their activation generally increases cell numbers by promoting cell proliferation and survival (30). It plays an important enough role that targeting its action as well as other members of the cascade has been the aim of several therapeutic approaches (31). The anticancer actions of both LDN and CBD alone are associated in part with changes to pERK signalling (2,32), and our results showed that combining the two both simultaneously or sequentially could still reduce its expression. There was no market increase in cell killing by using the two, but this was not surprising as both LDN and CBD are actually not cytotoxic agents, and even though pERK was reduced, the absence of an active 'kill' signal would mean death is unlikely to happen. Indeed, studies have shown that pERK is involved in the balancing of anti- and pro-apoptotic proteins, and as such its modification alone does not necessarily result in cell death, but instead modulates the likelihood of apoptosis occurring (33). Taken together, this suggests partnering a cytotoxic chemotherapy agent with LDN or CBD would be a way of increasing cytotoxicity, which is precisely something we and others have shown. Specifically, there are data showing CBD is effective with chemotherapy in a wide range of cancer types, with the MOAs underpinning synergy involving the modification to the balance of BAX:BCL2-related proteins downstream of pERK (34-36). Similarly, studies involving LDN and chemotherapy combinations report a similar MOA involving these apoptosis-determining proteins (2,37).

The results of the present study showed that using CBD and LDN together could also sensitise cells to the cytotoxic effects of two common chemotherapy agents *in vitro*. For example, the cytotoxic effect of GEM in HCT116 cells was enhanced by 40% when CBD and LDN were used up-front as a priming

agent (% cell death: 49±7.3 vs. 16±4.0% in GEM-treated cells without priming; P<0.01). Notably, using the two together was superior to using just one of the drugs individually. The priming effect was not clear cut in the studies with mice bearing a human tumour, and an expected divergence of tumour sizes in the primed and un-primed groups was not observed. Post experimental analysis of the data indicated that GEM generated an effect that was much higher than expected. For logistical reasons, an equivalent dosage for the mouse experiment was extrapolated from standard guides (38), based on previously published studies, and in accordance with our *in vitro* work targeted to be in the region of an IC_{20}. The actual dose turned out to be more effective than expected, and any benefit of the priming effect was swamped by GEM working at the more efficacious dose. Nevertheless, the mouse studies indicated that priming with CBD and LDN made GEM at this concentration more tolerable to mice. Specifically, the loss of body weight with GEM treatment was negated and in fact reversed when mice were pre-treated with CBD and LDN. This suggested that another possible benefit of using CBD and LDN especially *in vivo* would be an improvement in the capacity of patients to tolerate cytotoxic treatments (39).

In conclusion, these data reinforce the idea that CBD and LDN are drugs that have the capacity to enhance the action of other treatments. Although they have a minimal effect on their own on cell growth and death, their benefits lie in the way that they can enhance the activity of chemotherapy drugs. This effect is not only seen when LDN and CBD are used individually, but when used together following a treatment schedule that involves a sequenced-administration of the drugs. Our *in vivo* examinations recapitulated the laboratory studies and also showed that adding CBD and LDN as a priming agent resulted in animals bearing the chemotherapy much better. Finally, all our data reported here were based on cancer cell lines and did not take into consideration the tumour microenvironment and the inflammatory immune responses. We previously demonstrated that naltrexone blocks Toll-like receptors (TLR)-7, -8 and -9 that reduce the production of interleukin (IL)-6, which is a major determinant of cancer progression (40). Hence the anticancer effect may be even more important in the human clinical situation where significant responses have been reported with LDN alone (41). Overall, these studies provide evidence to support the role for LDN/CBD and chemotherapy in clinical trials, especially in those cancer that have an issue of high toxicity with standard chemotherapy regimens.

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Availability of data and materials

The datasets used in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

The study was conceived and written by WML and AGD. The experiments were performed by WML, NKH and HSYL. The data were analysed, examined, and assessed by WML. Interpretation of the data and studies and confirmation of the integrity of all data were performed by WML, FLH and AGD. All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

All animal experiments were performed according to the strict guidelines in accordance with the UK Animals (Scientific Procedures) Act and associated guidelines. All work was approved by the Ethics Committee of St George's University of London (SGUL), and performed at the Biological Research Facility of SGUL under project licence PP1419291.

Patient consent for publication

Not applicable.

Competing interests

WML and AGD are named inventors on patents (some that are pending) related to the use of LDN and/or cannabinoids as a potential cancer therapy. FLH is contracted by LDN Pharma Ltd. (London, UK)

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