Anticancer effects of phytocannabinoids used with chemotherapy in leukaemia cells can be improved by altering the sequence of their administration

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Abstract. Phytocannabinoids possess anticancer activity when used alone, and a number have also been shown to combine favourably with each other *in vitro* in leukaemia cells to generate improved activity. We have investigated the effect of pairing cannabinoids and assessed their anticancer activity in cell line models. Those most effective were then used with the common anti-leukaemia drugs cytarabine and vincristine, and the effects of this combination therapy on cell death studied *in vitro*. Results show a number of cannabinoids could be paired together to generate an effect superior to that achieved if the components were used individually. For example, in HL60 cells, the IC_{50} values at 48 h for cannabidiol (CBD) and tetrahydrocannabinol (THC) when used alone were 8 and 13 μM, respectively; however, if used together, it was 4 μM. Median-effect analysis confirmed the benefit of using cannabinoids in pairs, with calculated combination indices being <1 in a number of cases. The most efficacious cannabinoid-pairs subsequently synergised further when combined with the chemotherapy agents, and were also able to sensitise leukaemia cells to their cytotoxic effects. The sequence of administration of these drugs was important though; using cannabinoids after chemotherapy resulted in greater induction of apoptosis, whilst this was the opposite when the schedule of administration was reversed. Our results suggest that when certain cannabinoids are paired together, the resulting product can be combined synergistically with common anti-leukaemia drugs allowing the dose of the cytotoxic agents to be dramatically reduced yet still remain efficacious. Nevertheless, the sequence of drug administration is crucial to the success of these triple combinations and should be considered when planning such treatments.

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Introduction

The phytocannabinoids (CANN) are a group of related compounds extracted from the cannabis plant (1). The archetypal CANN that is most widely known is Δ9-tetrahydrocannabinol (THC). It is notorious for its psychoactive properties; however, it is arguably the only one to exhibit such an effect out of the >80 members. CANNs interact with the endocannabinoid system of the human body, and through this can consequently intrude into a number of physiological aspects such as appetite (2). Evidence from the 1970s (3) suggested CANNs possessed anticancer activity; and since then a large body of *in vitro* studies have been developed and performed to confirm this (4,5).

A number of cells express the cannabinoid receptor (CBR), of which there are a number of sub-types (e.g. CBR1, CBR2), and it is believed that signalling through this G-protein coupled receptor is required for CANN anticancer action. Most of the *in vivo* models of anticancer action of CANNs have focussed on cancers of the brain where there are high levels of CBR1, and a large proportion of these have indicated that CANN use is associated with decreased tumour growth and/or increased cell killing (6). These models have also shown the use of CANN can successfully support and enhance the action of other treatment modalities (7,8). Peripheral cells, mainly immune cells, can also highly express these receptors (9), in particular CBR2, and as a consequence the effects of CANNs on cancers emanating from these cells have also been studied.

Results from numerous *in vitro* studies have shown the importance of the CBR in the success of CANNs as effective anticancer agents. Signalling through the CBR gives CANN the ability to stimulate pro-apototic elements within the ceramide pathway (10), as well as being able to engage autophagy in cells (11). Additionally, CANNs can subsequently interfere/interact with other intrinsic intracellular signalling pathways such as PI3-K and ERK via downstream crosstalk, which offers a way in which they can also fundamentally manipulate key processes like cell growth and survival (4,12).

The necessity for receptors to be present in order to elicit these cell killing mechanisms may not, however, be absolute; anticancer activity has been seen in leukaemia cells that is independent of the receptors (13), and similarly, minor-occurring

CANNs, which have low binding affinities for these canonical receptors, are equally as active in these same cell types (14,15). Furthermore, there have been reports of CANN activity directly on cancer cells that do not usually express the receptors such as those of the breast and prostate (16). Together, this suggests the number of cancers that could respond to CANNs may not be limited to those expressing the receptors. Nevertheless, the evidence that a number of CANNs can be used to reduce the growth of leukaemia cells *in vitro* is exciting, and warrants further investigation.

As part of our ongoing research efforts investigating the potential benefits of CANNs in a leukaemia setting, we have examined further the effects of these drugs combined with others on cell growth and survival. We paired CANNs together and specifically examined the activity of these mixes in leukaemia cells, both alone and in combination with a number of common anti-leukaemia drugs. We have adopted a number of practical models to assess drug-drug interactions, and also assessed the importance of drug sequence in determining the overall efficacy of the differing treatments.

Materials and methods

Cell culture and drugs. The human cancer cell lines CEM (acute lymphocytic leukaemia) and HL60 (promyelocytic leukaemia) were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK), and grown in RPMI-1640 medium (Sigma-Aldrich Co., Ltd., Dorset, UK) supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine. All cell lines were incubated in a humidified atmosphere with 5% CO_2 in air at 37˚C, and discarded after ~12 passages. Authentication of the cell lines was performed by the service provider using the AmpFISTR Identifier Plus PCR amplification kit looking for the presence of <10 known loci for each of the cell lines.

Cytarabine (CYT: Sigma) and vincristine (VIN: Sigma) were reconstituted in PBS at a stock concentration of 10 mM, and kept at -20˚C for no more than four weeks. Cannabidiol (CBD), cannabigerol (CBG) and THC (all provided by GW Research Ltd., Cambridge, UK) were dissolved in ethanol to appropriate concentrations that ensured a final ethanol concentration in cell cultures <0.1%. For experiments with treatments, the amount of FBS in the cell culture medium was reduced to 5%. One aim of the current study was to investigate the benefit of using two different CANNs together in a pair. The combinations used here mimic a number of current and recent clinical trials where a proprietary product containing CBD and THC was used (www.clinicaltrials.gov - Identifier: NCT01812603 and NCT01812616). Consequently, our experiments involved using CANNs paired concomitantly at a 1:1 ratio, where the stated concentration for them reflected an equal amount of each CANN-component; for example, 10 μM CBD+THC contained 5 μM CBD and 5 μM THC. A similar approach was adopted and reported in our earlier studies (8).

Proliferation assays - CANNs alone. To study the effect of the CANNs on cell growth, leukaemia cells that were growing exponentially were seeded into 96-well plates at a density of $1.5x10^4$/well. CANNs were then added to the wells at various concentrations, ensuring an equal volume of 200 μl across the plate. Single-agent testing: Either CBD, CBG or THC alone was added to the wells at a concentration range of 1-50 μM. Paired-CANN testing: CBD+CBG, CBD+THC or CBG+THC was added to the wells at a concentration range for the paired CANNs of 1-50 μM. The molarity was based upon the total CANN in each pair. Cell number was assessed by using a methylthiazoletetrazolium (MTT)-based assay according to methods previously described (17), and by cell counting using trypan blue dye as a way of discriminating live and dead cells.

Combination studies - median-effect analysis. Cells ($1.5x10^4$/well) growing exponentially were reset in fresh culture medium and aliquoted into 96-well plates. A CANN-pair (either CBD+THC or CBD+CBG) was combined with CYT or VIN at concentrations that were equal ratios of their respective IC_{50} according to methodologies described previously (14,17,18). Cell number was then assessed after 72 h by the MTT-based assay, and a combination index (CI) calculated by using the median-effect equation (19).

Combination studies - modulatory effect. The ability of CANNs to modify the efficacy CYT and VIN was studied by assessing and comparing the IC_{50} of the anti-leukaemia drugs in the absence and presence of the CANNs. The CANNs tested were CBD+CBG and CBD+THC (the modulating drug in this setting), and these were used at a single total sub-optimal concentration of 1 μM in CEM and 5 μM HL60. Methodologically, cells ($1.5x10^4$/well) growing exponentially were reset in fresh culture medium and aliquoted into 96-well plates. Drugs were added (CYT and VIN over a range of concentrations) and cell number determined after 72 h. Parallel 6-well plates containing cells were also prepared and were cultured with the same treatment combinations described. These allowed for determination of cell cycle distribution at 72 h by flow cytometry utilising the nucleic acid stain propidium iodide (17).

Combination studies - drug sequence and the impact of a recovery phase. CEM and HL60 cells were seeded into 6-well plates at a density of $1x10^5$/well and then treated according to a culture schedule that lasted a total of 96 h. The treatment would involve two separate phases; each lasting 48 h. One set of drugs would be administered in the first 48 h phase and a second set of drugs in the following 48 h phase. The culture medium would be removed by centrifugation after the first treatment to be replaced with fresh medium in an attempt to remove the drugs used in the first phase of treatment. The drugs studied were either CBD+CBG (4 μM in CEM and 10 μM in HL60), CBD+THC (4 μM in CEM and 10 μM in HL60), CYT (10 nM), or VIN (0.1 nM). The effect of a recovery phase was assessed by keeping the second 48 h phase of treatment drug-free. Flow cytometry using propidium iodide staining was performed at the end of the experiment to assess the extent of cell death/apoptosis.

Immunoblotting analysis. Western blot analyses were performed as previously described (8). Primary antibody probing was performed with anti-cyclin B1 and anti-GAPDH (both from New England Biolabs, Hitchin, UK) and used at a dilution of 1:1,000. Appropriate HRP-conjugated secondary

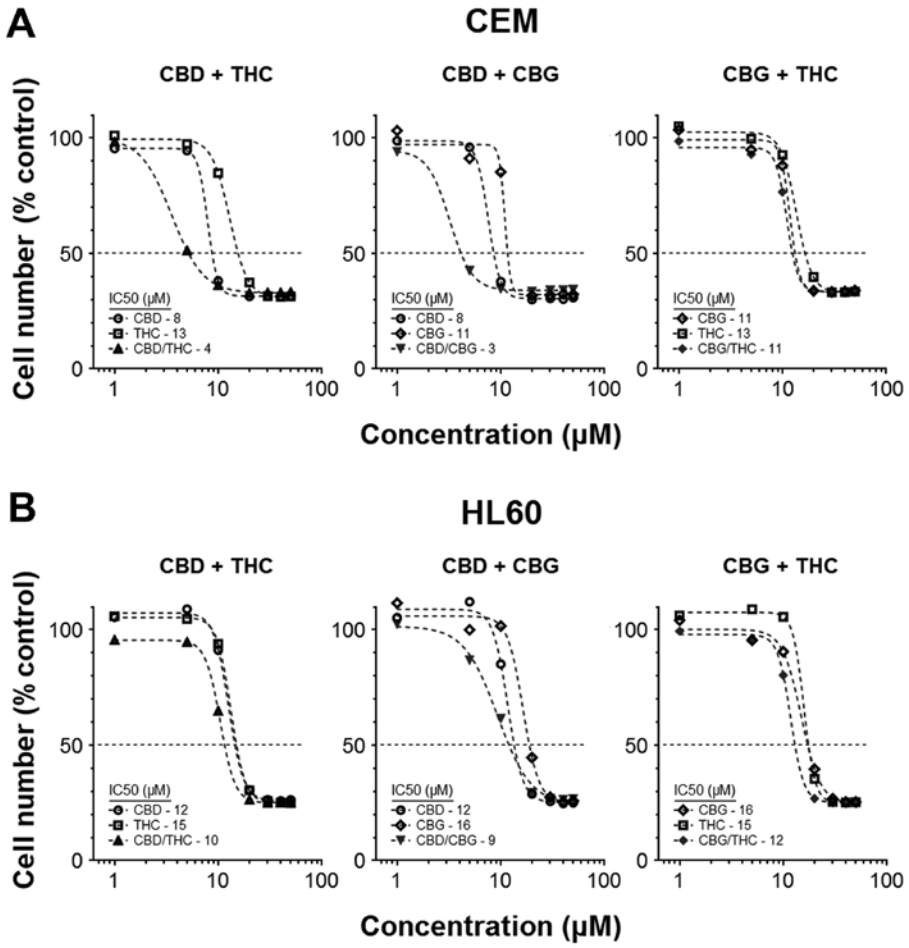

Figure 1. Dose response curves of single agent and cannabinoid combinations. CEM and HL60 cells were grown for 48 h in the presence of increasing concentrations of the three cannabinoids, THC, CBD and CBG, either as single agents or in dual combinations prepared at a 1:1 ratio. Thus, 10 μM of the CBD and THC combination would be made of 5 μM CBD + 5 μM THC. Cell number was assessed using the MTT assay and the concentration required to reduce the cell number by 50% (IC_{50}) for each condition in CEM (A) and HL60 (B) was calculated using GraphPad Prism. Each data point represents the mean of at least three separate experiments. SDs have been omitted for clarity.

antibodies were then used (New England Biolabs), and bands were visualised by the ECL-plus detection system (Amersham Biosciences Ltd., Little Chalfont, UK).

Statistical analysis. All statistical analyses were performed using GraphPad Prism or Microsoft Excel, and differences between treatments and control groups were determined by one-way ANOVA and subsequently by paired tests. Data values were presented as the means and SDs of at least three separate experiments.

Results

Combining CANNs can improve their overall activity. Our previous studies showed a small number of CANNs could be used together to induce a cytotoxic response that was hyper-additive in nature. We therefore expanded this initial work by pairing CBD, CBG and THC in different permutations, and assessing their effects on cell numbers after 72 h treatment. IC_{50} values for the individual CANNs were determined, and these were compared with IC_{50} achieved when the matching CANN-pair was used. Results showed the virtual IC_{50} of the mixtures were generally lower than those for the CANNs when used individually (Fig. 1A and B). For example, the IC_{50} in CEM cells was 7.8±0.21 μM for CBD alone and 13±0.49 μM for THC alone, compared to 3.6±0.19 μM when CBD and THC were used simultaneously at a ratio of 1:1 (Fig. 1A).

In this basic paired-model, CEM cells were more responsive to treatments, as the combination of two CANNs generally resulted in an improvement in activity. Moreover, combinations including CBD as one of the partners in a pair usually resulted in a greater reduction in cell number (IC_{50} values in CEM for CBD/THC, CBD/CBG and CBG/THC were 3.6±0.19, 2.8±0.24 and 11±0.55 μM, respectively) (Fig. 1A).

CANN-pairs can cooperate with anti-leukaemia agents to reduce cell numbers. Median-effect analyses were employed to assess the interactions between each CANN-pair and common anti-leukaemia drugs. Guided by our initial results showing CBD-containing pairs to be most efficacious, we selected the CBD/CBG and CBD/THC pairs and combined them with either CYT or VIN. CI-values were then calculated by using these results and used as a way of understanding the drug-interactions (14,18).

Results showed that outcome of the interactions were dependent upon both drug and cell line. They also hinted

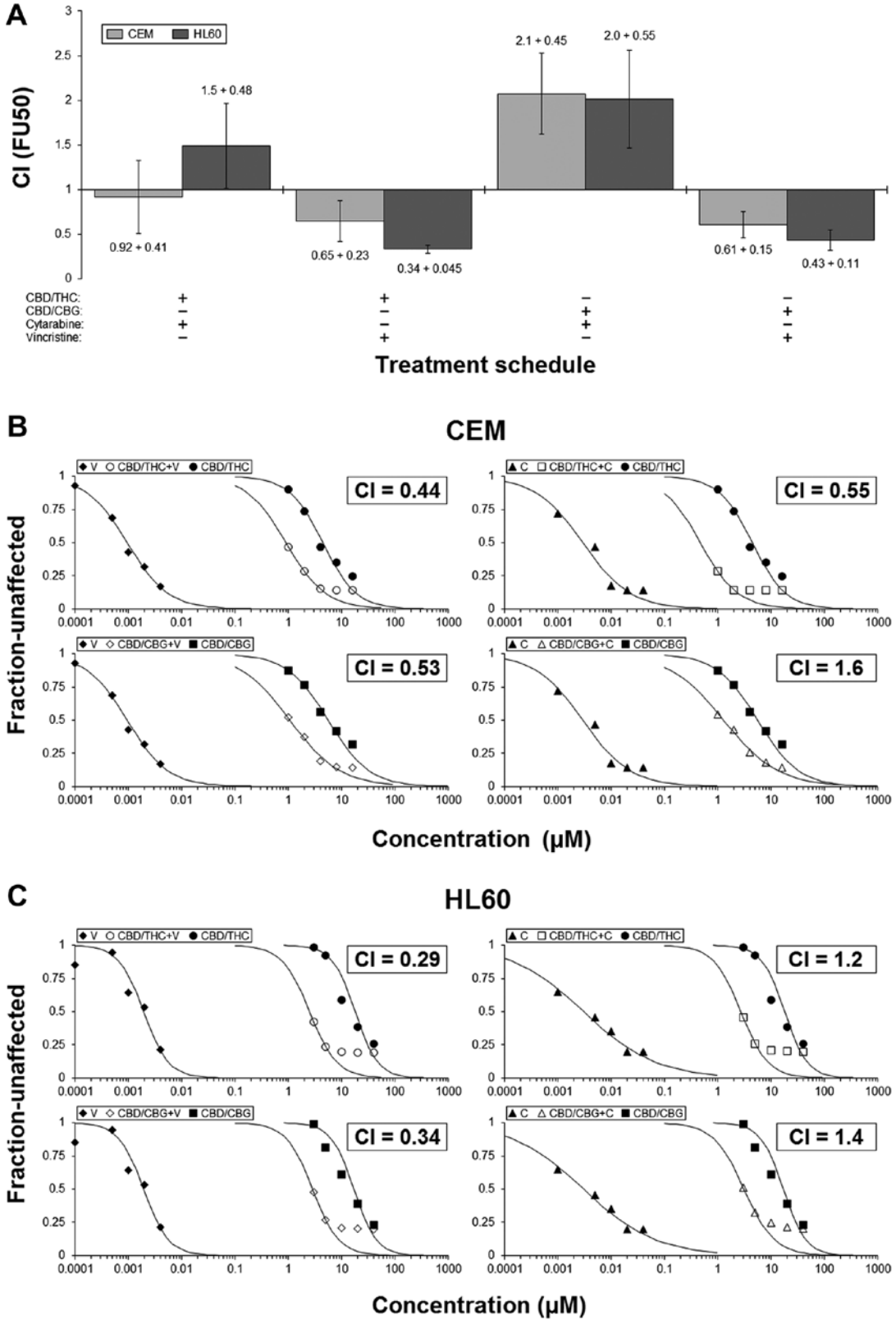

Figure 2. Median effect analysis of chemotherapy and cannabinoid combinations. CEM and HL60 cells were grown for 72 h in the presence of increasing concentrations of both cytarabine or vincristine and a cannabinoid-pair, combined at fractions of their respective IC_{50} values. CBD/THC and CBD/CBG were the two cannabinoid-pairs that were investigated, and were used at equal 1:1 ratios. Cell number was assessed at 72 h using the MTT assay and defined algorithms were then used to generate a combination index score (CI) which indicates the nature of the combination interactions (CI=1 = additivity; CI <1 = synergy; CI >1 = antagonism) (A). Representative data have also been included from experiments in CEM (B) and HL60 (C). Each data point in the column graph represents the mean and SD of at least three separate experiments.

that combinations involving VIN would more likely result in enhanced activity, whilst those with CYT may cause additivity/mild antagonism (Fig. 2A). Representative examples of the interactions are presented (Fig. 2B and C). Notably, in HL60

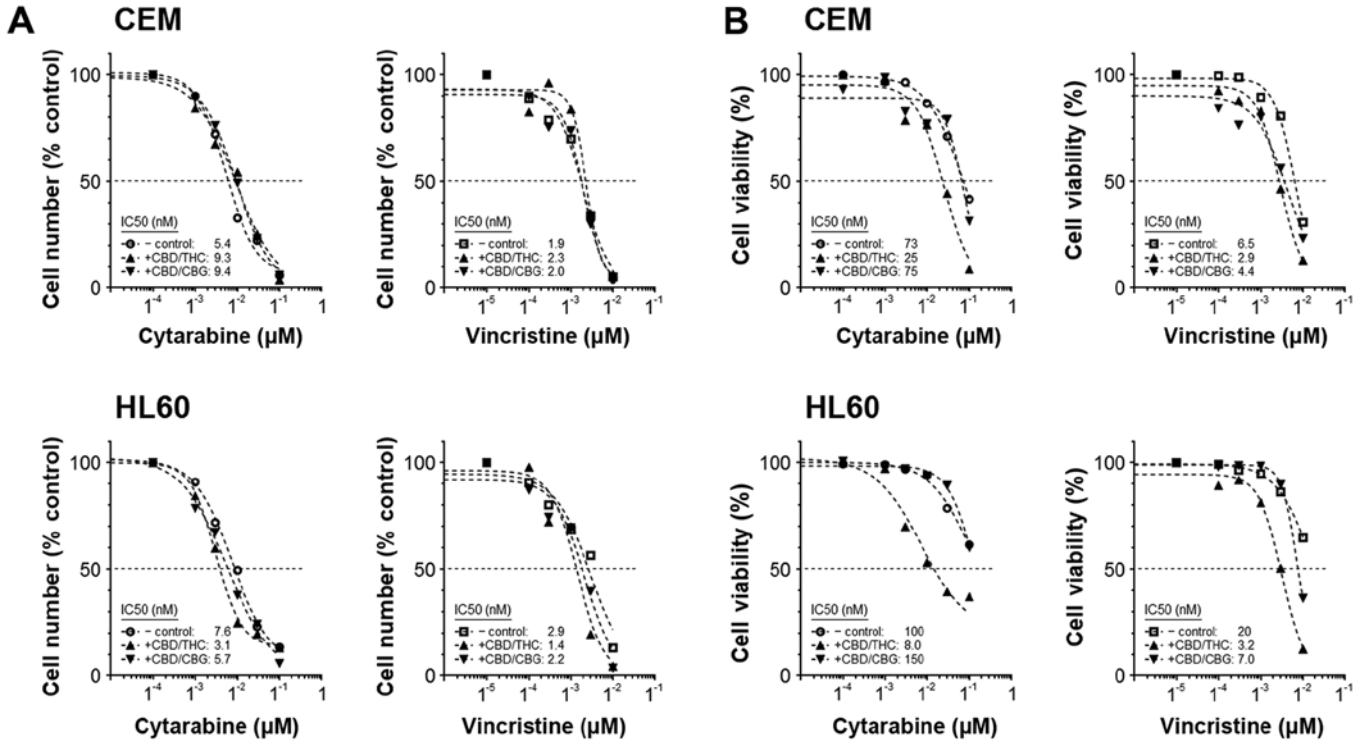

Figure 3. Sensitising chemotherapy action with low doses of cannabinoids. CEM and HL60 cells were grown for 72 h in the presence of increasing concentrations of cytarabine (CYT) or vincristine (VIN). The effect of a low dose of CBD/THC or CBD/CBG on the activity of CYT and VIN was also assessed. IC_{50} values for cell number (A) and percentage cell viability (B) were determined by Emax models.

cells for example, the IC_{50} for CBD/THC was 18 μM and for VIN was 1.9 nM; however, when these two were used concomitantly, the IC_{50} of this combination treatment was 2.5 μM. The calculated CI-value was 0.29, which indicated synergy (Fig. 2C). This result shows that in certain circumstances a combination approach can result in an equivalent level of action even though the concentrations of the agents used are much lower (in this instance CBD/THC and VIN were used at ~2.5 μM and ~0.25 nM, respectively).

CANN-pairs can sensitise cells to the effects of anti-leukaemia agents. A second model of drug interaction was employed in our studies. This experiment was designed to test the ability of a CANN-pair to sensitise cells to the effects of CYT or VIN. Specifically, the ability of a sub-effective concentration of CANN to alter the efficacy of CYT or VIN was determined by comparing the IC_{50} values of the chemotherapy agents in the absence or presence of the modulating CANN drug. Results showed adding CANNs to cells cultured with CYT or VIN only changed the cell number IC_{50} values for each chemotherapy drug to a small extent (Fig. 3A). Changes were most apparent in treatments where CBD/THC was the modulating drug. Conversely, however, there were clear changes in the IC_{50}, when examining the modulatory effect of CANNs on cell viability (Fig. 3B). For example, the IC_{50} for CYT in HL60 was 100 nM; however, this was reduced to 8 nM if CBD/THC was included to the cultures, but increased to 150 nM if CBD/CBG was used (Fig. 3B). These results generally agreed with those from the median-effect combination model, and suggest combinations of CANNs with VIN would result in hyperadditive interactions leading to reduced cell numbers.

The reduction in viability was associated with an increase in apoptosis, as shown by flow cytometry, which was generally higher in combinations involving CBD/THC (Fig. 4A). Cell death was not specific to any phase of the cell cycle, and the drug-induced arrest in the S-phase did not significantly impede the ability of cells to undergo death when CANNs were added (data not shown). The expression of cyclin B was used as a general marker of cell cycling, and levels increased when cells were cultured with CYT or VIN (Fig. 4B). However, this increase was negated or in the case with HL60, reduced when a CANN pair was included (Fig. 4B).

The sequence of drugs is important in determining overall activity. Having seen synergistic interactions between CANNs and anti-leukaemia drugs when they were used simultaneously, we next assessed the impact of using the drugs sequentially. Consequently, cells were cultured according to schedules that consisted of two rounds of treatment, each lasting 48 h. Each round of treatment was separated by a washing step to remove drug from the medium. The order of the drugs were swapped in equivalent experiments to assess the counter-order of drugs. In some cases, a treatment schedule could involve the use of a CANN-pair in the first round of treatment followed by no treatment in the second. This mimicked a 'recovery' schedule. The duration of each treatment phase was 48 h to ensure that cells were not overgrown by the end of the full treatment regimen, which lasted 96 h.

Results showed that, generally, the percentages of cells within the sub-G1 population of the cell cycle were low in CEM cells following any treatments (Fig. 5A); however, the order of administration of the drugs affected the amount of

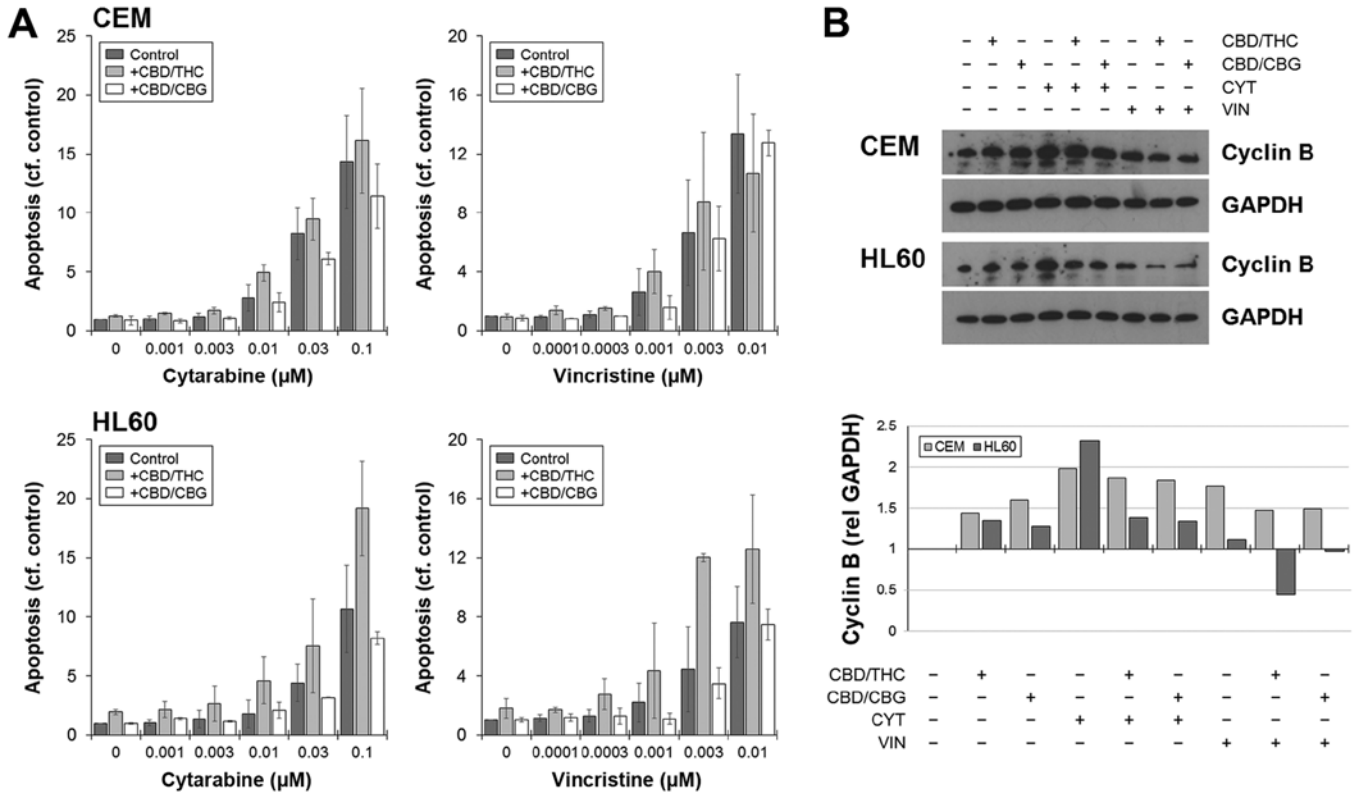

Figure 4. Sensitising chemotherapy action with low doses of cannabinoids. CEM and HL60 cells were grown for 72 h in the presence of increasing concentrations of cytarabine (CYT) or vincristine (VIN). The effect of a low dose of CBD/THC or CBD/CBG on the cell cycle distribution was assessed by flow cytometry, and the particular effects on the sub-G1 (apoptosis/cell death) fraction of cells (A) and on cyclin B expression (B) were studied more closely as read-outs for cytotoxicity and cytostasis, respectively. Each point in the column charts represent the mean and SD of three separate experiments, and SDs in (B) were omitted for clarity.

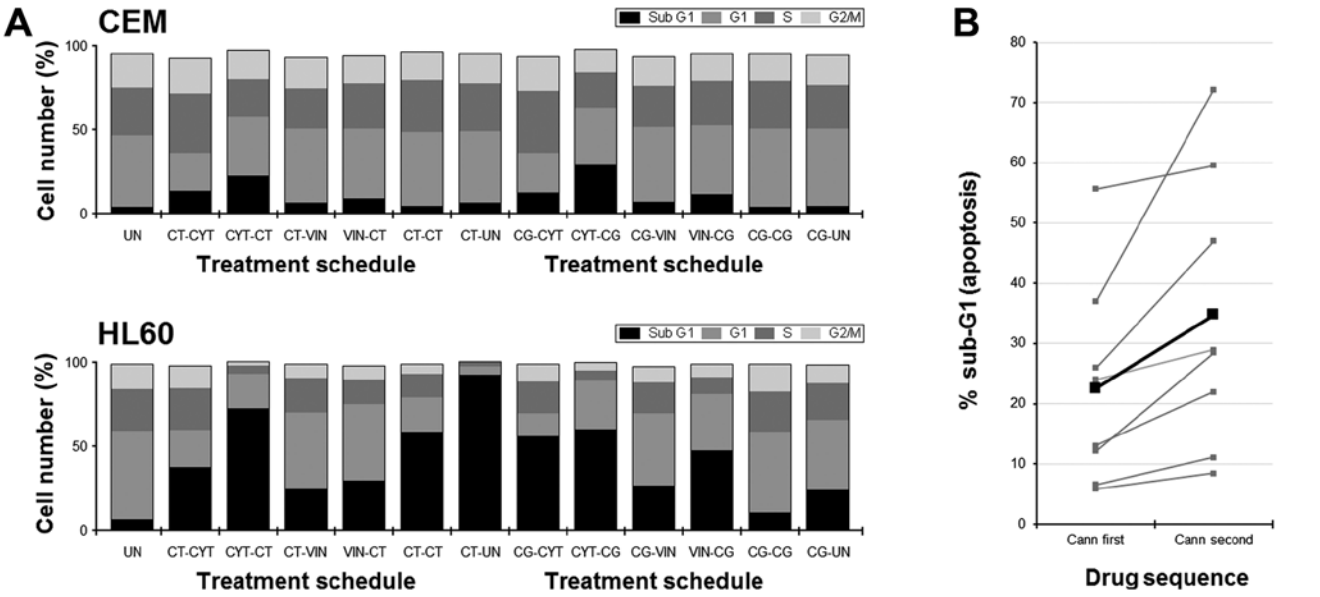

Figure 5. Effect of drug sequence on the cell cycle. CEM and HL60 cells were cultured according to schedules consisting of two distinct treatment stages lasting 48 h each. Treatments consisted of a cannabinoid - either CBD+THC (CT) or CBD+CBG (CG) in the first stage, followed by cytarabine (CYT) or vincristine (VIN) in the second. Parallel cultures were also performed in which the sequence of drugs was reversed. In some instances, cells were untreated (UN). Cell cycle distribution was then assessed by flow cytometry at 96 h (A). The specific effect on % sub-G1 cells using any regimen where a cannabinoid was used first was compared with those in which a cannabinoid was used second (B).

cells in sub-G1. Typically, using CYT or VIN before a CANN-pair resulted in a greater amount of cells in sub-G1 compared to schedules in which the order of drugs was reversed (Fig. 5A). In HL60 cells for example, % sub-G1 was 37% if CBD/THC

was used before CYT, but 72% if CBD/THC was used after CYT. Furthermore, paired t-test of all the data, irrespective of cell line and drug used, showed that significantly more apoptosis was seen if the order of treatment entailed a CANN after chemotherapy (Fig. 5B).

In accordance with our earlier published data, the greatest number of cells present in a sub-G1 population was seen following the schedule where HL60 cells were cultured with CBD/THC in the first phase of treatment followed by no treatment in the last phase (92%), in imitation of a recovery phase. This was considerably higher than the percentage seen when the cells were cultured with CBD/THC in both rounds of treatment (66%) (Fig. 5A).

Discussion

This work was a continuation study performed to investigate further the effects that CANNs may have on leukaemia cells. Our earlier studies showed that a number of CANNs were capable of eliciting death in cancer cells when used alone or in combination with each other; however, the benefits of using these with pre-existing chemotherapy drugs had not been investigated. In the current study, we showed that combining CANNs with the anti-leukaemia agents CYT and VIN resulted in enhanced overall activity. Furthermore, cooperation between CANN and chemotherapy was sequence-dependent, with a greater level of cell killing seen when the CANNs were used after the chemotherapy.

There is an increasing body of evidence showing that CANNs derived from the cannabis plant possess anticancer activity (12). A number of *in vitro* and murine models have shown that the CANNs CBD and THC can alter the way that tumour cells proliferate, as well as increase the capacity of these cells to undergo death by apoptosis and/or autophagy. These effects appear to be both dependent and independent of signalling via their cognate CBR (13,20). More recently, in the context of glioma, CANNs have also been shown to enhance the action of other treatment modalities such as chemotherapy and irradiation *in vivo* (7,8).

Although anticancer studies involving CANNs have rightly concentrated on cancers of the brain (21), a number have focused on their efficacy in leukaemias (13,22,23). Earlier investigations studied the action of THC alone in leukaemia, but limitations to the dosages that could be used in patients due to the psychoactivity associated with its use, made THC unattractive. As a result, this hindered its development as a putative form of therapy. Nonetheless, the concept of using cannabis-derived substances in leukaemia was revealed. Research using the non-psychoactive CANNs then rapidly followed, which recapitulated the results seen with THC. The above also revealed that combining a number of these minor CANNs could result in responses that were more active than if the individual CANNs were used separately (14,23).

These studies fully support the possibility that mixing CANNs could result in a product that is optimised for anticancer effect. Crucially, it is important to note that not all the individual components of a combination need to elicit a direct cytotoxic effect, but instead can merely support the effect of its corresponding pair/partner. This cooperative phenomenon has been described using a number of terms such as an entourage-effect, a bystander-effect and a compensatory-effect; however, the overall effect for a combination is simply to induce a measurable response that is greater than the sum of component's individual ones (24). The resulting synergy would be clinically attractive; not only because of an overall increase in general activity, but also because this improvement would have arisen concomitantly with a reduction in the dosages of the individual drugs. Associated with this reduction in dose is the potential easing of adverse effects that typically accompany the usage of the individual drugs. A number of recent clinical trials involving CANNs have tested the efficacy and/or safety of Sativex™, which is a proprietary product composed of equal amounts of CBD and THC (www.clinicaltrials.gov: NCT01812603 and NCT01812616). The consideration being that both CANNs possess anticancer action, and so using them concomitantly would maximise the chances of a positive effect. These trials are expected to report soon.

In our current studies, initial experiments were performed to assess the activity of various CANN pairs and to identify the most active mix. Our results suggested that pairs comprising CBD were most active. In agreement with our earlier results, pairing CBD with CBG was as active as CBD with THC. The mechanism of this cooperative interaction between CANNs is unknown, but may simply be a reflection of the sum of the anticancer properties of the individual agents used (13,25,26). However, there may also be activation of other unique processes following the use of two CANNs, as an earlier study of ours showed a distinct number of genes were activated only when CBD and THC were used together, and not when they were used separately (27). These involved a number of cell cycle and apoptosis genes, suggesting distinct pathways that may become engaged when the two CANNs were used. Understanding these interactions may offer ways of developing new treatment strategies and regimens to best utilise this class of drug. Generally, HL60 appeared the more sensitive of the cell lines tested. The reason for this difference could be due to the higher expression of CBR-2 in HL60 compared to CEM (13), or simply due to differences in the intrinsic background of intracellular signalling pathways in both, which we have previously shown to be different (14). This highlights the potential caveats of selecting the cancers best suited to CANN treatment.

After confirming that these CANNs could be paired without a loss of anticancer action, we next mimicked the current clinical path by assessing the effect of combining CBD/THC with common anti-leukaemia drugs. We first determined the value of using the CANN pair and chemotherapy at the same time, and results showed clear improvements in the cytotoxic response. This was indicated by significant improvements in the IC_{50} of CYT and VIN if CBD/THC was included in the treatment. For example, in HL60 cells, the IC_{50} for VIN was 20 nM; however, this was reduced to 3.2 nM if a sub-toxic dose of CBD/THC was used with it. Furthermore, improvements in the IC_{50} were associated with increases in apoptosis. Generally, substituting the CBD/THC pair with CBD/CBG had little effect on the IC_{50} for CYT and VIN; however, the IC_{50} values were reduced and chemotherapy efficacy improved in some instances.

The sequence in which certain drugs are administered can influence the overall activity of a treatment course for

a number of cancers (28,29). This should be an important consideration in any treatment plan as one drug can influence the action/activity of others. These interactions can be both beneficial and detrimental to the outcome of the treatment, and as such, the order of administration should be optimised. When these interactions are favourable, it is conceivable that one drug alters the biology of tumour cells to render them more susceptible to another. For example, it has been suggested that in response to the inhibition of topoisomerase I by the drug camptothecin, the related enzyme topoisomerase II is increased. Thus the use of the drug etoposide after camptothecin may be fruitful as the specific target of its action is topoisomerase II (30). In addition to this compensatory phenomenon, some drugs can work to prime cancer cells to the cytotoxic action of others by promoting apoptotic pathways. BH3 mimetics serve in such a manner, by removing the 'brakes' in the form of proteins such as BCL-2 and BCL-xL, that obstruct apoptosis (31). Therefore, a treatment regimen could be designed that uses these agents first to lower the threshold for apoptosis, before using a second drug specifically chosen to elicit a death signal. Equally, drugs that influence the cell cycle and modulate the restriction points within it, may increase the sensitivity of tumour cells to other treatments in a sequence-dependent manner (32). Importantly, these drugs can take the form of dedicated cell cycle inhibitors or those that, through their intrinsic mechanism of action, disturb the cell cycle (33).

In addition to their cytotoxic features (12), CBD and THC are able to directly impede the cell cycle through modulation of the cyclin-dependent kinase inhibitor $p21^{waf1}$ and p27 (14,34). We therefore hypothesised that any possible benefit by combining them with other drugs could be influenced by treatment sequence; specifically that these could influence any possible benefit of combining CANNs with other drugs, particularly those that act on the cell cycle like the anti-leukaemics. As such, we assessed the level of activity in the cell lines treated with CANNs and chemotherapy when used sequentially. Results showed sequence of administration was important, and that significantly greater amounts of apoptosis was seen when the CANN was used after the chemotherapy.

In summary, our data showed that a number of CANNs could be used together in pairs to generate anticancer responses that are greater than would be expected if the components were used separately. These CANN pairs can then also be combined synergistically with common anti-leukaemia agents. Importantly, results also suggested that the sequence of the drugs may be crucial in determining the clinical activity of combination treatment regimens involving CANNs. Specifically, our studies recommend that if CANNs are to be combined with other anti-leukaemia drugs, that they should be used either concomitantly or after them. In conclusion, evidence of CBD activity in patients with certain forms of cancer linked with a considerable body of evidence *in vitro*, support the overall concept that these plant-derived CANNs are valid therapeutic compounds. However, until clinical trials that test their value in an oncological setting are completed and reported, reticence will always remain. Ultimately, using information from evidence-led *in vitro* studies is the best way to predict and determine the treatment combinations and approaches for CANNs that have the best chance to translate successfully to the clinic.

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