

Enhancing the Activity of Cannabidiol and Other Cannabinoids *In Vitro* Through Modifications to Drug Combinations and Treatment Schedules

KATHERINE ANN SCOTT, SINI SHAH, ANGUS GEORGE DALGLEISH and WAI MAN LIU

Department of Oncology, Division of Clinical Sciences, St. George's, University of London, London, U.K.

Abstract. *Cannabinoids are the bioactive components of the Cannabis plant that display a diverse range of therapeutic qualities. We explored the activity of six cannabinoids, used both alone and in combination in leukaemic cells. Cannabinoids were cytostatic and caused a simultaneous arrest at all phases of the cell cycle. Re-culturing pre-treated cells in drug-free medium resulted in dramatic reductions in cell viability. Furthermore, combining cannabinoids was not antagonistic. We suggest that the activities of some cannabinoids are influenced by treatment schedules; therefore, it is important to carefully select the most appropriate strategy in order to maximise their efficacy.*

“Cannabis” is an umbrella term for the chemically diverse products that are derived from the *Cannabis* sp. plant. For many centuries, it has been used as a herbal remedy for a variety of ailments such as gout and rheumatism, and in more contemporary times has been recognised as a potent sedative with anaesthetic quality and as an anti-emetic (1). Recent years have also seen growing evidence supporting a role for cannabis-derived substances in cancer therapy (2). These substances that are collectively referred to as the cannabinoids, number over 100 chemicals that possess varying degrees of biochemical activity (3). Although a number of these chemicals are precursors of the major cannabis component, Δ^9 -tetrahydrocannabinol (THC), they also display their own biological activity (4).

It is widely known that THC possesses potent psychoactive properties that results in cannabis being used recreationally (5). However, it and other prominent cannabinoids such as

cannabidiol (CBD), which is non-psychoactive, also have anticancer properties, which make them effective in a wide spectrum of tumour cells both *in vivo* and *in vitro*. This includes potent effects in leukaemia both as single agents and in combination with conventional chemotherapies (6, 7). Although this feature of cannabinoids has been known for some time, their exact mechanisms of action remain largely unknown. A number of possible mechanisms of action have been postulated, which involve the complex interplay between intracellular signalling pathways that underpin cellular proliferation, survival and death. These pathways include signalling cascades such as mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) (8) and phosphoinositide-3 kinase (PI3-K) (9), and disruptions in their functioning can result in profound effects that impede cell functioning. Not only are cell growth and death disrupted, the effect of cannabinoids on these influential signal transduction pathways can also affect diverse processes such as angiogenesis (10) and inflammation (11), which are reliant upon competent signalling through these pathways.

There is some doubt as to the importance of the cannabinoid receptors in mediating drug action in the context of cancer. The effects of the cannabinoids are both dependent and independent of their cognate receptors, of which there are a number, and the degree to which cell function is influenced and determined by them *via* each route is unclear (6, 12, 13). Given that psychoactivity has been associated with receptor activation (14), minimising this route of action may be favoured; however, it is unknown whether this would hamper any therapeutically-desirable effect. Fortunately, individual cannabinoid have different binding affinities for the cannabinoid receptors (15), and so it is possible that different cannabinoids could be utilised more carefully to ensure that useful anticancer action is maintained whilst simultaneously minimising the undesirable effects associated with cannabis in general. This may include combination strategies with these cannabinoids, where similar physiological effects could be maintained whilst reducing the doses of each individual drug.

Correspondence to: Wai Liu, Ph.D., Dept of Oncology, Division of Clinical Sciences, St. George's, University of London, 2nd Floor, Jenner Wing, London, SW17 0RE, U.K. Tel: +44 02087255037, e-mail: w.liu@sgul.ac.uk

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Although there have been a number of studies reporting the anticancer effect of prominent cannabinoids such as THC and CBD, information regarding the minor cannabinoids are lacking. The current study was, therefore, initiated to examine the cellular effects of a collection of cannabinoids used alone and with each other in a leukaemia setting. This included alterations to the combinations of the cannabinoids as well as changes in the sequence of administration. Additionally, the underlying molecular mechanism of actions of the cannabinoids was also investigated.

Materials and Methods

Cell culture. The human cancer cell lines CEM (acute lymphocytic leukaemia) and HL60 (promyelocytic leukaemia) were purchased from the European Collection of Cell Cultures (Salisbury, UK), and grown in RPMI-1640 (Sigma-Aldrich Company Ltd., Dorset, UK) medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin. All cell lines were incubated in a humidified atmosphere with 5% CO₂ in air at 37°C, and discarded every six weeks.

Drugs. A total of six cannabinoids were studied, CBD, cannabigerol (CBG) and cannabigeravarin (CBGV) in their neutral forms and their acid form consisting a carboxy group on position 6 of the benzene ring of the cannabinoid framework (designated with an 'A'; all provided by GW Pharmaceuticals Ltd., Salisbury, UK). All cannabinoids were dissolved in ethanol and final solvent concentrations in cell cultures were <0.1%.

Proliferation assays. To study the effect of each agent on cell growth, cells growing exponentially were added to 96-well plates at a density of 1.5×10⁴/well. Cannabinoids (0.1-100 μM) were then added to the wells, ensuring an equal volume of 200 μl across the plate. Cell number was measured at 24, 48 and 72 h using a methylthiazolotetrazolium (MTT)-based assay according to methods previously described (16). In addition to these MTT assays, cells growing exponentially were seeded at a density of 1×10⁵/ml in the presence of each of the cannabinoids used at a concentration capable of inhibiting cell growth by 50% (IC₅₀); these were established by the earlier MTT assays. Aliquots were removed daily for the assessment of cell number and viability by the method of trypan blue dye exclusion and cell cycle distribution by flow cytometric analysis.

Flow cytometric analysis of the cell cycle. Cells were harvested and washed in ice-cold PBS prior to fixation in 70% (v/v) ethanol in PBS. Following an incubation period of at least 30 min, cells were re-washed and re-suspended in 1 mg/ml propidium iodide and 1 mg/ml RNase A (both from Sigma-Aldrich). Acquisition of data was performed within one hour using a Becton Dickinson FACSCalibur instrument (BD Biosciences Ltd., Oxford, UK), and gating on fluorescence width and area was employed to remove doublet artefacts and to discriminate cells from debris. The percentages of cells in each phase were determined using WinMDI v2.9 (<http://facs.scripps.edu/software.html>).

Immunoblotting analysis. Western blot analyses were performed as previously described (16). Primary antibody probing was performed

with anti-p21^{WAF1}, anti-total and phosphorylated AKT, anti-total and phosphorylated ERK, anti-CBR2 or anti p2AX. All primary antibodies were obtained from New England Biolabs, Hitchin, UK, and used at a dilution of 1:1,000, and bands were visualised by the ECL-plus detection system (Amersham Biosciences Ltd., Little Chalfont, UK).

Combination studies: fixing the ratio of the concentration of the cannabinoids. Cells (1.5×10⁴/well) growing exponentially were re-suspended in fresh culture medium and aliquoted into 96-well plates. CBD was the primary partner and was combined with each of the other cannabinoids at an equal ratio of their respective IC₅₀ (e.g. CBD at ½x IC₅₀ was combined with CBG at ½x IC₅₀). Cell numbers were then assessed after 72 h by the MTT assay as described above. The activity of each of the combinations was established by comparing optical density readings from the treated wells with the control wells with no drug treatment, and the nature of drug:drug interactions then assessed by calculating a combination index (CI) by using the median-effect equation.

Recovery studies. CEM cells growing exponentially were re-suspended at 1×10⁵/ml in the presence of 10 μM CBD (~IC₅₀). On day 3, the medium was washed-off, and the cells re-suspended in fresh culture medium with or without CBD (10 μM), and incubated for a further three days. Cell numbers and viability, cell cycle profiles and p21^{WAF1} levels were assessed daily for the five-day period. The impact of different cannabinoid sequences were also studied by culturing CEM cells according to a treatment schedule that incorporated two rounds of 2-day exposures to a cannabinoid. The periods were separated by a washing step that allowed for the removal of the first cannabinoid and a subsequent use of the same or another cannabinoid. Some treatments involved combining two different cannabinoids, in which case, each were used at half their IC₅₀ value. Cell numbers and percentage cell viability were assessed on days 2 and 4.

Results

Cannabinoids reduce cell number by being cytostatic. IC₅₀ values for each of the six cannabinoids were assessed by emax-modelling of the data attained from the MTT assays. These values were for 24, 48 and 72 h, and results showed that generally, the neutral form of the molecules were more active than their respective acid partner (Figure 1). The reductions in cell numbers caused by these compounds were corroborated by cell counting using microscopy and viability discrimination by trypan blue exclusion (Figure 1), which also showed that the reductions were not associated with loss of cell viability, suggesting the cannabinoid effects were primarily cytostatic in nature. Flow cytometric analyses revealed culturing cells with the cannabinoids resulted in no significant changes in the DNA profile (Figure 1). It was only when higher concentrations of CBD (~5x IC₅₀) were used when any increases in the sub-G₁ population were observed, which indicated cell killing (apoptosis). Taken together, these data suggested a simultaneous arrest in cell progression at all phases of the cell cycle.

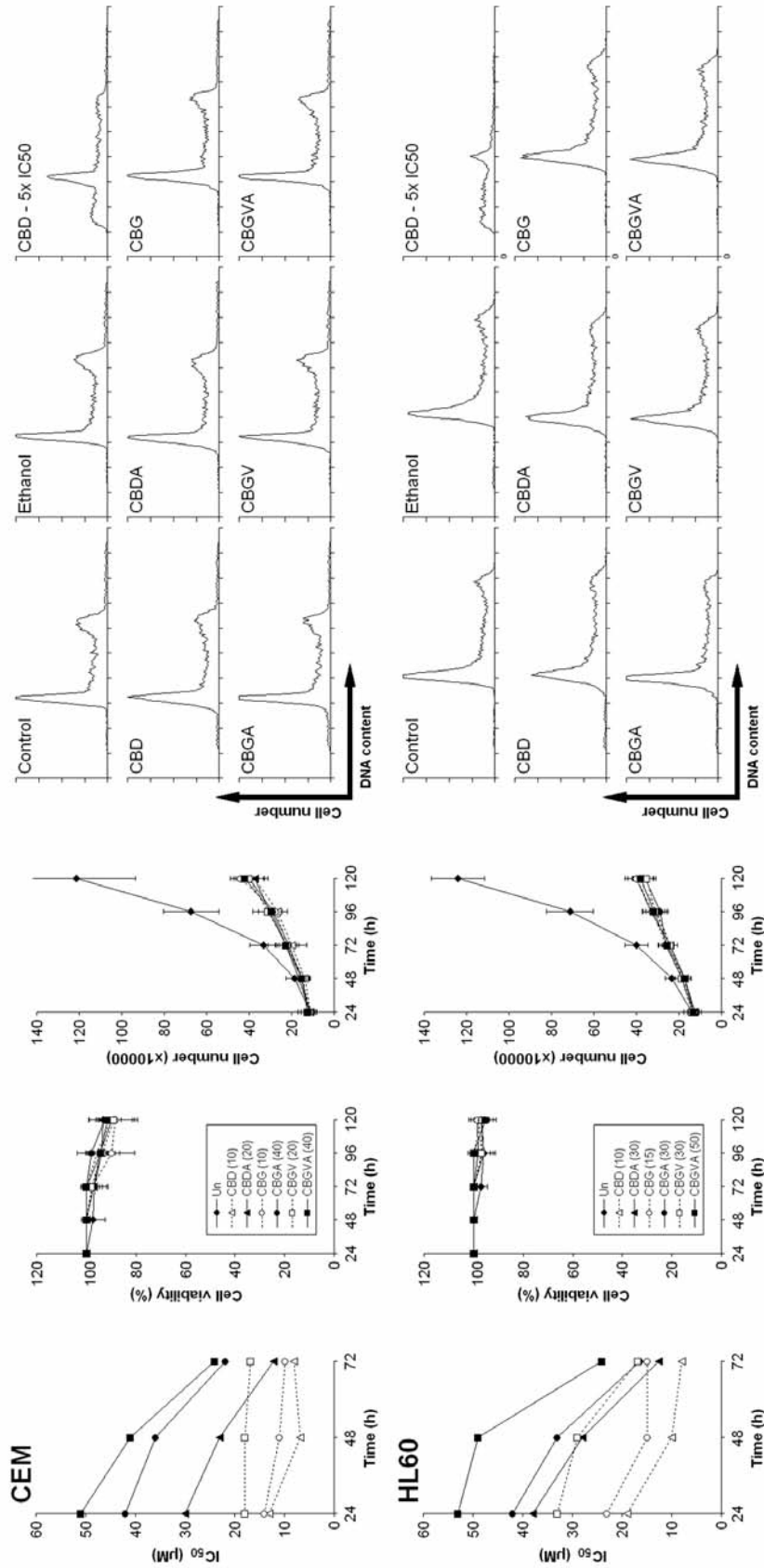


Figure 1. Effect of the cannabinoids on cell viability, cell proliferation and cell-cycle dynamics. CEM (upper panel) and HL60 (lower panel) cells were cultured with each of the cannabinoids at a concentration equivalent to their respective IC₅₀ value. This was 10, 20, 10, 40, 20 and 40 μM for Cannabidiol (CBD), Cannabidiolic acid (CBDA), Cannabigerol (CBG), Cannabigerolic acid (CBGA), Cannabigerarin (CBGV) and Cannabigerarinic acid (CBGVA) respectively for CEM, and 10, 30, 15, 30, 30 and 50 μM for HL60 cells. Cell number and viability were assessed by cell counting using trypan blue dye discrimination. Each data point is the mean and SDs of three separate experiments. Cell cycle profiling was achieved through propidium iodide staining, which revealed no clear changes to the profiles for CEM and HL60 cells treated with the cannabinoids. This suggested a general global cell-cycle arrest. However, a clear increase in the sub-G₁ population (apoptosis/necrosis) was observed in the cell lines when CBD was used at a higher concentration. Representative histograms from three independent replicates are presented.

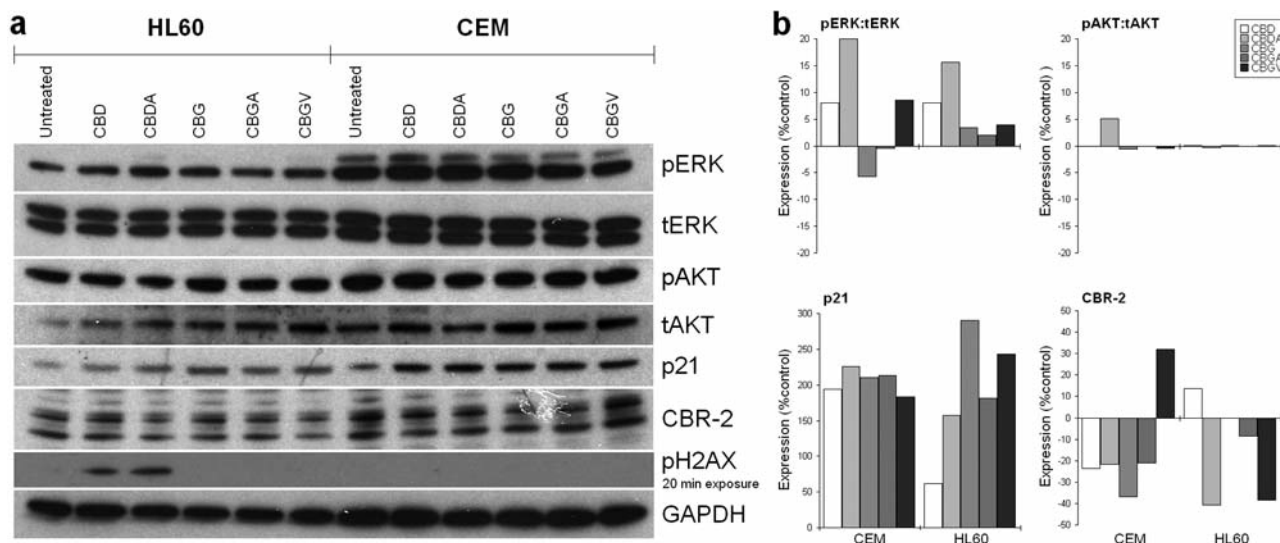


Figure 2. Effect of the cannabinoids on cell signalling proteins. CEM and HL60 cells were cultured with each of the drugs at the IC_{50} concentration for 48 h prior to western blotting for the proteins indicated. Representative blots (a), and the results of densitometric analyses (b) are presented. Each data point is the mean of three separate experiments and SDs have been omitted for clarity, but coefficient of variances for most points were <10%.

Cannabinoids alter cell signalling proteins. Cells were cultured with equi-efficacious IC_{50} doses of each compound for two days prior to assessment of the proteins by western blotting. By assessing the ratio of phosphorylated to total levels of ERK and AKT, we were able to gauge the effects that these compounds had on these central signalling cascades. The results show the cannabinoids generally increased ERK in the two cell lines, but had no significant effect on AKT (Figure 2). The most striking result was the dramatic increase in p21^{WAF1} levels in the cell lines following exposure to any of the cannabinoids. Furthermore, these increases were not necessarily in response to DNA damage, as blots also showed that apart from CEM cells treated with CBD and CBDA, pH2AX was virtually absent.

Cytotoxicity can be achieved through a break in treatment (recovery phase). Previous studies of our group have described the ability of some drugs to interfere with the capacity of cells to undertake cell death through a protracted drug-mediated-cell cycle arrest (16). We, thus, cultured CEM cells with CBD for two days before removal of the drug, allowing for cell recovery in CBD-free medium for a further three days. Results showed re-culturing CBD-treated CEM cells in CBD-free medium caused significant decreases in cell viability (viability on day 3: $81 \pm 4.4\%$ vs. $95 \pm 3.4\%$ in control cells where the medium with CBD was refreshed; $p < 0.001$) (Figure 3a). These reductions occurred as soon as cells were allowed to recover and were associated with significant increases in sub-G₁ events and a concomitant fall in the number of cells with the G₁-phase of the cell cycle

(Figure 3b). Furthermore, p21^{WAF1} levels in the cells on the days post-recovery were significantly lower than in the cells remaining in medium containing CBD (Figure 3c).

CBD acts non-antagonistically with other cannabinoids to reduce cell number. We used MTT assays to establish for cannabinoid-combination activity, and calculated CI-values as a way of determining the nature of any interactions. CBD was our principal drug partner, and was combined with the one of the other cannabinoids at equi-active doses governed by fractions of their IC_{50} s. For example, in CEM cells, the IC_{50} s for CBD and CBGA were ~ 10 and ~ 40 μM respectively; and thus they were combined at a fixed ratio of 1:4. Dose-response curves for the agents used separately and combined together were then generated and IC_{50} -values input into the median effect equation for the generation of CI-values (Figure 4a). The results indicate the combinations tested were generally additive in nature, and that some combinations were mildly synergistic (Figure 4b).

Cannabinoid activity is influenced by drug combination and treatment schedule. Having shown an advantage of a recovery phase in increasing the cytotoxicity of CBD, as well as the possible synergy from combining cannabinoids, we next tested the benefits of treatment schedules that consolidated these approaches. The results show that in any of the schedules that included a cannabinoid, the numbers of cells on day 4 were significantly reduced compared to that seen in the untreated control schedule (Figure 4c). Furthermore, there was no significant difference in cell

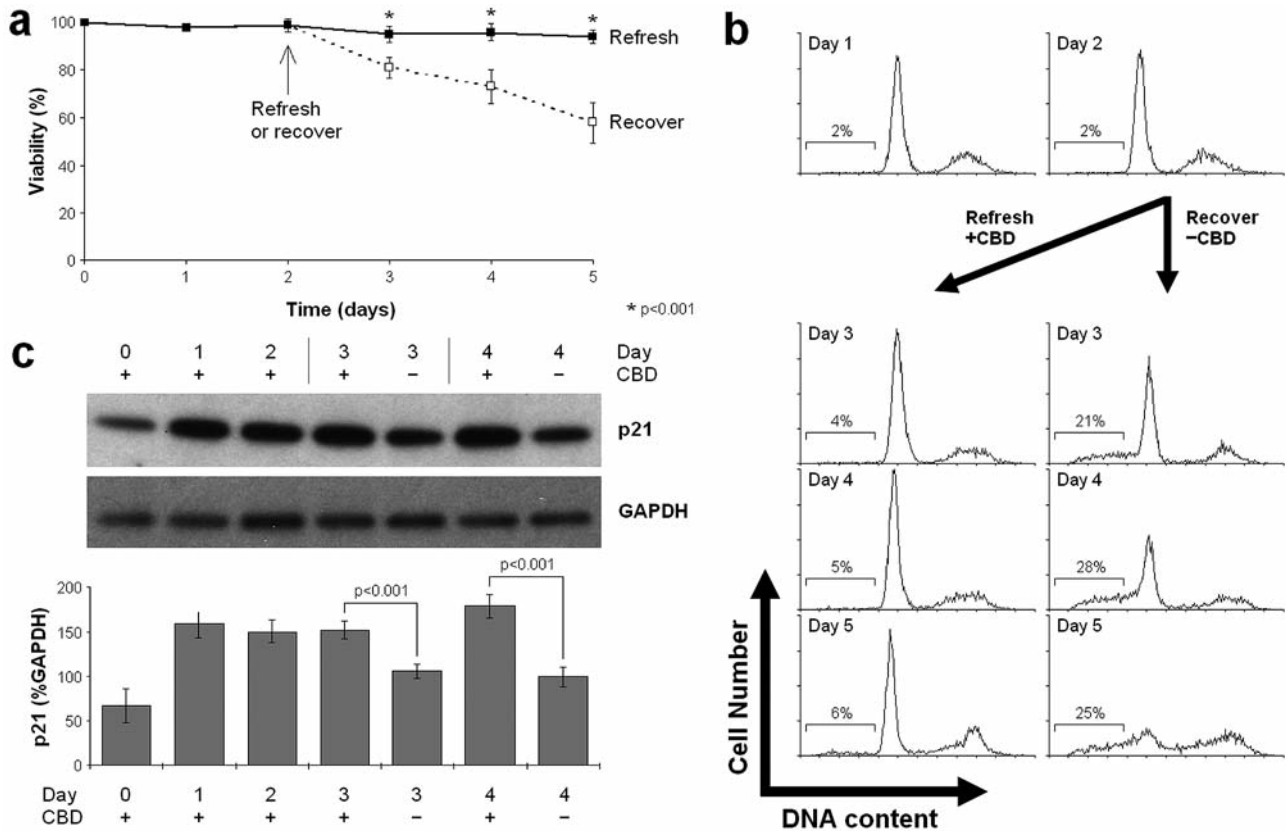


Figure 3. Effect of a drug-free period following exposure to Cannabidiol (CBD). CEM cells were cultured with 10 μ M CBD for two days before removing the drug, and then re-culturing cells in fresh culture medium supplemented with (Refresh) or without (Recover) 10 μ M CBD for three days further. Aliquots were removed daily for the analysis of cell viability (a), cell-cycle distribution (b) and p21^{WAF1} expression (c). Each data point in (a) and (c) are the means and SDs of three separate experiments. Percentages in (b) indicate the proportion of cells within the sub-G₁ (apoptotic/necrotic) population.

numbers between these cannabinoid-containing schedules. As indicated previously, cell viabilities were generally >95% in the schedules apart from those treatment schedules that incorporated a recovery phase in the last two day period of treatment (Figure 4d).

Discussion

This study was undertaken to explore the cytotoxic effect of a number of prominent cannabinoids on two leukaemia cell lines, and had a particular interest in examining the impact of drug-scheduling in determining activity. The most significant finding of the current study is that removing cells from medium-containing cannabinoids, and allowing them to recover in drug-free medium results in a dramatic increase in cytotoxicity. Furthermore, there was value in using some of the cannabinoids concomitantly, as this combination method results in additive/mildly synergistic interactions.

In the first part of our investigation, we established the IC₅₀s for each of the cannabinoids in the two cell lines. Six

cannabinoids were selected, three in their neutral and acid forms, which unlike THC, lack psychoactivity. Our principal cannabinoid, CBD, has been shown to be a good therapeutic candidate that displays good anticancer qualities for a number of solid tumour types (17-19). In the cannabis plant, CBD exists primarily in the precursor acid form which bears a carboxyl moiety, which undergoes de-carboxylation into the more familiar and active cannabinoid. However, recent studies have shown that CBDA also possesses bioactivity in its own right, being a potent cyclo-oxygenase-2 inhibitor (20), and having the capacity to inhibit the migration of breast cancer cells (21). Our studies show that in all cases, the neutral form of the cannabinoids were more active than their respective acid counterpart, and that the two cannabinoids with the greatest activities were CBD and CBG, with IC₅₀s at 48 h of about 7 and 10 μ M, respectively. These were, thus, taken forward into our subsequent investigations. The lesser studied cannabinoids, CBGA, CBGV and CBGVA, also possessed activity at micromolar concentrations.

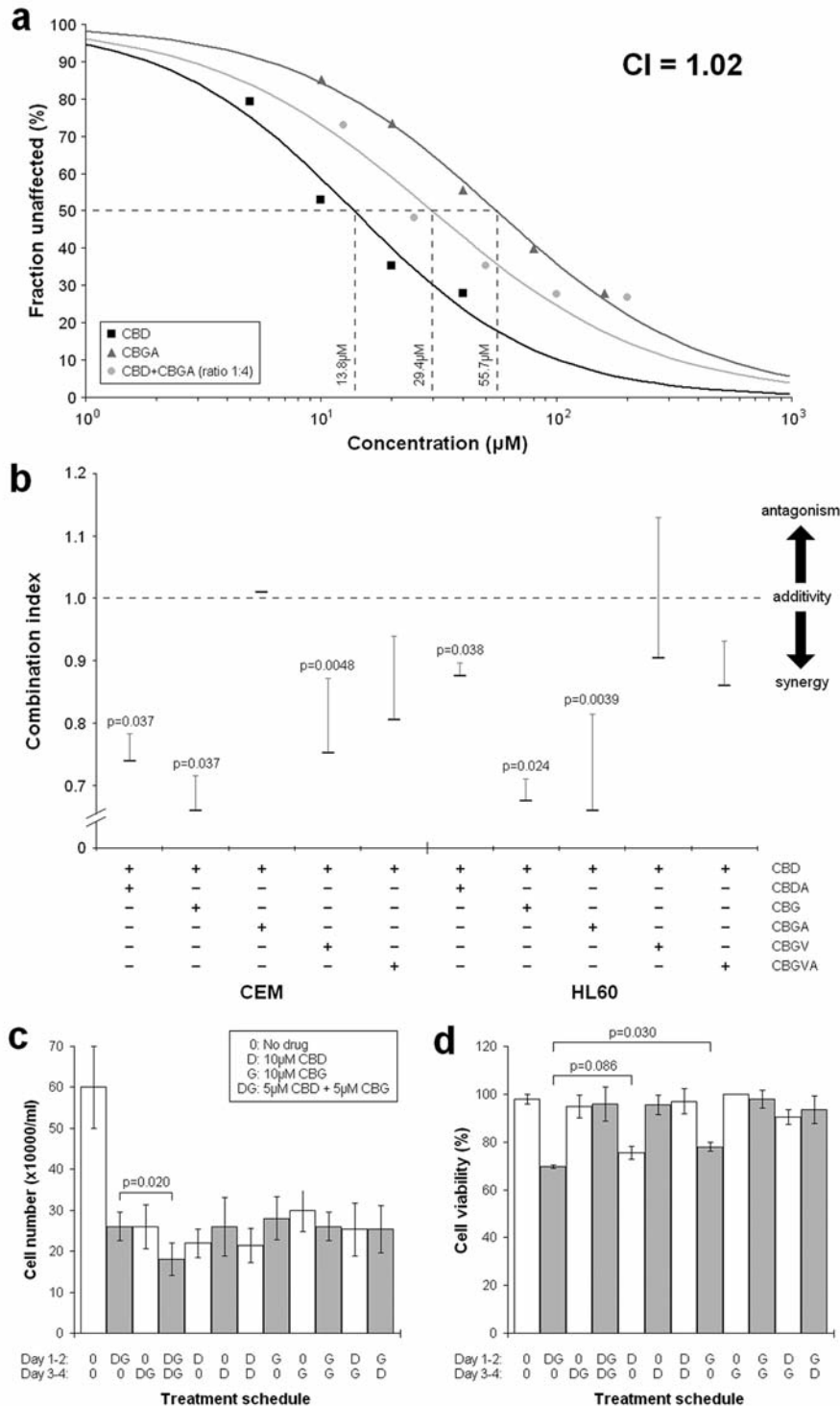


Figure 4. Effect of combining Cannabidiol (CBD) with other cannabinoids in CEM and HL60 cells. Cells were cultured concomitantly with CBD and one other cannabinoid for 48 h before assessing cell numbers. Non-exclusive combination index (CI) values for the 50% unaffected fraction (FU) were calculated. CI-values >1 indicates antagonism; CI=1 additivity and CI<1 synergy. Representative response curves in CEM cells and the extrapolated IC_{50} -values for CBD, Cannabigerolic acid (CBGA) and a combination of the two drugs (combined at equi-molar fractions of respective IC_{50} values, which was at a ratio of 1:4) are shown (a). The complete data set for the combinations in CEM and HL60 cells are presented (b). CEM cells were also cultured according to twelve distinct treatment schedules involving 10 μM CBD (D), 10 μM CBG (G) or a combination of 5 μM of each (DG). Each schedule consisted of two rounds of treatment separated by a wash step, and each round lasted for two days. Cell number (c) and cell viability (d) were assessed on the final day of treatment. Each data point represents the mean and SD of three separate experiments. P-values are for paired t-tests.

The reduction in cell number was due to a cytostatic effect, which was supported by our data showing increased levels of p21^{WAF1}. This cyclin-dependent kinase inhibitor is primarily transactivated by p53 in response to DNA damage. However, cannabinoids are not known to damage DNA directly (6), and this was a point we verified by probing for pH2AX, a marker of DNA damage (22). Crucially, the two cell lines we used were either null or mutated in p53 expression, so increases in p21^{WAF1} would be independent of p53. In addition to modulating the cell cycle, p21^{WAF1} is also intimately involved in regulating cell growth and death (23), and its overall function is controlled by intracellular signalling (24, 25). We, thus, inspected the expressions of pAKT and pERK to see if alterations could account for the changes in p21^{WAF1} expression. Our results showed pAKT levels to be unchanged in cells treated with cannabinoids; however, pERK levels were altered by them. Furthermore, the extent to which pERK was altered varied with the cannabinoid used.

We have previously shown that cell-cycle arrest can actually be maintained inadvertently by treatment to the extent that cell death cannot proceed (16, 26). In these situations, cytotoxicity can be restored if the treatment regimen is altered to include a 'recovery' phase by culture in drug-free medium, as this allows the cell to re-engage cell cycling and thus undergo cell death. Results showed that reculturing CEM cells that were initially treated for two days with a cannabinoid in drug-free medium resulted in a decrease in cell viability. This suggests that including a recovery phase in treatment schedules alters the mechanism causing a reduction in cell number from a cytostatic mechanism to a cytotoxic one. This re-activation of cell killing presents the possibility that sequential administration of these cannabinoids with conventional cytotoxic agents may enhance cell killing. Our own studies in leukaemia cells already support the combining of THC with conventional anti-leukaemia agents (6, 7), and so testing these approaches by substituting THC with CBD and the other cannabinoids would be interesting.

The purpose of combining drugs in therapeutic regimens is to achieve an overall effect that is greater than the sum of the individual effects of each agent. Combinations that result in no loss of overall effect (antagonism) would also be clinically valuable, as a similar level of activity would be achieved at lower doses of the individual drugs. This lowering of doses could also lead to fewer adverse events. Although the main activities of CBD seem to imitate those of THC, they occur independently of the receptors. This suggests that combining CBD with THC should result in a good level of anticancer activity exerted by CBD and THC, and a diminished psychotropic effect of THC through antagonism of the receptor by CBD. Even though there is a crossover in effects between CBD and THC, there appears

to be sufficient redundancy and diversity in their activities that allows for improved activity when used together (27). Equally, the similarities in functions indicate combinations should involve the other cannabinoids. The results of our median-effect approach (28) indicate there was no antagonism between any of the cannabinoids; this combination approach suggests that equal activity can be achieved by using smaller doses. For example, to achieve 50% cell kill in CEM cells, ~14 μ M of CBD or ~56 μ M of CBGA would be required if used on their own. However, if CBD and CBGA were used together, the concentrations required to achieve the same 50% cell kill would only be ~6 μ M of CBD and ~24 μ M of CBGA.

In conclusion, this study adds further support to the idea that cannabinoids can have a role in the cancer setting, not only as single agents, but also in combination with each other. Our findings indicate that cannabinoids act with each other in a way such that doses for therapy could be reduced without a significant loss of activity. Additionally, these data also showed the cytostatic nature of the cannabinoids hampered cell killing; however, cytotoxicity was restored when a pulsed-schedule was adopted. It is important that this observation be expanded so the benefits of recovery phases are more fully understood, which will ultimately help us to fashion ways of developing new treatment strategies that utilise this class of compound.

Conflicts of Interest

KAS, SS and AGD have no conflict of interests. WML is in receipt of research funding from GW Pharmaceuticals Ltd.

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