Cannabidiol and Cannabigerol Cooperatively Regulation Autophagy affect Caco-2 Cell Viability

JinJuan Li[1]

1. Faculty of Human Sciences, Waseda University,2-579-15 Mikajima, Tokorozawa 359-1192, Japan;

jinjuanli1214@gmail.com;

Abstract

Cannabidiol (CBD) and Cannabigerol (CBG) are non-psychoactive cannabinoids known to affect both cancerous and non-cancerous cells. Autophagy is a critical regulator of cell survival and death; however, the impact of CBD and CBG on cell viability through autophagy remains limited. In this study, we show that low-dose combinations of CBD and CBG synergistically enhance Caco-2 cell proliferation, achieving effects comparable to those observed at higher doses. Both cannabinoids—whether applied individually at high concentrations or in low-dose combinations—activate autophagy. Correlation analyses between cell viability and autophagic flux, along with comparative assessments of wild-type and ATG9-deficient Caco-2 cells, demonstrate that the survival-promoting effects of CBD and CBG are closely associated with autophagy activation. Overall, these findings reveal that both individual and combined treatments significantly modulate Caco-2 cell viability under conditions with or without autophagy activation, emphasizing the substantial role of cannabinoid-regulated autophagy in influencing cell survival.

Keywords: Cannabidiol (CBD), Cannabigerol (CBG), autophagy, combination, cell survival

Highlights:

1. Low-dose combinations of CBD and CBG synergistically enhance Caco-2 cell proliferation.

2. Both high-dose individual treatments and low-dose combinations of CBD and CBG activate autophagy.

3. CBD- and CBG-mediated autophagy paly beneficial role in supporting Caco-2 cell survival.

Introduction

Cannabis sativa (hemp), an annual herbaceous plant of the Cannabaceae family, has been used since ancient times to relieve pain, fever, anxiety, and even to exert antitumor effects[1]. The Cannabis plant produces over 100 distinct cannabinoids. These compounds—either purified from the plant or synthetically produced—are collectively referred to as cannabinoids, including Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN), cannabichromene (CBC), and cannabicyclol (CBL). According to the U.S. Department of Agriculture (USDA) FoodData Central database, over 680 registered commercial food products contain cannabis seed derivatives, such as oils, extracts, flours, or powders [2]. This highlights the growing interest and need for in-depth studies on cannabinoids and their biological functions. Cannabinoids are widely employed for therapeutic purposes, including the management of chemotherapy-induced nausea, cancer-related pain, seizure disorders (e.g., in multiple sclerosis), and appetite stimulation in cancer patients [3]. Concerns regarding the psychoactive effects of certain cannabinoids have driven recent interest toward non-psychoactive compounds, particularly CBD and CBG. CBG, often referred to as the "mother of all cannabinoids," is a precursor molecule for the biosynthesis of many other cannabinoids[4].

Early studies suggest that oral administration of CBD and CBG improves gut health [2]. Recent studies suggest that naturally extracted CBD exhibits higher biological activity than its synthetic counterpart, possibly due to the presence of trace amounts of other cannabinoids such as CBG. This observation has sparked growing interest in the bioactivity of CBD–CBG mixtures and their potential to produce a synergistic "entourage effect" [5, 6]. Most existing research has focused on the use of high concentrations of these compounds in cancer models, where they modulate multiple signaling pathways involved in tumor progression and exhibit notable anticancer effects[7, 8]. However, few studies have investigated the cell-activating regulatory effects of low-concentration CBD and CBG or their combinations. This gap in research may limit their broader therapeutic applications. Cannabinoid combinations may yield additive, antagonistic, or synergistic effects depending on concentration and cellular context, but the underlying mechanisms remain poorly understood. Further investigation is needed to elucidate how CBD and CBG interact at sub-cytotoxic concentrations to influence cell fate, especially under stress conditions. Synergy quantification models, such as the Zero Interaction Potency (ZIP) model, are commonly used to assess drug interactions by comparing observed combination effects against a predicted baseline[9]. The ZIP model assumes synergy when the dose–response curve of one compound remains unaffected by the addition of another. Despite its utility, no studies to date have applied the ZIP model to evaluate the interaction between CBD and CBG, leaving an important gap in understanding their combined pharmacodynamics.

CBD and CBG have also been reported to regulate autophagy, a cellular degradation and recycling process that plays complex roles in both normal and cancer cells[7, 10]. Autophagy involves the formation of double-membraned autophagosomes that deliver cytoplasmic materials, including damaged organelles, to lysosomes for degradation[11]. This process allows cells to recover essential energy and maintain homeostasis under metabolic stress [12]. Therefore, significant metabolic differences are expected between conditions with and without autophagy activation. However, limited studies have compared the effects of various concentrations of CBD and CBG on cell viability under autophagy-

inducing versus non-inducing conditions. The precise relationship between cannabinoid-induced autophagy and cell viability remains poorly understood[13]. Some studies report that CBD activates protective autophagy. For instance, autophagy induced by CBD has been shown to enhance cell survival in SH-SY5Y neuroblastoma cells [14, 15], and to involve p53-dependent protective mechanisms in HCT116 cells [16]. Meanwhile, CBG has been reported to induce autophagic cell death via inhibition of the EGFR–RAS signaling pathway in pancreatic cancer cells[17]. Despite these findings, most current research has focused on the effects of CBD- and CBG-induced autophagy in cancer cells, while their impact on non-cancerous cells remains poorly understood. Understanding the functional consequences of CBD- and CBG-mediated autophagy is therefore crucial. However, no studies to date have employed autophagy-deficient models, such as ATG gene knockouts, which limits our ability to determine whether CBD- and CBG-regulated autophagy exerts a protective or detrimental effect on cell survival.

This study utilized Caco2 (colorectal adenocarcinoma) cell lines to evaluate the effects of CBD and CBG across various cell types. Autophagic flux was analyzed in multiple cell types expressing the GFP-LC3-RFP probe. A checkerboard assay was conducted in Caco2 cells to assess how CBD and CBG regulate combination autophagy and cell viability activation. The Zero Interaction Potency (ZIP) model was employed to quantify the synergistic or antagonistic interactions between CBD and CBG in combination. Correlation analyses between cell survival and autophagic flux, along with experiments using Caco-2 ATG9-deficient cells, were performed to evaluate the impact of CBD- and CBG-regulated autophagy on cell survival. These findings provide insights into how CBD and CBG, at different concentrations and alone or in combination, modulate Caco-2 cell viability under both autophagy-activated and non-activated conditions.

Method

Reagents

Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L glucose), a stabilized penicillin-streptomycin solution, bovine serum albumin, a cocktail of protease inhibitors, and a cocktail of phosphatase inhibitors were sourced from Nacalai Tesque Inc (Kyoto, Japan). Puromycin dihydrochloride (AG-CN2-0078) was acquired from Adipogen Life Sciences (San Diego, CA, USA). Fetal bovine serum (FBS**)** and FluoroBrite™ DMEM (A1896701) were obtained from Thermo Scientific Inc. (Waltham, MA, USA). BCA Protein Assay Kit were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Bafilomycin A1 and Torin1 were sourced from Cayman Chemical Co (Ann Arbor, MI, USA). Lipofectamine™ 2000 Transfection Reagentwas acquired from Invitrogen (Carlsbad, CA, USA). pMRX-IP-GFP-LC3-RFP (RDB14601) were purchased from the RIKEN BRC DNA BANK (Tsukuba, Japan). Artificially synthesized pure CBG as well as CBD (Nihonbashi Odenmacho, Chuo-ku, Tokyo, Japan), were dissolved in DMSO. Phospho-p70 S6K (#97596) and 4EBP1 (#9644) were obtained from Cell Signaling Technology Inc (Beverly, MA, USA). The mouse polyclonal antibody for β-actin (C4; sc-4778) were sourced from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). HRP-conjugated antibodies specific to mouse or rabbit IgG were purchased from Millipore Inc (Billerica, MA, USA).

Drug matrix design

The checkerboard assay is a widely utilized drug screening method that evaluates the interactions between two compounds by systematically arranging their concentration gradients in a cross-pattern format. In this study, CBD (0.01, 0.1, 1, 10, 100 μM) and CBG (0.01, 0.1, 1, 10, 100 μM) were also assessed individually or in combination in the checkerboard assay for 24 hours to evaluate their effects on Caco2 cells.

Cell culture and transfection

Caco2 (human colon cancer cells) (ATCC, Manassas, VA, USA) were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Additionally, the Caco2 cell medium was supplemented with 2 mM L-glutamine. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and 95% humidity.

The expression plasmids containing ATG9 complementary DNA were purchased form Obio (Shanghai, China). Lentivirus was generated by transfecting HEK293T cells with a lentiviral vector, psPAX2 (provided by D. Trono, Ecole Polytechnique Federale de Lausanne), and pCMV-VSV-G (provided by R.A. Weinberg, Whitehead Institute for Biomedical Research). For retrovirus transfection, HEK293T cells were transiently transfected with a retroviral vector, pCG-VSV-G, and pCG-gag-pol (gifts from T. Yasui, Osaka University), and the virus was collected from the supernatant as described. Plasmid transfections were conducted using Lipofectamine 2000 (11668019; Thermo Fisher Scientific) following the manufacturer’s instructions. Following retrovirus or lentivirus infection, stable transformants were

selected with puromycin (Sigma-Aldrich).

WST-8 assay

WST-8 (also known as 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,3,5-tetramethyl-1H-pyrazol-1-yl) tetrazolium chloride) (CCK-8, Sigma Aldrich) is added to the cell culture medium. Viable cells with active dehydrogenase enzymes reduce WST-8 to an orange-colored formazan dye. The amount of formazan dye produced is directly proportional to the number of viable cells.

Caco2 cells (0.5×10^4 cells/mL) and their ATG9-deficient cells were seeded in 100 µL of 10% FBS-fresh medium in each well of 96-well plates (BioTek Instruments, Winooski, VT, USA) and incubated for 24 hours at 37 °C with 95% humidity and 5% CO_2. Subsequently, the medium was replaced with 100 µL of medium containing various concentrations of CBD and CBG, selected based on range-finding tests. Subsequently, the plates were incubated at 37 °C for 24 hours. For each experiment, the following controls were included: a background control (wells containing only medium, but no cells to measure the absorbance of the WST-8 reagent alone), a low control (wells containing cells exposed only to the medium, to determine baseline absorbance), a high control (wells containing cells treated with a known cytotoxic agent, to establish maximum absorbance), and solvent controls (2.5% methanol). Subsequently, 10 µL of WST-8 solution, a colorimetric reagent, was added to each well incubated for two hours at 37 °C. The WST-8 reagent is reduced to a water-soluble formazan dye by cellular dehydrogenases, which correlates with the number of viable cells. The absorbance of the formazan dye was quantified spectrophotometrically at a wavelength of 570 nm. To account for any non-cellular contributions, the absorbance values from the background control wells were subtracted from all other measurements.

Generation of GFP-LC3-RFP Expressing Caco2 Cells for Autophagic Flux Analysis

HEK293FT cells were co-transfected with pMRX-IP-GFP-LC3-RFP, puro-ATG9, pCG-VSV-G, and pCG-gag/pol [18]. After 72 hours of cultivation, the medium containing the virus was collected and incubated with Caco2 cells for 48 hours. As a positive controls, 1µM Torin (an autophagy inducer) was used, and as a negative control, 200µM Bafilomycin (an autophagy inhibitor) was used. After treatment with drugs or solvent, cells were collected, and fluorescence intensity was measured using the Cellometer® Vision (Nexcelom Bioscience LLC, Lawrence, MA, USA). In this probe , the lipidation of microtubule-associated protein 1 light chain 3 (LC3-II) and visualize the tandem green fluorescent protein (GFP)-LC3-red fluorescent protein (RFP) [19].When autophagy is activated, the probe is cleaved by endogenous ATG4, leading to GFP-LC3-II degradation, which enables quantification of autophagic activity. The stable RFP-LC3-II serves as an internal control, allowing autophagic flux assessment by comparing GFP/RFP fluorescence. To calculate the fluorescence intensity of the GFP-LC3-RFP probe inside the cells, the GFP/RFP ratio (GFP: λ ex: 460–490 nm, λ em: 500–550 nm; RFP: λ ex: 530–560 nm, λ em: 570–650 nm) was used as an index of autophagic flux. As a negative control, the late-stage autophagy inhibitor Bafilomycin A1 (200 µM) blocks autophagosome-lysosome fusion, resulting in a GFP/RFP ratio > 1[20]. Conversely, the mTORC1 inhibitor Torin 1 (1 µM) was used as a positive control for autophagy induction, its treated yielding a GFP/RFP ratio < 1 [21].

Statistical analysis

The data were presented as mean ± SEM and statistical analysis was performed using an unpaired Student's t-test for comparisons between two independent groups, one-way analysis of variance (ANOVA) followed by Tukey-Kramer's post hoc multiple comparison tests for multiple comparisons. The threshold for statistical significance was set at $p < 0.05$. The synergistic effects were calculated using the Synergy finder and Zero Interaction Potency (ZIP) methods. The ZIP score was utilized to represent synergism (ZIP score >0) and antagonism (ZIP score < 0), calculated according to the standard is bologram equation[22].

Result

CBD and CBG exert dose-dependent effects on cell viability in Caco2 cells

Previous studies have primarily focused on the cytotoxic effects of high concentrations of CBD and CBG, with limited exploration of their activity at lower concentrations. To investigate whether CBD and CBG's biological activity in many doses, we first evaluated their effects on Caco2 cell viability over a 24-hour period at concentrations of 0.01, 0.1, 1, 10, and 100 μM. The results showed that CBD and CBG alone promoted Caco2 cell proliferation at concentrations of 0.01 to 10 μM, while both CBD and CBG induced cell death at 100 μM (Figure 1A).

Enhanced cell survival with low dose CBD and CBG combination compared to higher single doses

Previous studies have reported the use of CBD and CBG in cancer treatment, highlighting their potential efficacy in combination[8]. However, the activity of these compounds at lower concentrations remains underexplored. To better understand the effects of low-dose combinations of CBD and CBG, we further analyzed their impact on cell viability. A dose variation of CBD or CBG (0.01, 0.1, 1, 10 μM) was applied in combination using a checkerboard method. As shown in Figure 2A, compared to the control group, the combination of 0.01 μM CBD or 0.01 μM CBG with varying doses (0.01, 0.1, 1, 10 μM) of the counterpart significantly enhanced cell viability ($p < 0.05$). In the 0.1 μM CBD or 0.1 μM CBG combination groups, adding varying doses of the counterpart also significantly increased Caco-2 cell viability (Figure 2B). In the 1 μM CBD or 1 μM CBG combination groups, cell survival was also significantly increased compared to the control group (Figure 2C). As shown in Figure 2D, in the 10 μM CBD or CBG combination groups, adding 0.01, 0.1, and 10 μM CBD significantly increased cell survival compared to the control group ($p < 0.05$). These results demonstrate that low-dose CBD-CBG combinations significantly regulate Caco-2 cell metabolism. Furthermore, we ranked the Caco2 cell viability values for individual CBD or CBG treatments and their combination groups. As shown in Figure 2E, the ranking was as follows: CBD 0.01 μM + CBG 10 μM > CBD 1 μM + CBG 0.1 μM > CBD 10 μM + CBG 0.1 μM > CBD 10 μM + CBG 10 μM > CBD 0.01 μM + CBG 0.1 μM > CBD 10 μM + CBG 0.01 μM > CBD 1 μM + CBG 10 μM > CBD 0.01 μM + CBG 1 μM > CBD 0.1 μM + CBG 0.1 μM > CBD 0.01 μM + CBG 0.01 μM > CBD 10 μM + CBG 1 μM > CBD 0.1 μM + CBG 10 μM > CBD 0.1 μM + CBG 1 μM > CBD 1 μM + CBG 1 μM > CBD 1 μM > CBD 0.01 μM > CBG 1 μM > CBG 0.1 μM > CBG 0.01 μM > CBD 10 μM > CBD 0.1 μM > CBG 10 μM. The results indicate that some low-concentration combinations, particularly the 0.01 and 0.1 μM groups, resulted in higher Caco-2 cell survival rates compared to individual treatments with 1 μM or 10 μM CBD or CBG.

The low-dose combination of CBD and CBG may synergistically promotes Caco2 proliferation, with a more pronounced effect than higher concentrations in alone.

Low dose CBD or CBG combined with high dose attenuates high dose induced cell death

Prior experimental findings have shown that low concentrations of CBD and CBG, when combined, promote Caco2 cell survival more efficiently than either compound at higher individual doses. These findings underscore the therapeutic potential of low-dose CBD/CBG combinations. To further explore

the therapeutic potential of low-dose CBD/CBG in combinations, we examined their effects on cell survival when combined with higher doses. In Figure 3A, for the 100 μM CBD or CBG combination, adding 0.01~10 μM CBD slightly increased cell survival compared to 100 μM CBG alone while insignificantly. 1 μM CBG was added to 100 μM CBD, cell death increased significantly. The combination of 0.01 μM CBG + 100 μM CBD showed significantly higher cell survival compared to 100 μM CBD alone.

This leads us to speculate that there may be synergistic, additive, or antagonistic effects between CBD and CBG in combination. ANOVA evaluates group mean differences by partitioning variance but does not account for CBD-CBG interactions or quantify their effects. This study utilized the checkerboard method combined CBD-CBG treated Caco2 cells, enabling interaction quantification their combination effect via synergy model. Therefore, based on CBD and CBG alone or combination teated cell viability, we further employed ZIP (Zero Interaction Potency) synergy metrics model to assess the potential synergistic and antagonistic interactions between CBD and CBG (red squares represent synergy and green squares indicate antagonism). Uploaded the CBD and CBG alone or in combination treated cell viability to the SynergyFinder software, the result quantified synergistic and antagonistic effects within the regulatory matrix confirmed the synergy of the CBG-CBD combination (ZIP score: 23.204) (Figure 3B).

These results suggest that low concentrations of CBD and CBG exert a synergistic effect when combined, whereas low-dose co-treatment may attenuate the cytotoxic effects induced by higher concentrations, indicating a potential antagonistic interaction.

Dose-dependent autophagy induction by CBD and CBG in Caco2 cells

Autophagy is a key molecular mechanism that regulates cellular metabolism and influences cell viability[12]. CBD and CBG possess autophagy-activating properties. While numerous previous studies have focused on the autophagy-inducing activity of CBD and CBG at relatively high concentrations, their potential effects at concentrations below 1 μM have not been thoroughly investigated. To determine whether lower concentrations of CBD and CBG can activate autophagy, we treated Caco-2 cells expressing the GFP-LC3-RFP probe with varying doses (0.01, 0.1, 1, 10,100 μM) of each compound. Our preliminary findings indicate that 100 μM CBD and CBG significantly enhance autophagy in Caco-2-GFP-LC3-RFP cells. In contrast, 10 μM CBD and CBG exhibit a trend toward autophagy activation, although the effect does not reach statistical significance. At concentrations below 1 μM, neither CBD nor CBG displays detectable autophagy-inducing activity (Figure 4A).

Low doses CBD or CBG in combination can promote autophagy in Caco2 cells

The combination of compounds can fundamentally reshape the cellular environment, influencing the activity of each component and leading to either synergistic or antagonistic effects[23]. In our preliminary analysis of cell viability, we observed that low concentrations of CBD and CBG exert a synergistic effect in promoting cell survival when applied in combination, whereas an antagonistic interaction was observed between high and low concentrations. To further investigate the modulatory effects of combined CBD and CBG on autophagy, we evaluated their ability to induce autophagic flux

in Caco-2 cells expressing the GFP-LC3-RFP-LC3 probe, using a checkerboard combination approach.

Low concentrations of CBD and CBG (0.01, 0.1, 1, 10 μM) combined with varying doses of the counterpart in a checkerboard method, were applied to Caco-2 cells for 24 hours. As shown in Figure 5A, the combination of 0.01 μM CBD with different CBG concentrations resulted in a potential increase in autophagic flux compared to 0.01 μM CBG alone ($p < 0.05$), with a notable enhancement observed at 10 μM CBD + 0.01 μM CBG. Similarly, adding varying doses of CBG to 0.01 μM CBD also increased autophagic flux, though the effect was not statistically significant. In the 0.1 μM CBD or CBG combination groups, the addition of 10 μM of the counterpart significantly increased autophagic flux compared to the control (Figure 5B). In the 1 μM CBD or CBG combination groups, the combinations of 1 μM CBG + 10 μM CBD and 1 μM CBD + 10 μM CBG resulted in a significant increase in autophagic flux compared to the control (Figure 5C). However, no significant changes were observed when adding doses ranging from 0.01 μM to 10 μM of the counterpart. Similarly, in the 10 μM CBD or 10 μM CBG combination groups, the combination of 10 μM CBD + 10 μM CBG significantly enhanced autophagic flux compared to the control (Figure 5D).

These results indicate that although low concentrations of CBD and CBG combination with 10 μM CBD or CBG have autophagy active protential in Caco-2 cells.

Low dosages of CBD or CBG enhance high dose induced autophagic flux in Caco2 cells

Based on previous experimental results, we found although the overall molar concentration of their combinations changed remain low (e.g., 10 μM + 0.1 μM), the combined treatment significantly enhanced autophagic flux in Caco-2 cells. Therefore, we further investigated whether low concentrations of CBD or CBG could also enhance the autophagic flux induced by high concentrations of CBD or CBG. Compared to the DMSO control group, adding low doses (0.01, 0.1, 1, and 10 μM) of CBD or CBG to a high concentration of the other compound resulted in significant autophagy activation when dose-adjusted CBG was added to 100 μM CBD. However, when dose-adjusted CBD was added to 100 μM CBG, only the 1 μM and 100 μM combinations showed significant differences (Figure 6A).

Reduced Survival of Caco-2 ATG9-KO Cells Compared to Caco-2 WT upon CBD and CBG Treatment

Preliminary results from this study demonstrate that high concentrations (100 μM) of CBD and CBG induce autophagy activation, accompanied by a reduction in cell viability. Interestingly, low concentrations (0.01–10 μM) of CBD or CBG, when combined with 10 μM of the other compound, synergistically promoted Caco-2 cell proliferation, and this combination also exhibited a trend toward autophagy activation. These findings suggest that CBD and CBG, either individually or in combination, regulate autophagy in a complex manner that influences Caco-2 cell survival. In detail, the relationship between Related GFP/RFP ratio and cell viability. A certain degree of correlation was observed between autophagic activity and cell viability when 10 μM CBD was combined with dose-varying CBG (R^2 = 0.6040, P = 0.1219). Similarly, the combination of 10 μM CBG with dose-varying CBD yielded R^2 = 0.7908 (P = 0.0435). By contrast, combinations involving 100 μM CBD or CBG showed weaker

correlations (R^2 = 0.0671 and 0.1113, respectively) (Figure 7A).

To further clarify the relationship between cannabinoid-induced autophagy and cell viability, we next performed comparative assays using both wild-type (WT) and autophagy-deficient (ATG9-knockout) Caco-2 cell models. This approach allowed us to determine whether the observed survival-promoting effects of CBD and CBG are dependent on functional autophagy machinery. Caco2-ATG9-KO cells and compared their viability to Caco2 cells following treatment with varying concentrations of CBD and CBG (0, 0.01, 0.1, 1, 10, and 100 μM). 0.1 μM CBD showed some toxicity in Caco2-ATG9-KO cells, although the differences compared to Caco2-WT cells were not statistically significant. In contrast to 10 μM CBD, Caco2-WT cell survival was significantly higher than that of Caco2-ATG9-KO cells in the 10 μM CBG group ($p < 0.05$). Compared to Caco2-WT cells, 100 μM CBD or CBG caused a significant increase in cytotoxicity in Caco2-ATG9-KO cells (Figure 7B).

This result suggests that autophagy activated by CBD or CBG may benefits Caco2 cell survival.

Discussion

Cannabidiol (CBD) and Cannabigerol (CBG) are non-psychoactive cannabinoids known to regulate autophagy in both cancerous and non-cancerous cells. In this study, low-dose combinations of CBD and CBG synergistically enhanced Caco-2 cell proliferation. Moreover, high concentrations of either compound alone, or their low-dose combinations, strongly activated autophagy. By comparing treatments under conditions with and without autophagy induction, we found that combinations exhibiting autophagy-activating tendencies promoted Caco-2 cell viability more effectively than single treatments that did not trigger autophagy. Analyses using correlation analysis method and autophagy-deficient cells further confirmed that CBD- or CBG-induced autophagy plays a beneficial role in supporting Caco-2 cell survival.

Our analysis of the effects of CBD and CBG in Caco-2 cells revealed a clear dose-dependent response. Specifically, low concentrations of CBD and CBG (0.01–10 μM) significantly promoted cell proliferation, suggesting a potential cytoprotective or proliferative effect. In contrast, higher concentrations (100 μM) of either compound induced notable cytotoxicity, indicating a shift from beneficial to detrimental effects at elevated doses. Importantly, when lower concentrations (0.01 or 0.1 μM) were combined with 100 μM of either CBD or CBG, cell viability significantly increased compared to treatment with 100 μM alone, with the combination of CBD 100 μM + CBG 0.01 μM showing particularly strong effects. This suggests that low concentrations of CBD or CBG may mitigate the cytotoxic effects of higher doses, highlighting a potential protective interaction at submicromolar levels. Furthermore, ranking of viability outcomes for single and combined treatments revealed that certain low-dose combinations (0.01 or 0.1 μM) led to higher cell survival than 1 or 10 μM alone. These findings consistently support the hypothesis that low-dose CBD and CBG synergistically enhance Caco-2 cell survival. Given that Caco-2 cells are widely used as an in vitro intestinal model, these results may have broader implications for intestinal epithelial protection. However, studies specifically focusing on CBD and CBG concentrations below 1 μM remain limited, warranting further mechanistic investigation. Given this gap, elucidating the receptor-mediated mechanisms, particularly those involving the ECS, may provide important insights[24]. These effects are likely mediated, at least in part, by the endocannabinoid system (ECS), which regulates intestinal homeostasis and inflammation through CB1/CB2 signaling [25]. In vitro studies have shown that CB1 activation promotes wound closure in colonic epithelium, suggesting potential relevance in inflammatory bowel disease (IBD) [26]. Apical administration of CB1 agonists to Caco-2 cells has also been reported to enhance intestinal permeability. In vivo, the CB1 antagonist rimonabant reduced plasmatic lipopolysaccharide (LPS) levels in a leaky gut model, demonstrating the ECS's role in maintaining barrier integrity [27-29]. CBD and CBG have also been shown to exert anti-inflammatory effects, partly by modulating CB1/CB2 signaling in intestinal epithelial cells[30, 31] , and their activity through the ECS has been confirmed across several animal and preclinical models[32]. While the present study cannot definitively attribute the synergistic effects of low-dose CBD and CBG to ECS-mediated pathways, the existing literature provides circumstantial support for this possibility.

Although the molecular composition of CBD and CBG mixtures remained constant, the biological effects varied depending on their relative proportions—indicating that CBD + CBG and CBG + CBD are

not functionally identical. Building upon previous observations that low concentrations of CBD or CBG can attenuate the cytotoxic effects induced by higher concentrations of the other compound, we further examined whether these interactions were synergistic or antagonistic in a dose- and ratio-dependent manner. To quantitatively assess these interactions, we employed a checkerboard assay, a standard approach for evaluating drug interactions, which calculates synergy or antagonism using the fractional inhibitory concentration index (FICI). Traditional models such as the Concentration Addition (CA) and Combination Index (CI) have been widely used in cannabinoid synergy studies [33, 34]. The Concentration Addition (CA) model assumes that all components in a mixture act through similar mechanisms and occupy the same target site, whereas the Independent Action (IA) model is suited for combinations in which the components have distinct modes of action (MOA) [23]. In contrast, the Zero Interaction Potency (ZIP) model, developed based on the four-parameter logistic function and median-effect principle, has emerged as a robust tool for evaluating drug interactions. The ZIP model quantifies deviations from purely additive effects, thereby identifying synergistic or antagonistic interactions with high sensitivity[35]. Previous studies have evaluated the synergistic activity of THC-CBD combinations using CI methods[36]. Whereas previous studies primarily focused on molecular signaling analyses of CBD–CBG synergy[25, 37], we applied ZIP synergy scoring, to analyze viability data from Caco-2 cells treated with varying CBD-CBG combinations. The ZIP model fitted our experimental data well, capturing nuanced dose-interaction dynamics between CBD and CBG. Our ZIP synergy analysis revealed that combinations of CBD and CBG at low concentrations (0.01 and 0.1 μM) significantly enhanced Caco-2 cell viability, with the synergy score at 0.01 μM exceeding that observed at 10 μM. This suggests a potential receptor saturation effect, where increasing concentrations beyond a certain threshold fails to further augment the proliferative benefit. Furthermore, we observed that low concentrations of either CBD or CBG antagonized the cytotoxic effects induced by high concentrations of the same or the other compound, reflecting a dose-dependent shift from synergism to antagonism.In contrast to many prior reports describing predominantly cytotoxic outcomes for CBD–CBG mixtures, our findings identify specific concentration ratios that maximize viability while minimizing toxicity. This study thus provides a novel demonstration of both synergistic and antagonistic interactions between CBD and CBG in colorectal epithelial cells, offering new insight into their potential therapeutic window.

As we all known, autophagy is a critical adaptive response activated by various cellular stressors such as nutrient deprivation or compound exposure, functioning to maintain homeostasis and promote survival under adverse conditions[38]. Functionally, autophagy is classified into four categories: cytoprotective (promoting cell survival), cytotoxic (promoting cell death), cytostatic (affecting cell growth arrest), and nonprotective (having no contribution to cell death or survival) [39]. To further clarify the cellular mechanisms underlying these effects, we next investigated whether autophagy contributes to the observed changes in Caco-2 cell viability. Autophagy-regulating activity of CBD and CBG in Caco-2 cells, our findings reveal a clear concentration-dependent pattern. High concentrations (100 μM) alone or combination of either compound significantly induced autophagy. Moreover, previous studies have mainly focused on the autophagy-inducing effects of high-dose CBD or CBG, they have largely overlooked their potential cooperative effects at lower concentrations. In this study, we innovatively explored the combined effects of CBD and CBG at low concentrations (<10 μM), where treatment with either compound alone (0.01–10 μM) did not significantly enhance autophagic flux. Additive effects

emerged when these sub-effective doses were combined. For example, co-treatment with 0.01 μM CBD and 10 μM CBG induced markedly stronger autophagy than 10 μM CBG alone, and a similar enhancement was observed with the combination of 1 μM CBD and 10 μM CBG. To our knowledge, this is the first report demonstrating that the co-administration of CBD and CBG at concentrations below 1 μM can effectively modulate autophagic flux in Caco-2 cells. Consistent with these findings, viability assays showed that under autophagy-activating conditions—such as treatment with 10 μM CBD or CBG combined with 0.01 μM of the counterpart compound—Caco-2 cell viability was significantly higher than that observed with 10 μM CBD or CBG alone, where autophagy was not activated. Notably, this enhancement occurred despite only a minimal increase (0.01–0.1 μM) in total cannabinoid concentration, emphasizing the beneficial role of CBD/CBG-regulated autophagy in promoting Caco-2 cell survival. Taken together, our findings indicate that low-dose combinations of CBD and CBG activate autophagy and promote Caco-2 cell survival, whereas high-dose exposure triggers excessive autophagy and compromises viability. These results suggest that CBD- and CBG-mediated autophagy, or their modulation of basal autophagy, acts as a context-dependent regulator of Caco-2 cell fate.

This co-occurrence of autophagy activation and complex cytotoxicity effect raised a critical question: does the autophagy induced by CBD or CBG contribute to cell survival or cell death? To clarify this issue, we analyzed the correlation between cell viability and autophagic flux (related GFP/RFP ratio) under different CBD and CBG combination treatments (10 μM and 100 μM). In the condition where 10 μM CBD was combined with dose-varying CBG, a strong negative correlation was observed between cell viability and the GFP/RFP ratio (R^2 = 0.6040, P= 0.1219), indicating that lower GFP/RFP ratios (reflecting higher autophagic activity) were associated with increased Caco-2 cell survival. Approximately 60.4% of the variation in cell viability could be explained by changes in autophagic flux, suggesting that autophagy activation substantially contributes to the enhanced survival observed under this combination. Similarly, when 10 μM CBG was combined with dose-varying CBD, a strong correlation between cell viability and autophagy was detected (R^2 = 0.7908, P= 0.0435), again supporting the notion that enhanced autophagy induced by the combination treatment promotes cell survival. In contrast, combinations involving higher concentrations (100 μM) showed weaker associations. The correlation between cell viability and GFP/RFP ratio for 100 μM CBD combined with varying CBG was minimal (R^2 = 0.0671, P= 0.6202), and similarly low for 100 μM CBG combined with varying CBD (R^2 = 0.1113, P= 0.5182). These results suggest that while moderate autophagy activation contributes positively to cell viability.

To further elucidate the relationship between cannabinoid-induced autophagy and cell survival, we used an autophagy-deficient model to dissect whether autophagy functions in a protective or destructive manner under cannabinoid treatment. We investigated whether the observed autophagy activation plays a causative or compensatory role in regulating cell survival by an autophagy dysfunction cells. Among the core autophagy-related (ATG) proteins, ATG9 is unique as the only transmembrane protein and is essential for the formation of the pre-autophagosomal structure (PAS) during early autophagosome biogenesis [40, 41]. In this study, we generated ATG9 knockout (ATG9-KO) Caco-2 cells to determine the functional relevance of autophagy in CBD and CBG treated cells. Comparison between Caco-2 wild-type and ATG9-KO cells revealed that the loss of ATG9 markedly reduced viability upon 100 μM CBD or CBG exposure, indicating that the autophagy induced under these conditions is cytoprotective rather

than cytotoxic. Treatment with 10 μM CBG resulted in a significant difference in cell viability between Caco-2 WT and Caco-2 ATG9-KO cells. This finding also underscores that, in the combination of low-concentration CBD with 10 μM CBG, the autophagy induced by the CBD–CBG combination continues to exert a positive effect on cell survival. These findings are consistent with our correlation analysis between cell viability and autophagic activity under combined CBD and CBG treatment, indicating that the autophagy induced by these compounds exerts a beneficial effect on Caco-2 cell survival. Substantial evidence supports the efficacy of CBD and CBG in inhibiting cancer cell proliferation and inducing autophagy-dependent cell death in various studies; however, our findings in Caco2 cells challenge the prevailing understanding of their role in autophagy-mediated cell death, as observed in multiple myeloma, cholangiocarcinoma, and glioma cells [36]. However, these studies only suggest that CBD influences the co-expression of autophagy-related and cell death proteins. Such as CBD has been reported to reveal that Beclin1 regulates apoptosis and autophagy in breast cancer cells [5]. Autophagy is a complex process that cannot be determined solely by protein levels. Autophagic cell death encompasses two distinct yet interrelated types: autophagy-dependent cell death (ADCD) and autophagy-mediated cell death (AMCD). These two forms of autophagy-associated cell death differ in their reliance on autophagy. They may occur concurrently during cell death processes and can potentially transition between these modes under certain circumstances[42]. It is well known that ADCD relies heavily on the autophagy mechanism, with the cell death process being reversible through genetic or pharmacological inhibition of autophagy and independent of other forms of programmed cell death. In contrast, AMCD involves autophagy driving different modes of cell death and serves as a basis for the initiation of other death pathways[43, 44]. Therefore, previous studies investigating the effects of CBD and CBG on autophagy and cell death can be interpreted as indicating that these cannabinoids induce AMCD rather than ADCD, whereas our comparative analysis of autophagy-deficient and wild-type cells suggests a shift toward autophagy-mediated cell survival in Caco-2 cells. Moreover, although the cytoprotective effect of CBD-induced autophagy observed in Caco-2 cells is consistent with the findings reported by Fei Wang et al[16], it should be noted that Caco-2 cells carry the R273H mutation in p53, resulting in a non-functional protein. In contrast, the HCT116 cells used in their study express wild-type p53, which remains functionally active and tightly regulates both cell cycle arrest and autophagy induction under stress conditions, including cannabinoid exposure [1,2]. Our results demonstrate that in Caco-2 cells, CBD and CBG can induce cytoprotective autophagy independently of p53 activity, differing from the p53-dependent mechanism reported in HCT116 cells [16]. Since p53 status influences mitochondrial metabolism and ROS production, which are closely related to the nature of autophagy being protective or destructive [45, 46], these findings suggest that CBD- and CBG-mediated autophagy promote colon epithelial cell survival through a p53-independent pathway.

In summary, this study highlights the positive contribution of autophagy activation to cell survival under CBD and CBG alone or combination exposure in Caco-2 cells. Notably, by comparing CBD and CBG treatments under both autophagy-activated and non-activated conditions, our findings reveal that even at extremely low concentrations, co-treatment can induce a measurable activation of autophagy accompanied by a marked increase in cell viability. Although additional in vivo investigations are needed to elucidate how cellular, tissue-specific, and environmental factors contribute to the differential outcomes observed in vitro. It is important to acknowledge the limitations inherent to the cell model employed in this study. Further studies are warranted to determine whether CBD and CBG can achieve

459 therapeutic efficacy without impairing normal physiological functions. Future research should extend
460 these findings through preclinical and clinical evaluations and explore the molecular mechanisms
461 underlying the dual and context-dependent actions of cannabinoid-induced autophagy.

462

Author Contributions

C Conceptualization, L.J.J.; Data curation, L.J.J.; Formal analysis, L.J.J.; Methodology, L.J.J.; Investigation, L.J.J.; Visualization, L.J.J.; Writing—original draft preparation, L.J.J.; Writing—review and editing, L.J.J.

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Conflicts of Interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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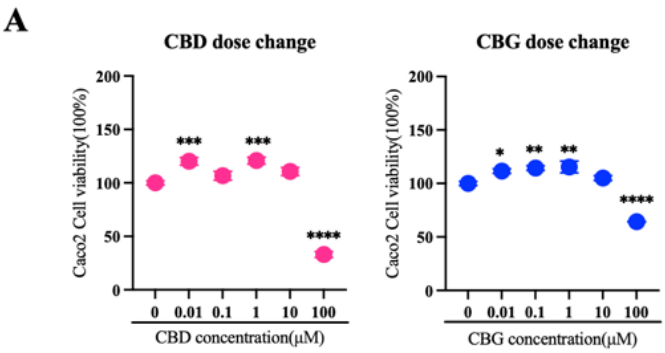
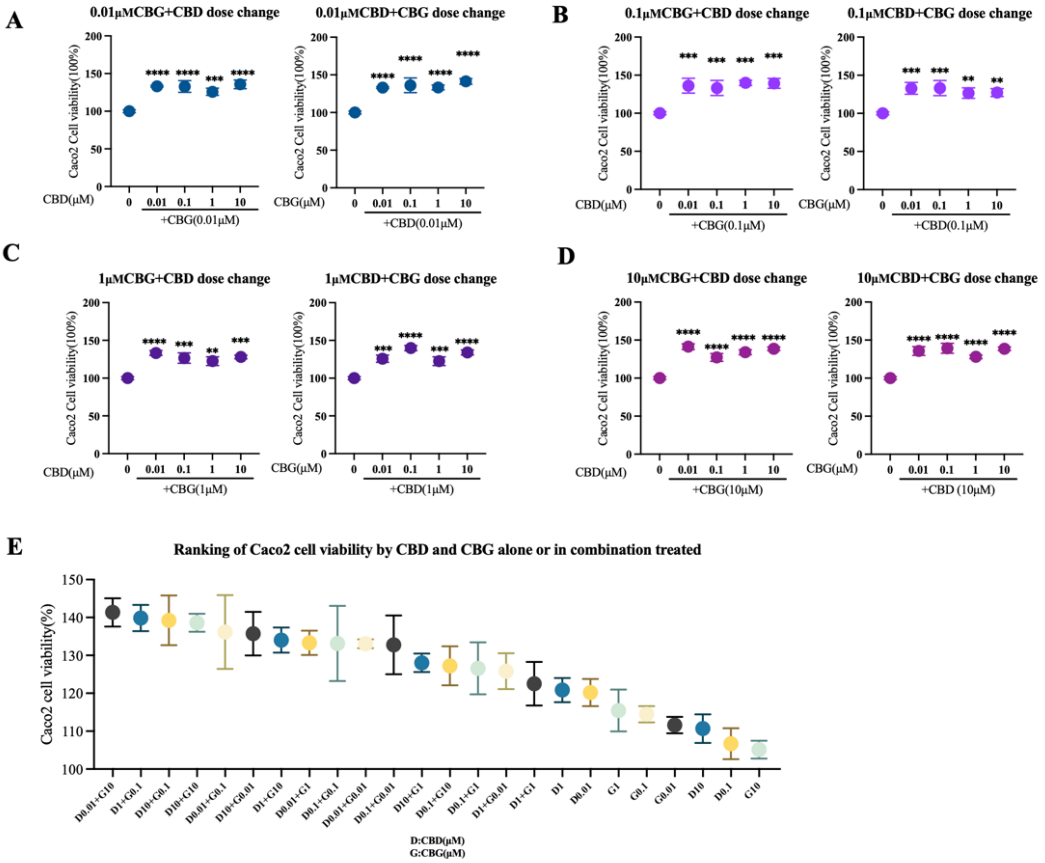


Figure 1. Differential effects of CBD and CBG concentrations on Caco-2 cell viability. (A) Cell viability of Caco-2 cells treated with increasing concentrations of CBD or CBG (0.01, 0.1, 1, 10, and 100 μM). Low concentrations promoted cell proliferation, whereas high concentrations reduced viability, indicating a dose-dependent shift from pro-survival to cytotoxic effects.

Cell viability values are normalized to respective DMSO controls. Data represent the mean ± SD of three biological replicates. Statistical significance was determined by one-way ANOVA. P values are indicated as follows: n.s. = $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.



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658 **Figure 2. Low-dose combinations of CBD and CBG synergistically enhance Caco-2 cell survival.**
659 (A–D) Cell viability of Caco-2 cells treated with CBD and CBG combinations at varying concentrations.
660 Each panel represents a fixed concentration of one compound (A: 0.01 μM, B: 0.1 μM, C: 1 μM, D: 10
661 μM) combined with dose gradients (0.01, 0.1, 1, and 10 μM) of the other.
662 (E) Comparative ranking of cell viability values for single and combined treatments of CBD (D) and
663 CBG (G) at the indicated concentrations, arranged from highest to lowest. (D indicates CBD; G indicates
664 CBG; numerical values represent the concentration of each compound.)

665 Cell viability values were normalized to DMSO-treated controls. Data represent the mean ± SD of three
666 independent biological replicates. Statistical significance was determined using one-way ANOVA.
667 n.s., $P > 0.05$; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$.

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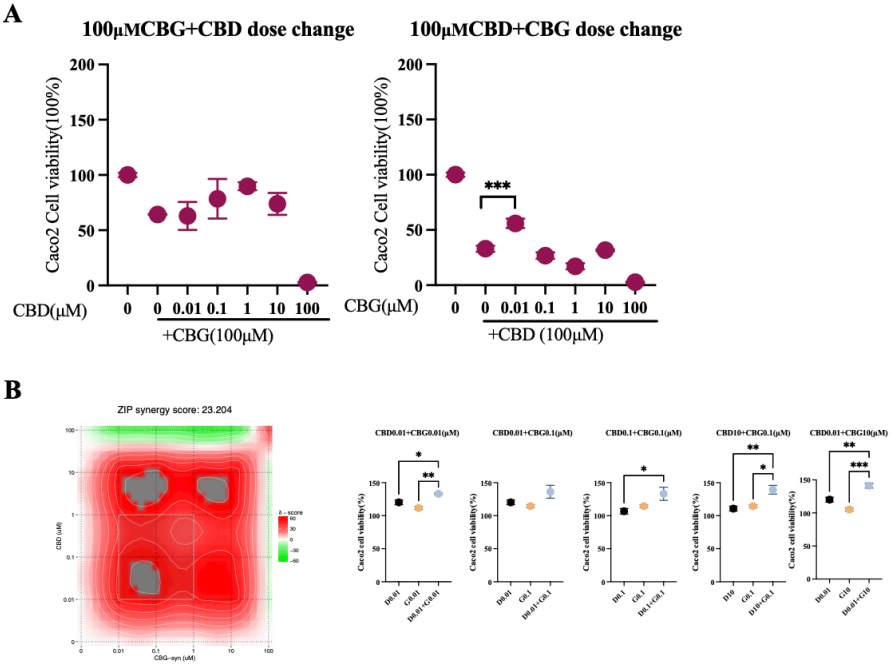

Figure 3. Low-dose CBD or CBG attenuates high-dose-induced cytotoxicity through combinatorial treatment.

(A) Cell viability of Caco-2 cells treated with 100 μM CBD or CBG in combination with dose-varied counterparts (0.01, 0.1, 1, 10, 100 μM), analyzed by one-way ANOVA ($p < 0.05$, *$p < 0.01$, **$p < 0.001$ vs. control).

(B) Cell survival matrix of CBD/CBG combination treatment analyzed using the ZIP model to evaluate synergistic effects, with a Synergy ZIP score of 23.204. Synergy maps were generated using *SynergyFinder*.

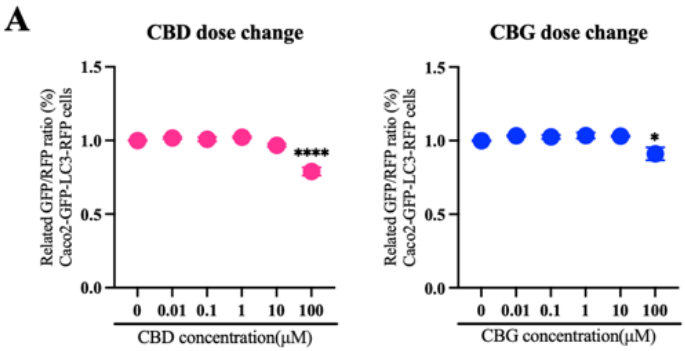

Figure 4. High concentrations (100 μM) of CBD and CBG induce autophagy, whereas lower concentrations fail to activate autophagic flux in Caco-2 cells. (A) Autophagic flux in Caco-2 GFP-LC3-RFP reporter cells treated with increasing concentrations of CBD or CBG (0.01, 0.1, 1, 10, 100 μM). The RFP/GFP fluorescence ratio was quantified and normalized to the DMSO control. Data represent the mean ± SD of biological triplicates. One-way ANOVA was applied. *P* values are displayed as follows: n.s. = $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Autophagy was markedly increased only at 100 μM, indicating a concentration-dependent activation pattern.

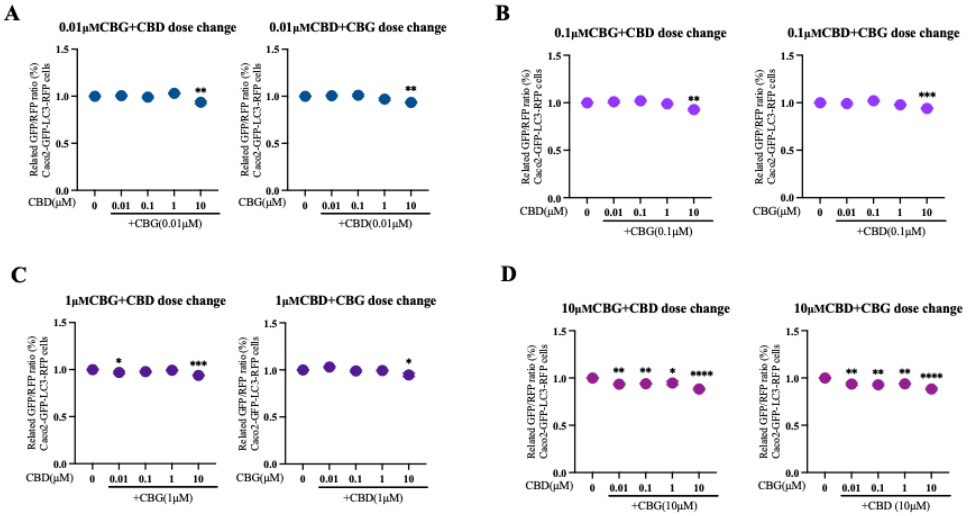

Figure 5. Low-dose combinations of CBD and CBG enhanced autophagic flux in Caco-2 cells.

(A–D) Autophagic flux of Caco-2 GFP-LC3-RFP cells treated with CBD and CBG combinations at varying concentrations. Each panel represents a fixed concentration of one compound (A: 0.01 μM, B: 0.1 μM, C: 1 μM, D: 10 μM) combined with dose gradients (0.01, 0.1, 1, and 10 μM) of the other.

Autophagic flux values were normalized to DMSO-treated controls. Data represent the mean ± SD of three independent biological replicates. One-way ANOVA was applied. *P* values are displayed as follows: n.s. = $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

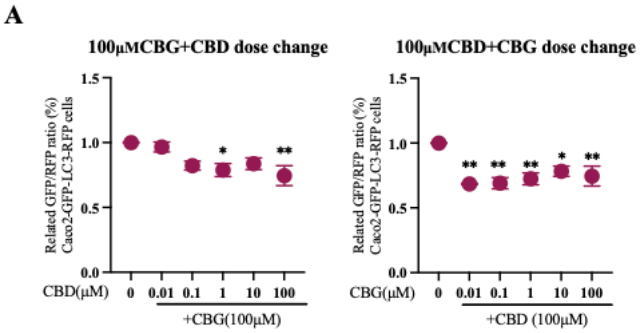

Figure 6. Low non-autophagic activation doses of CBD or CBG enhance high-dose-induced autophagic flux in Caco-2 cells.

(A) Autophagic flux of Caco-2 GFP-LC3-RFP cells treated with 100 μM CBD or CBG combined with varying concentrations (0.01, 0.1, 1, 10, 100 μM) of the other compound.

Autophagic flux values were normalized to DMSO-treated controls. Data represent the mean ± SD of three independent biological replicates. Statistical significance was determined using one-way ANOVA. n.s., $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

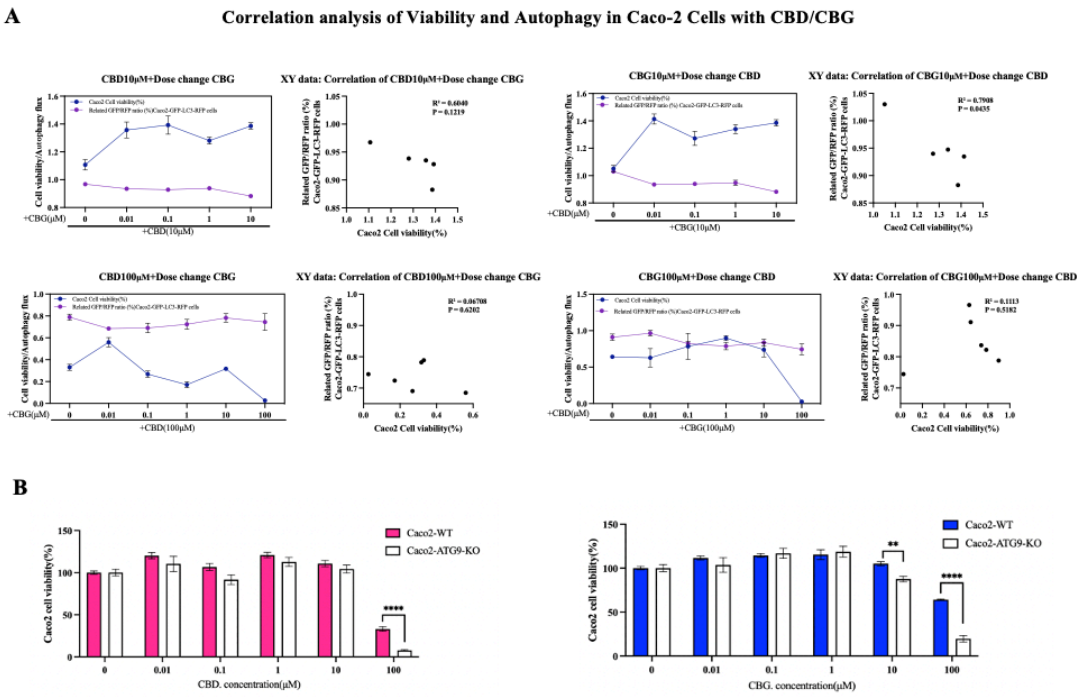

Figure 7. CBD and CBG modulate autophagy in a manner that may promote Caco-2 cell survival.

(A) Line plots show the dose-dependent changes in relative GFP/RFP ratio and cell viability (%) in *Caco2-GFP-LC3-RFP* reporter cells or parental *Caco-2* cells treated with 10 μM CBD or CBG in combination with dose-varied concentrations of the other compound. Both parameters were measured under identical conditions to evaluate the relationship between autophagic flux and cell viability. Correlation analysis between the relative GFP/RFP ratio and cell survival rate revealed a notable association between autophagic activity and cell viability. Pearson correlation coefficients (r) and determination coefficients (R^2) were calculated using GraphPad Prism.
(B) Comparison of autophagy-related responses in *Caco2-WT* and *Caco2-ATG9-KO* cells treated with CBD or CBG (0.01, 0.1, 1, 10, 100 μM). The autophagy-deficient *ATG9-KO* cells exhibited markedly lower cell viability compared with wild-type cells, indicating that ATG9-dependent autophagy contributes to maintaining cell survival under cannabinoid treatment.

Autophagy flux and cell viability are normalized to respective DMSO conditions. Data represent the mean ± SD of biological triplicates. One-way ANOVA is applied. P values are displayed as follows: n.s. = $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.