Cooperative Effects of Cannabidiol and Cannabigerol on Autophagy in Epithelial Cells

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Abstract

Cannabidiol (CBD) and cannabigerol (CBG) are non-psychoactive cannabinoids that exert diverse biological activities in both normal and cancerous epithelial cells. Although autophagy plays a pivotal role in maintaining cellular homeostasis, the effects of combined CBD–CBG treatment on autophagic regulation across epithelial cell types remain largely unexplored. In this study, GFP-LC3-RFP reporter assays and ATG9-deficient cell models were employed to examine the influence of CBD and CBG on autophagy in Ca9-22 and HaCaT cells. Certain concentrations of either compound alone failed to induce autophagy and, in some cases, appeared to suppress autophagic activity. In contrast, their combined administration markedly enhanced autophagic flux in both cell lines. Low-dose CBG or high-dose CBD promoted differential greater cell survival in HaCaT-WT cells compared to their ATG9-KO counterparts. Collectively, these findings provide novel insights into the cooperative regulation of autophagy by CBD and CBG, underscoring their combined effects on cellular autophagic responses in cancer or normal epithelial cells.

Keywords: Cannabidiol (CBD), Cannabigerol (CBG), autophagy, combination, synergy

Highlights:

1. In both Ca9-22 and HaCaT cells, certain doses of CBD alone failed to induce autophagy, whereas CBG at some concentrations showed a trend toward autophagy suppression.

2. Sub-effective doses of CBD and CBG in combination enhance autophagic flux in Ca9-22 and HaCaT cells, with some combinations exceeding the flux induced by higher doses of either compound alone.

3. CBD and CBG exhibit distinct dose-dependent effects on the survival of HaCaT ATG9-deficient cells compared with HaCaT-WT cells, indicating differential ATG9-dependence.

Introduction

Cannabis sativa is a medicinal plant known for its long-standing use in relieving pain, inflammation, and anxiety, as well as for its emerging antitumor properties[1, 2]. Cannabidiol (CBD) and cannabigerol (CBG) have attracted particular interest due to their non-psychoactive nature and broad therapeutic potential[3]. These compounds have been reported to alleviate chemotherapy-induced nausea, reduce cancer-related pain, and modulate immune responses. CBG serves as a biosynthetic precursor to other major cannabinoids, though it exists in low abundance and has historically been understudied[4]. With growing concern over the adverse effects of psychoactive cannabinoids like THC, attention has increasingly shifted toward non-psychoactive extracts such as CBD and CBG for their potential roles in cancer treatment and supportive care. Although it constitutes less than 10% of the cannabinoid profile in Cannabis sativa, and was historically overlooked, emerging research supports its therapeutic relevance[5]. Beyond oral administration, various delivery routes for cannabinoids have been developed, including transdermal and transmucosal applications[6]. Current research on the combined use of CBD and CBG has primarily focused on their ability to inhibit tumor cell proliferation[7]. While early-stage studies on cannabinoid polypharmacy have largely emphasized survival outcomes, growing evidence suggests that these compounds exert multifaceted antitumor effects. A comprehensive meta-analysis by Bachari et al. (622 studies) demonstrated that combinatorial cannabinoid regimens enhance melanoma control by simultaneously suppressing proliferative and metastatic signaling pathways [8]. At the molecular level, synergistic interactions between CBD and CBG have been shown to activate distinct cell death pathways—such as caspase cascade potentiation in glioblastoma models [9]and modulation of Bcl-2 family proteins to favor mitochondrial apoptosis [10]. These findings suggest that the co-administration of CBD and CBG alters the cellular environment in a manner that can lead to either synergistic or antagonistic effects. Recent studies have shown that cannabinoids contribute to cells through the endocannabinoid system (ECS) via multiple mechanisms, such as inhibiting epidermal keratinocyte proliferation, modulating pro-inflammatory cytokine production, and regulating reactive oxygen species generation[11, 12].However, the combination biological effects of cannabinoids in epithelial systems remain incompletely understood, primarily due to the complexity of their molecular targets—including the diverse and context-dependent activity of the ECS and its associated receptors.

Autophagy, a vital intracellular degradation pathway involving the formation of double-membraned autophagosomes, plays critical roles in maintaining cellular homeostasis and regulating cancer cell fate[13]. Both CBD and CBG have been individually reported to modulate autophagy across various cellular contexts[14]. For instance, CBD has been shown to induce autophagy via ERK1/2 activation in neural cells, suggesting a protective cellular response under stress conditions [15]. In placental trophoblasts, CBD disrupts normal autophagic flux, highlighting its potential to interfere with tightly regulated autophagy-related homeostasis in non-cancerous tissues [16] These findings underscore the context-dependent and concentration-sensitive nature of CBD-mediated autophagy regulation. Importantly, cannabinoid combinations have also been implicated in autophagy-related cellular responses in cancer models. For example, a combination of cannabinoids was shown to induce cytoplasmic vacuolation—a hallmark of dysregulated autophagy—in MCF-7

breast cancer cells, suggesting that co-administration of phytocannabinoids may modulate autophagic cell fate in tumors[17]. Furthermore, synergistic anti-cancer effects of cannabinoids with conventional chemotherapeutics, such as carfilzomib in multiple myeloma, have been reported to involve alterations in autophagy[18]. These observations collectively point to the relevance of both individual and combined cannabinoid treatment in modulating autophagy, particularly in the context of cancer therapy. However, to date, no systematic investigation has been conducted on their combined effects in epithelial system. Specifically, it remains unclear: (1) how co-administration of CBD and CBG regulates autophagy, (2) whether their interaction in modulating autophagic flux is synergistic, antagonistic, or merely additive. Given that autophagy plays a fundamental role in epithelial homeostasis, inflammation, and stress responses, the lack of mechanistic insight into how CBD and CBG co-administration modulates autophagy may limit the optimization and safe application of cannabinoid-based interventions in clinical and therapeutic dermatology. Clarifying these synergistic or antagonistic effects is thus crucial for maximizing the efficacy and safety of cannabinoid combinations in epithelial cells management.

This study employed two epithelial cell lines, Ca9-22 (gingival squamous cell carcinoma) and HaCaT (immortalized keratinocyte), to assess the effects of cannabidiol (CBD) and cannabigerol (CBG) in normal versus cancerous epithelial cells. Autophagic flux was monitored using the GFP-LC3-RFP reporter system. The effects of CBD and CBG combinations at 10, 20, and 40 μM were further evaluated in both cell lines. To explore differential autophagy regulation, HaCaT cells with ATG9 knockout were used to compare the impact of these cannabinoids on cell survival relative to HaCaT-WT cells. Our findings demonstrate that CBD and CBG exert cooperative effects on autophagy in normal and cancerous epithelial cells.

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

In this study, CBD (10, 20, 40 μM) and CBG (10, 20, 40 μM) were tested individually or in combination using Ca9-22 and HaCaT cells over a 24-hour period.

Cell culture and transfection

HaCaT (human keratinocyte cells; CLS Cell Lines Service GmbH, Eppelheim, Germany), HCT116 (human colorectal carcinoma cells; ATCC, Manassas, VA, USA), Ca9-22 (gingival squamous cell carcinoma; JCRB Cell Bank, Japan) were cultured in high-glucose Dulbecco's Modified Eagle Medium (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).

ATG9-expressing plasmids were obtained from Obio Technology (Shanghai, China). Lentiviral particles were produced by co-transfecting HEK293T cells with a lentiviral expression vector, psPAX2 (a gift from D. Trono, École Polytechnique Fédérale de Lausanne), and the envelope plasmid pCMV-VSV-G (a gift from R.A. Weinberg, Whitehead Institute for Biomedical Research). Retroviral particles were generated by transiently transfecting HEK293T cells with a retroviral vector along with pCG-VSV-G and pCG-gag-pol (gifts from T. Yasui, Osaka University). Viral supernatants were collected and used to infect target cells following standard protocols. Plasmid transfections were carried out using Lipofectamine 2000 (Thermo Fisher Scientific; Cat. No. 11668019) according to the manufacturer's instructions. Stable cell lines were established through

selection with puromycin (Sigma-Aldrich).

WST-8 assay

WST-8, also known as 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,3,5-trimethyl-1H-pyrazol-1-yl) tetrazolium chloride (CCK-8; Sigma-Aldrich), was used to assess cell viability. This water-soluble tetrazolium salt is reduced by intracellular dehydrogenases in metabolically active cells to produce an orange-colored formazan product, the quantity of which is directly proportional to the number of viable cells.

HaCaT cells (1.0×10^4 cells/well), and their respective ATG9-deficient counterparts were seeded in 96-well plates (BioTek Instruments, Winooski, VT, USA) in 100 µL of complete medium (DMEM supplemented with 10% FBS) per well and incubated for 24 h at 37 °C under 5% CO_2 and 95% humidity. After incubation, the medium was replaced with 100 µL of fresh medium containing varying concentrations of CBD and CBG, based on prior dose-finding assays. The cells were then incubated for an additional 24 h under the same conditions. Experimental controls included: a background control (medium only, no cells, to measure the baseline absorbance of WST-8 reagent), a low control (cells with medium only, to determine basal metabolic activity), a high control (cells treated with a known cytotoxic agent, to establish maximum inhibition), and a solvent control (2.5% methanol). Following treatment, 10 µL of WST-8 solution was added to each well and incubated for 2 h at 37 °C. The absorbance of the resulting formazan was measured at 570 nm using a microplate reader. Background absorbance values were subtracted from all wells to correct for non-cellular interference.

Generation of GFP-LC3-RFP Expressing HaCaT, and Ca9-22 Cells for Autophagic Flux Analysis

HEK293FT cells were co-transfected with the pMRX-IP-GFP-LC3-RFP reporter plasmid, puro-ATG9, pCG-VSV-G, and pCG-gag/pol plasmids[19]. After 72 hours of culture, the virus-containing supernatant was collected and used to infect HaCaT and Ca9-22 cells for 48 hours. For assay validation, Torin 1 (1 µM; an mTORC1 inhibitor and autophagy inducer) was used as a positive control, while Bafilomycin A1 (200 µM; a late-stage autophagy inhibitor) served as a negative control. Following treatment with test compounds or solvents, cells were harvested, and fluorescence intensity was measured using the Cellometer® Vision system (Nexcelom Bioscience LLC, Lawrence, MA, USA). This tandem fluorescent probe enables visualization and quantification of autophagic flux based on the lipidation and degradation of LC3. In this system, GFP-LC3 and RFP-LC3 are expressed as a single fusion protein and subsequently cleaved by endogenous ATG4. Upon autophagy activation, GFP-LC3-II is delivered to autolysosomes and degraded, whereas RFP-LC3-II remains relatively stable due to its resistance to lysosomal quenching. Thus, the ratio of GFP to RFP fluorescence serves as a quantitative index of autophagic flux. Fluorescence signals were measured using appropriate filter sets (GFP: excitation 460–490 nm, emission 500–550 nm; RFP: excitation 530–560 nm, emission 570–650 nm). A high GFP/RFP ratio (>1) indicates impaired autophagic flux, as seen with Bafilomycin A1 treatment, while a low GFP/RFP ratio (<1) reflects

166 enhanced autophagic activity, as observed with Torin 1[20, 21].

167 **Statistical analysis**

168 The data were presented as mean ± SD and statistical analysis was performed using an unpaired
169 Student's t-test for comparisons between two independent groups, one-way analysis of variance
170 (ANOVA) followed by Tukey-Kramer's post hoc multiple comparison tests for multiple
171 comparisons. The threshold for statistical significance was set at $p < 0.05$.

172

Result

Dose-dependent autophagy induction by CBD and CBG in Ca9-22 cells

To determine cannabidiol (CBD) and cannabigerol (CBG) autophagy activation in Ca9-22 (gingival squamous cell carcinoma) cells, we treated Ca9-22 cells expressing the GFP-LC3-RFP probe with varying doses (10, 20, and 40 μM) of each compound. The results indicated that 10 μM CBD did not significantly activate autophagy in the Ca9-22 GFP-LC3-RFP cell line. Conversely, 10 μM CBG exhibited a trend toward inhibiting autophagy. In contrast, both CBD or CBG in 20 μM or 40 μM significantly induced autophagy in Ca9-22 GFP-LC3-RFP cells (Figure 1A).

Combination of Sub-effective Doses of CBD and CBG Enhances Autophagic Flux in Ca9-22 Cells

Although the combined application of CBD and CBG has been widely studied for their effects on cancer cell viability, their cooperative regulation of autophagy remains largely unexplored. To systematically investigate how CBD and CBG jointly modulate autophagy, we employed Ca9-22 cells stably expressing the GFP-LC3-RFP autophagy reporter and quantified autophagic flux in response to a range of CBD–CBG combination (10, 20, and 40 μM).

In Ca9-22 cells, treatment with either compound alone produced limited autophagy activation. In contrast, combinations of 10 μM and 20 μM CBD or CBG with their dose-adjusted counterparts induced markedly higher autophagic flux (Figure 2A–B). Notably, 10 μM CBG alone failed to activate autophagy; however, its combination with 20 μM CBD resulted in an autophagic response exceeding that of 40 μM CBD or CBG administered individually. Specifically, the comparison of combination versus single-agent treatments revealed the following trends in autophagic flux: CBD 10 μM + CBG 20 μM > CBG 40 μM, CBD 20 μM + CBG 10 μM > CBD or CBG 40 μM, CBD 20 μM + CBG 20 μM > CBD or CBG 40 μM, and CBD 10 μM + CBG 10 μM > CBG 20 μM (Figure 2C). Combinations involving 40 μM CBD or CBG with their counterparts were not evaluated due to pronounced cytotoxicity, which prevented the collection of sufficient viable cells for reliable assessment (see Supplementary Figure).

Overall, these results indicate a trend toward synergistic enhancement of autophagy by certain CBD–CBG combinations in Ca9-22 cells.

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

To assess the autophagy activation of CBD and CBG in a different type of non-cancerous cell line, HaCaT keratinocytes stably expressing the GFP-LC3-RFP reporter were treated with varying concentrations (10, 20, and 40 μM) of each compound. In HaCaT-GFP-LC3-RFP cells, no significant autophagy activation was observed at 10 μM for either cannabinoid. In contrast, treatment with 20 μM and 40 μM CBD, as well as 40 μM CBG, resulted in marked autophagy induction. Interestingly, 20 μM CBG did not enhance autophagy and instead exhibited a slight inhibitory trend (Figure 3A).

Combinatorial effects of sub-effective doses of CBD and CBG on autophagy in HaCaT cells

Previous findings showed that combinations of CBD and CBG enhanced autophagic flux in Ca9-22 cells. To investigate whether similar combinatorial effects occur in non-cancerous epithelial cells, HaCaT-GFP-LC3-RFP cells were treated with varying concentrations (10, 20, and 40 μM) of CBD and CBG in combination.

As illustrated in Figure 4A, the combination of 10 μM CBD or CBG combine with dose change each other significantly increased autophagic flux compared to the DMSO control (Figure 4A). Neither 10 μM nor 20 μM of CBG alone induced significant autophagy relative to control, whereas their combine with CBD markedly enhanced autophagic flux in HaCaT cells (Figure 4B). Combinations involving 40 μM CBD or CBG were excluded from analysis due to excessive cytotoxicity and insufficient cell recovery (Figure Supplement). Comparative analysis further revealed that while 10 μM and 20 μM CBG alone did not significantly activate autophagy, their combination with 20 μM CBD produced autophagic flux exceeding that of 40 μM CBD or CBG alone. Additionally, the CBD 10 μM + CBG 20 μM and CBD 20 μM + CBG 10 μM groups exhibited a trend toward higher autophagic flux than 40 μM CBD alone ($p < 0.1$), indicating a cooperative effect of CBD and CBG on autophagy in HaCaT cells (Figure 4C).

Differential Cytotoxicity of CBG and CBD in HaCaT-ATG9-KO versus HaCaT Wild-Type Cells

Given the observed cooperative trends of CBD and CBG in promoting autophagy in Ca9-22 and HaCaT cells, we next aimed to investigate whether these cannabinoids differ in their autophagy regulation. To this end, HaCaT wild-type (WT) and ATG9-knockout (ATG9-KO) cells were treated with varying concentrations of CBD or CBG, and cell viability was assessed as an indirect readout of autophagy modulation (Figure 5A). Exposure to 40 μM of either cannabinoid resulted in pronounced cytotoxicity, precluding reliable assessment. Following 24-hour treatment, 10 μM CBD did not show marked differences between WT and ATG9-KO cells, whereas 10 μM CBG exhibited slightly reduced viability in ATG9-KO cells relative to WT. At 20 μM, CBD induced greater reduction in ATG9-KO cell viability compared to WT, while 20 μM CBG showed minimal difference between the two cell types. Collectively, these observations suggest that CBD and CBG differ in their reliance on ATG9.

Discussion

Our findings indicate that cannabidiol (CBD) and cannabigerol (CBG) can cooperatively modulate autophagy in both neoplastic and non-transformed epithelial cell systems. While treatment with either cannabinoid alone at specific concentrations was insufficient to robustly induce autophagy, certain combinations of CBD and CBG produced a clear enhancement of autophagic flux in Ca9-22 (oral squamous cell carcinoma) and HaCaT (immortalized keratinocyte) cells. Moreover, the differential effects on cell viability observed between HaCaT-WT and HaCaT-ATG9-KO cells support the notion that the cooperative activation of autophagy by CBD and CBG is at least partially dependent on ATG9-mediated pathways. These results collectively suggest that specific CBD–CBG combinations can synergistically promote autophagy, providing new insights into their combined regulation of autophagic processes in epithelial cells.

Previous studies have shown that both CBD and CBG can suppress tumor progression through diverse signaling mechanisms, often accompanied by autophagy activation [22, 23]. However, while the autophagy-inducing roles of individual cannabinoids have been widely reported, little is known about how they may interact to regulate autophagy cooperatively. Consequently, they rely more heavily on autophagy as a survival mechanism under these conditions[24]. Autophagic activity is commonly assessed by monitoring the accumulation and redistribution of LC3-II following lysosomal inhibition under nutrient-rich conditions[25]. Many existing studies evaluating the autophagy-inducing effects of CBD and CBG have employed autophagy inhibitors such as bafilomycin A1 to interpret LC3-II levels as a surrogate marker for autophagic flux. However, this approach has limitations. Since LC3-II is lipidated and degraded during autophagy, its accumulation can result from either enhanced autophagosome formation or impaired autophagic degradation. Moreover, LC3 is not entirely specific to autophagosomes—it can also associate with single-membrane structures such as phagosomes during LC3-associated phagocytosis (LAP) [26]. Therefore, quantifying LC3-II alone is insufficient to precisely evaluate autophagic activity, particularly when assessing the effects of cannabinoid treatments. To overcome these limitations, our study utilized a dual-fluorescent GFP-LC3-RFP probe system, which enables more accurate measurement of autophagic flux. This system allows for the direct comparison of GFP-LC3 degradation (which is sensitive to lysosomal activity) against the stable RFP-LC3 signal, providing a more reliable assessment of autophagosome turnover. This approach allowed us to distinguish true autophagic flux from lysosomal blockage or LC3-associated phagocytosis, providing a more precise assessment of autophagy under cannabinoid treatment. We applied this system to HaCaT and Ca9-22 cells to evaluate the effects of CBD and CBG. In this study, we further examined their combined regulatory impact in epithelial cell models relevant to dermatological applications, where both cannabinoids are frequently utilized. We selected oral squamous carcinoma cells (Ca9-22) and immortalized keratinocytes (HaCaT) to investigate the combinatorial effects of CBD and CBG on autophagy. Using concentrations of 10 μM, 20 μM, and 40 μM, either alone or in combination, we observed that low doses of CBG alone (10 μM) were insufficient to activate autophagy. Notably, when 10 μM or 20 μM CBG was combined with dose-adjusted CBD, the resulting autophagic flux exceeded that observed with 40 μM of either compound alone, highlighting the potential of these combinations to enhance autophagy beyond the effects of individual treatments. At higher

concentrations (20 μM and 40 μM CBD, or 40 μM CBG), autophagy was induced in both cell types. Interestingly, lower concentrations of CBG tended to suppress autophagic activity in Ca9-22 and HaCaT cells. Overall, our findings indicate that while individual low doses of CBD or CBG exert limited or suppressive effects on autophagy, specific combinations of sub-effective doses can substantially enhance autophagic flux, suggesting concentration-dependent cooperative effects in epithelial cells. To date, no studies have systematically examined the autophagy-regulating effects of CBD and CBG co-treatment, particularly regarding their potential for synergistic or antagonistic interactions. While most prior studies have focused on the autophagy-inducing properties of CBD and CBG in cancer cells, our study is the first to report that, depending on concentration and cell context, these compounds may also suppress autophagy—as observed in Ca9-22 oral squamous carcinoma cells.

The potential mechanisms underlying the synergistic regulation of autophagy by CBD and CBG remain largely unexplored. Most existing studies on their combined effects have focused on protein-based signaling pathways [9, 10], including Beclin-1–mediated regulation of autophagy and apoptosis [22]. However, extensive preclinical evidence indicates that both cannabinoids exert many of their biological effects through the endocannabinoid system (ECS) [27, 28]. The ECS is a lipid-based signaling network composed of cannabinoid receptors (CB1 and CB2), endogenous ligands such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and associated enzymes and transporters, which are broadly expressed in epithelial tissues and oral mucosa, playing key roles in homeostatic and inflammatory regulation [29]. CBD and CBG interact with the ECS through distinct receptor affinities: CBD acts primarily as a negative allosteric modulator of CB1 and an indirect antagonist of CB2, whereas CBG displays partial agonistic activity at both receptors[30, 31]. Notably, CB1 inhibition has been reported to enhance autophagy, while CB2 activation may suppress it [32, 33]. These opposing activities may together establish a receptor-level balance that facilitates autophagy activation via complementary ECS-mediated signaling. In our study, low concentrations of CBG alone exhibited a mild inhibitory trend on autophagy in Ca9-22 and HaCaT cells, whereas co-administration of CBD and CBG significantly enhanced autophagic flux. This synergistic increase may arise from the combined receptor modulation by CBD and CBG, allowing differential engagement of CB1- and CB2-related pathways that converge on autophagy regulation. Although direct mechanistic evidence remains to be established, the reproducible synergy observed in both epithelial cell models provides compelling indirect support for an ECS-mediated cooperative mechanism.

Both previous literature and our data indicate that CBD and CBG may engage distinct signaling pathways to regulate autophagy, resulting in synergistic rather than additive effects. Given that ECS-related receptor signaling may account for part of this synergy, we further hypothesized that core autophagy components could also play a role. In particular, we examined whether ATG9, a critical regulator of autophagosome formation, is involved in mediating these cooperative effects by comparing CBD- and CBG-treated wild-type and ATG9-knockout cells. Using ATG9-knockout HaCaT cells, we found that high-dose CBD (40 μM) and low-dose CBG (10 μM) induced significantly greater cytotoxicity under ATG9-deficient conditions compared with wild-type cells. This suggests that low-dose CBG may still contribute to the regulation of basal autophagy. Basal

autophagy is essential for cellular homeostasis by removing damaged organelles and aggregated proteins [34], while induced autophagy is typically triggered in response to exogenous stressors such as nutrient deprivation or pharmacological agents[35]. Interestingly, combinations of low-dose CBD or CBG with moderate concentrations (20 μM) induced higher autophagic flux than 40 μM of either compound alone. A possible explanation is that moderate CBG concentrations, which can mildly inhibit autophagy or impose moderate stress, when combined with low-dose CBD that maintains basal autophagy, may trigger a compensatory autophagic response stronger than that elicited by higher concentrations alone. This observation implies that a potential crosstalk between basal and induced autophagy pathways may be involved when low-dose CBD is combined with moderate concentrations of CBG, resulting in enhanced autophagy. Although this remains a hypothesis requiring molecular validation, it provides a plausible basis for the observed synergy between CBD and CBG. These observations prompted us to further investigate whether ATG9-dependent pathways underlie this cooperative autophagy activation.

ATG9, the only transmembrane component among core autophagy regulators, functions as both a lipid scramblase and a vesicular transporter essential for autophagosome formation and phagophore expansion [36]. Beyond this canonical role, ATG9 also participates in non-classical processes such as Golgi stress adaptation, plasma membrane repair, and oxidative stress regulation[37]. Previous reports have shown that ATG9 interacts with TNFR-associated factor proteins to activate JNK signaling in response to reactive oxygen species (ROS), whereas ROS-induced autophagy disrupts this interaction to restore redox balance[38]. Building upon these insights, we propose that CBD may modulate ATG9-associated oxidative stress pathways, thereby sensitizing the intracellular environment for subsequent CBG-induced autophagy. Considering that both cannabinoids influence redox metabolism, their combined use may trigger a feedback mechanism in which CBD primes ATG9-dependent oxidative responses, facilitating enhanced autophagic activation by CBG. This model provides a plausible explanation for the observed ATG9-dependent synergy between CBD and CBG in epithelial cells, integrating oxidative stress signaling with autophagy regulation.

In summary, this study demonstrates that certain combinations of sub-effective doses of CBD and CBG promote enhanced autophagy activation in both Ca9-22 and HaCaT cell lines. CBD- and CBG-induced autophagy exhibits differential dependence on ATG9 in modulating the viability of HaCaT cells. This observation suggests that: (1) CBD and CBG differentially regulate autophagy, and (2) their synergistic autophagic activation likely occurs through ATG9-related pathways. While further investigation is required to elucidate the precise molecular crosstalk underlying CBD-CBG combinatorial effects, our findings provide novel insights into their cooperative regulation of autophagy and highlight their potential as therapeutic agents targeting autophagy pathways. This allowed us to assess whether their autophagic effects extend beyond cell models and play a role in skin or oral-related therapeutic contexts.

Author Contributions

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. The sponsors had no role in the design, execution, interpretation, or writing of the study.

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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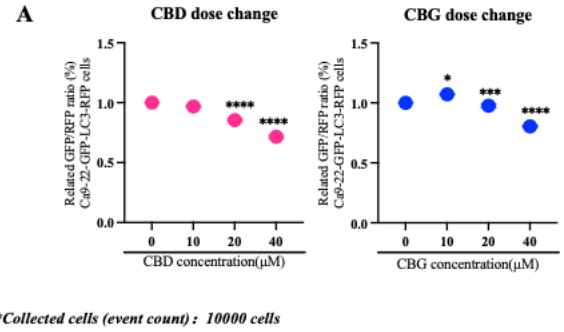

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511 **Figure 1: Dose-dependent modulation of autophagy by CBD and CBG in Ca9-22 GFP-LC3-**
512 **RFP cells.** High-dose CBD and CBG (40 μM) induced autophagy, whereas low-dose CBG (10 μM)
513 tended to suppress autophagic flux. Cells were treated with CBD or CBG at 10, 20, and 40 μM
514 individually. Autophagic flux is normalized to the respective DMSO control. Data represent the
515 mean ± SD of three independent biological replicates, with each replicate collected by FACS at
516 10,000 cells per sample. Statistical analysis was performed using one-way ANOVA. P values are
517 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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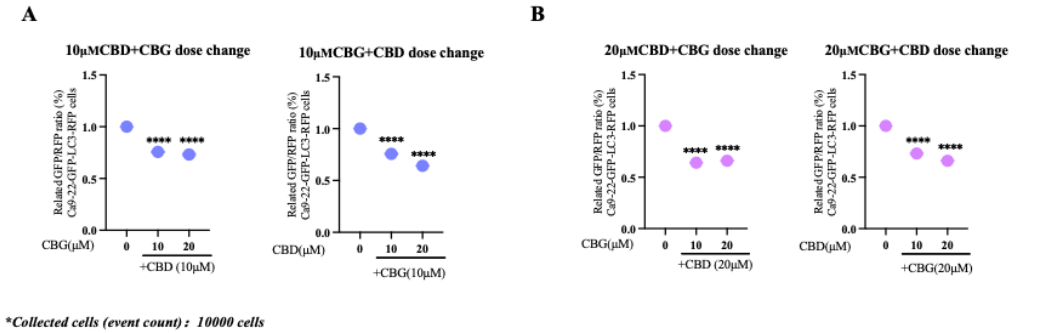

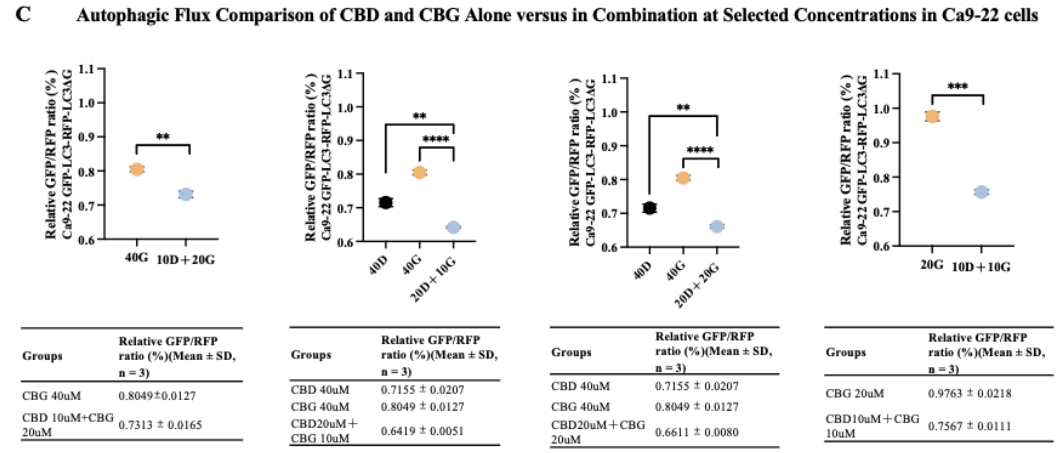

Groups	Relative GFP/RFP ratio (%)(Mean ± SD, n = 3)
CBG 40uM	0.8049±0.0127
CBD 10uM+CBG 20uM	0.7313 ± 0.0165

Groups	Relative GFP/RFP ratio (%)(Mean ± SD, n = 3)
CBD 40uM	0.7155 ± 0.0207
CBG 40uM	0.8049 ± 0.0127
CBD20uM+CBG 10uM	0.6419 ± 0.0051

Groups	Relative GFP/RFP ratio (%)(Mean ± SD, n = 3)
CBD 40uM	0.7155 ± 0.0207
CBG 40uM	0.8049 ± 0.0127
CBD20uM+CBG 20uM	0.6611 ± 0.0080

Groups	Relative GFP/RFP ratio (%)(Mean ± SD, n = 3)
CBG 20uM	0.9763 ± 0.0218
CBD10uM+CBG 10uM	0.7567 ± 0.0111

521

522 **Figure 2: Dose-dependent effects of CBD and CBG combinations on autophagy in Ca9-22 GFP-**
523 **LC3-RFP cells. Certain concentrations of CBD or CBG alone did not significantly induce**
524 **autophagy, whereas specific combinations of CBD and CBG resulted in greater autophagic flux**
525 **than higher doses of either compound alone.**
526 (A) Autophagic flux in cells treated with 10 μM CBD or CBG combined with a dose-adjusted
527 counterpart.
528 (B) Autophagic flux in cells treated with 20 μM CBD or CBG combined with a dose-adjusted
529 counterpart.
530 (C) Comparison of autophagic flux between low-dose CBD–CBG combinations and high-dose
531 individual treatments.
532 Autophagic flux is normalized to the respective DMSO control. Data represent the mean ± SD of three
533 biological replicates. Statistical analysis was performed using one-way ANOVA. P values are indicated
534 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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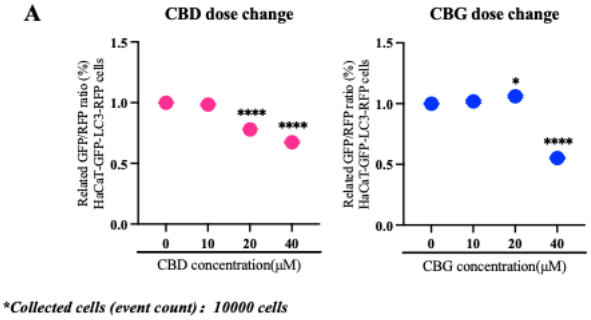

536

537 **Figure 3: Dose-dependent effects of CBD and CBG on autophagic flux in HaCaT GFP-LC3-RFP**
538 **cells.** Treatment with CBD (20–40 μM) or CBG (40 μM) increased autophagic flux, whereas CBG at 20
539 μM showed a slight inhibitory trend. Autophagic flux was measured in HaCaT cells treated with CBD
540 or CBG alone at 10, 20, and 40 μM. Flux values are normalized to respective DMSO controls. Data
541 represent mean ± SD of biological triplicates. One-way ANOVA was applied. P values are indicated as
542 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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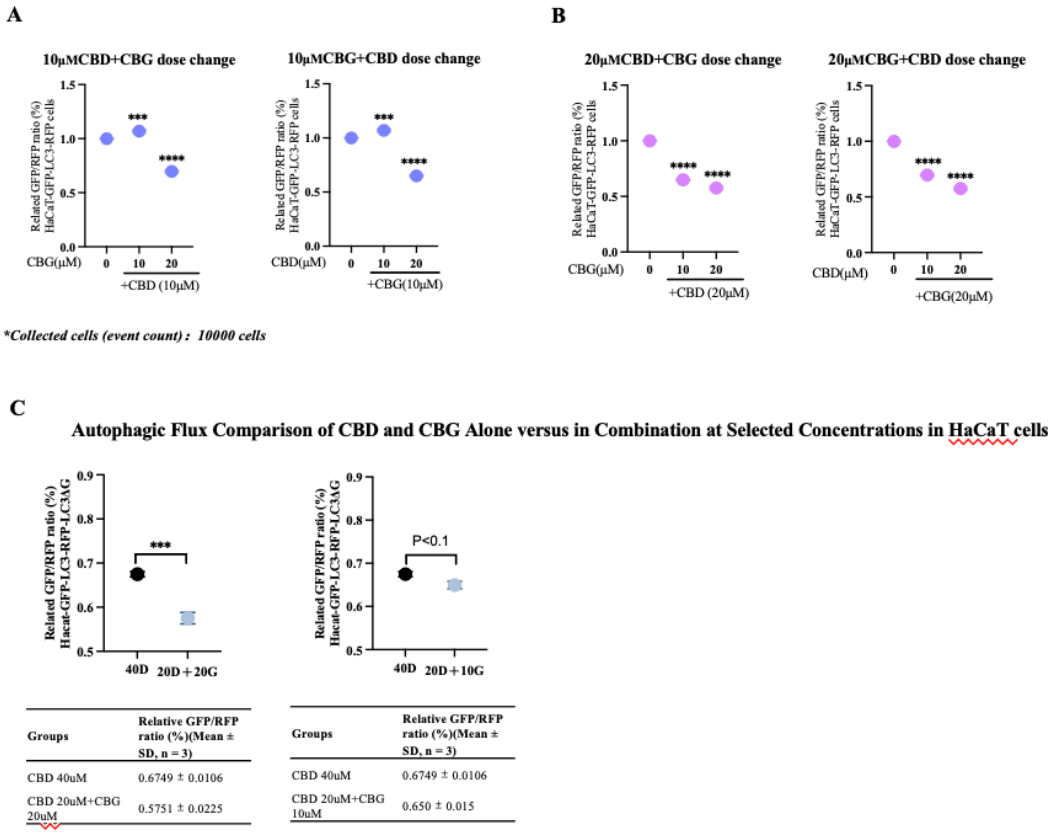

Groups	Relative GFP/RFP ratio (%)(Mean ± SD, n = 3)
CBD 40uM	0.6749 ± 0.0106
CBD 20uM+CBG 20uM	0.5751 ± 0.0225

Groups	Relative GFP/RFP ratio (%)(Mean ± SD, n = 3)
CBD 40uM	0.6749 ± 0.0106
CBD 20uM+CBG 10uM	0.650 ± 0.015

Figure 4: Dose-dependent enhancement of autophagic flux by combined CBD and CBG treatment in HaCaT GFP-LC3-RFP cells. While individual treatment with certain concentrations of CBD or CBG alone did not significantly activate autophagy, combinations of CBD and CBG led to greater autophagic flux than higher doses of either compound alone.

(A) Autophagic flux in HaCaT cells treated with 10 μM CBD combined with dose-adjusted CBG.
(B) Autophagic flux in HaCaT cells treated with 20 μM CBD combined with dose-adjusted CBG.
(C) Comparison of autophagic flux between low-dose CBD–CBG combinations and high-dose individual treatments.
Autophagic flux is normalized to respective DMSO controls. Data represent mean ± SD of biological triplicates. One-way ANOVA was applied. P values are indicated as follows: n.s. = $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

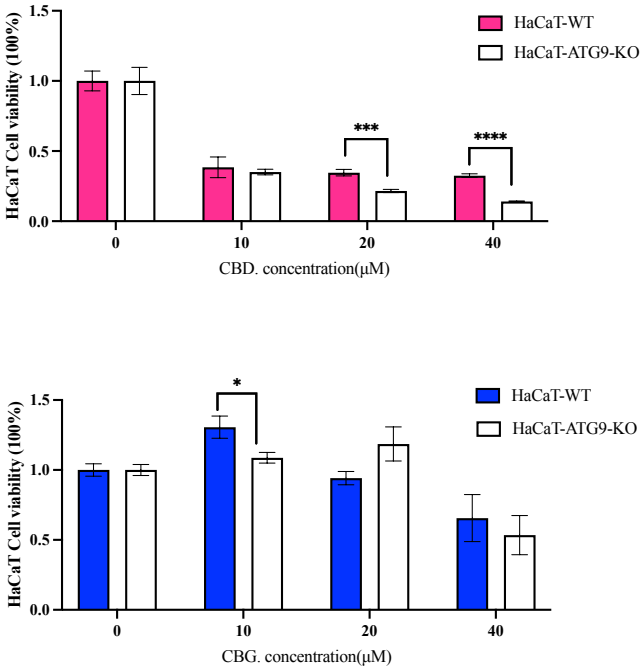

Figure 5: CBD and CBG modulate HaCaT cell survival in an autophagy-dependent manner. HaCaT-WT and HaCaT-ATG9-KO cells were treated with CBD or CBG at 10, 20, or 40 μM. Cell viability was assessed to determine the contribution of autophagy to cell survival. Data are normalized to respective DMSO controls and represent mean ± SD of biological triplicates. Two-tailed t-test was applied. P values are indicated as follows: n.s. = $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.