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# In Vitro and In Vivo Effects of Melatonin-Containing Combinations in Human Pancreatic Ductal Adenocarcinoma

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## ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) has poor prognosis and high mortality rates. Therefore, it is necessary to identify new targets and therapeutic strategies to improve the prognosis of patients with PDAC. Integrative therapies are increasingly being used to boost the efficacy of the known anticancer therapeutic approaches. Hence, this study aimed to evaluate the effects of a novel combination of different potential anticancer molecules, melatonin (MLT), cannabidiol (CBD), and oxygen–ozone ($O_2/O_3$) to treat PDAC using in vitro and in vivo models of human PDAC. The effect of this combination was investigated in combination with gemcitabine (GEM), the most common chemotherapeutic drug used for PDAC treatment. The combination of MLT + CBD + $O_2/O_3$ was more effective than the individual treatments in inhibiting PDAC cell viability and proliferation, inducing cell death, and modulating the RAS pathway protein levels. Moreover, different combinations of treatments reduced tumor mass in the PDAC mouse model, thus promoting the effect of GEM. In conclusion, a mixture of MLT + CBD + $O_2/O_3$ could serve as a potential adjuvant therapeutic strategy for PDAC.

## 1 | Introduction

Pancreatic cancer has the worst prognosis among common solid malignancies, and the 5-year overall survival rate is approximately 10%. Exocrine tumors account for 95% of all pancreatic cancers, with pancreatic ductal adenocarcinoma (PDAC) being the most common [1]. The high mortality rate is due to the lack of timely tumor detection, early metastases, the inability to apply aggressive treatment strategies, and resistance to chemotherapeutic agents, such as gemcitabine (GEM) and 5-fluorouracil [1, 2]. Therefore, it is necessary to identify new targets and therapeutic strategies to improve the prognosis of patients with PDAC. Considering the known anticancer potential of melatonin (*N*-acetyl-5-methoxytryptamine, MLT) in preclinical investigations [3], its effect is being assessed in clinical trials to prevent or treat the side effects of chemotherapy and radiotherapy [4–6]. In PDAC, the effects of MLT have been extensively studied in terms of tumor growth inhibition, improvement in chemotherapeutic drug efficacy, and regulation of tumor-associated immune cells [7–10]. Thus,

Laura Zeppa and Cristina Aguzzi contributed equally to this work.

MLT could have a primary role as an integrative therapy in PDAC [7, 10, 11]. The anticancer properties of cannabidiol (CBD), a non-psychomimetic compound derived from *Cannabis sativa*, have been extensively analyzed in preclinical and clinical studies. It is also a component of medicinal cannabis drugs approved by the Food and Drug Administration [12–15]. CBD reduces PDAC cell growth in vitro and in vivo and works synergistically with GEM, suggesting the use of a CBD–GEM combination to improve outcomes in patients with PDAC [16]. Furthermore, the in vitro anticancer effects of CBD alone or in combination with oxygen–ozone ($O_2/O_3$) and GEM further enhance the efficacy of the CBD–GEM combination [17]. $O_2/O_3$ therapy is an integrative approach for patients with cancer because of its ability to reduce pain, fatigue, and musculoskeletal symptoms [18]. Moreover, it is currently being evaluated clinically to determine its impact on the quality of life of patients with symptoms such as chemotherapeutic drug toxicity and chemotherapy-induced peripheral neuropathy [19]. Although the direct antitumor effects of $O_2/O_3$ were first reported in 1980 [20], its therapeutic applicability is still unexplored [21, 22]. Thus, this study aimed to assess the anticancer effects of MLT combined with CBD and $O_2/O_3$ in vitro, at the biological and molecular levels, and in a xenograft mouse model of PDAC. The study findings suggest that the different combination treatments inhibited PDAC cell line viability, modulated the RAS signaling pathway, and inhibited PDAC growth in vivo, thus highlighting their potential in PDAC therapy.

## 2 | Materials and Methods

### 2.1 | Cell Lines

Human PANC-1 (RRID:CVCL 0480) and MIAPaCa-2 (RRID:CVCL_0428) PDAC cell lines (Sigma Aldrich, Milan, Italy) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; EuroClone, Milan, Italy) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg streptomycin, and 1 mM sodium pyruvate. Cell lines were maintained at 37°C with 5% $CO_2$ and 95% humidity.

### 2.2 | Reagents

MLT (Cayman Chemical, Ellsworth, MI, USA) was prepared fresh by dissolving in 70% ethanol at 40 mg/mL. Pharmaceutical-grade CBD crystals were purchased from Cayman Chemical and solubilized in 70% ethanol at 15.7 mg/mL (50 mM). Luzindole (LUZ; Sigma Aldrich) was solubilized in dimethyl sulfoxide (DMSO) at 14.6 mg/mL (50 mM). GEM (50 mg/mL), supplied by Sigma-Aldrich, was dissolved in water. Aliquots were prepared and stored at −20°C; each aliquot was used at one time point.

### 2.3 | $O_2/O_3$ Treatment

Cell lines seeded on 96- or 12-well plates were pre-cultured in normoxia for 24 h. Subsequently, the plates were exposed to $O_2/O_3$ treatment in a hypoxia incubator chamber (Stemcell Technology, Vancouver, BC, Canada) by injecting $O_2/O_3$ (80 μg/mL) for 5 min after chamber saturation, using an E100 Ozonline machine (Eco3 s.r.l., Torino, Italy). The plates were then placed back in the 37°C incubator with 5% $CO_2$ and 95% humidity and incubated for 72 h before performing the experiments.

### 2.4 | Cell Viability Assay

To determine cell viability, $3 \times 10^4$ cells/mL were seeded in 96-well plates in a final volume of 100 μL/well. After incubation for 1 day, the treatments were added, and six replicates were used for each treatment. All experiments were repeated thrice. After 72 h, cell viability was analyzed by adding 0.8 mg/mL of 3-[4,5-dimethylthiazol-2-yl]−2,5 diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) to the media. After 3 h, the supernatant was separated, and the pellet of salt crystals was solubilized with 100 μL/well of DMSO. The absorbance of the sample against the background control was measured at 570 nm using an enzyme-linked immunosorbent assay microplate reader (BioTek Instruments, Winooski, VT, USA).

### 2.5 | Cell Death Assay

Propidium iodide (PI) staining, followed by fluorescence-activated cell sorter (FACS) analysis, was used to evaluate cell death. Cells ($3 \times 10^4$ cells/mL) were seeded in 12-well plates and after incubation for 1 day, the treatments were added. Cells were stained with PI (20 μg/mL) for 10 min at room temperature; the percentage of PI–positive cells was analyzed using a BD Accuri C6 plus flow cytometer and the BD Accuri C6 plus software (BD Biosciences, Rome, Italy). All experiments were performed in triplicates.

### 2.6 | Proliferation Assay

CellTrace Cell Proliferation Kit (Thermo Fisher Scientific, Rome, Italy) was used to analyze the proliferation of PDAC cells. PANC-1 and MIAPaCa-2 were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 20 min at 37°C, then seeded $3 \times 10^4$ cells/mL, and cultured for 72 h in a culture medium containing specific treatment. At the end of the treatment, fluorescence was analyzed using FACS. All experiments were performed in triplicates.

### 2.7 | Western Blot

Cell lysates obtained using lysis buffer (Tris 1 M pH 7.4, NaCl 1 M, EGTA 10 mM, NaF 100 mM, deoxycholate 2%, EDTA 100 mM, Triton X-100 10%, glycerol, SDS 10%, $Na_2P_2O_7$ 1 M, $Na_3VO_4$ 100 mM, PMSF 100 mM, cocktail of enzyme inhibitors, and $H_2O$) were separated on a sodium dodecyl sulfate-polyacrylamide gel and transferred onto Hybond-C extra membranes (GE Healthcare, Chicago, IL, USA) using a Bio-Rad system. Nonspecific binding sites were blocked with 5% low-fat dry milk in phosphate-buffered saline (PBS) containing

0.1% Tween-20 for 1 h at room temperature. The membranes were then reacted with mouse anti-MTNR1A/B (1:1000, sc-398788, Santa Cruz Biotechnology, Heidelberg, Germany) and mouse anti-β-actin (1:1000, sc-47778, Santa Cruz Biotechnology) were used. Antibodies were incubated 1 h or overnight, according to the manufacturer's protocol, followed by incubation with horseradish peroxidase–conjugated anti-mouse (1:2000, #7076, Cell Signaling, Danvers, MA, USA) antibodies for 1 h. Peroxidase activity was visualized with the LiteAblotPLUS or TURBO (Euro-Clone) kit and densitometric analysis was performed using Chemidoc and the Quantity One software version 4.6 (Bio-Rad, Milan, Italy).

### 2.8 | Milliplex Multiplex Assay

The levels of total RAS, pBRAF, pCRAF, and pMEK1 were measured using a RAS-RAF Oncoprotein Panel 6-Plex Magnetic Bead Kit 96-well plate (EMD Millipore Corporation, Billerica, MA, USA) following the manufacturer's protocol. Data were analyzed using a Luminex 200 instrument with xPONENT software (Luminex Corporation, Austin, TX, USA).

### 2.9 | Drug Interaction

Drug interaction was evaluated with SynergyFinder version 3.0 using the Bliss–Loewe model [23]. The Bliss–Loewe model combines Bliss, Loewe, and the highest single-agent (HSA) models. A synergy score larger than 10 is considered synergistic, a score from −10 to 10 is considered additive, and a score less than −10 is considered antagonistic.

### 2.10 | Evaluation Using an Orthotopic Pancreatic Tumor Mouse Model

The orthotopic pancreatic tumor mouse model was established by inoculating PANC-1 tumor cells in athymic nude mice. Female 5-week-old athymic nude-Foxn1nu mice were supplied by Envigo RMS SARL (Gannat, France). All the procedures involving the animals were conducted by the Department of Experimental and Clinical Medicine of the University of Florence (Florence, Italy) in accordance with national and international laws on experimental animals (d.l. March 4, 2014, No. 26, Implementation of Directive No. 2010/63/UE), and the experimental protocol was approved (Authorization No. 844/2021-PR). Animals were maintained at a temperature of 22 ± 2°C under a daily 12 h photoperiod in a ventilated cabinet. Following acclimatization for 13 days, 40 animals were anesthetized with isoflurane (induction at 4% and maintenance at 2%) and inoculated with PANC-1 tumor cells ($1 \times 10^6$ cells in 20 μL PBS), which were orthotopically injected into the tail of the pancreas using an echo-guided procedure. Tumor size was determined using ultrasound imaging; on reaching a tumor volume of approximately 10 $mm^3$ (19 days after inoculation), 30 out of 40 animals were divided into the following six groups ($n = 5$ per group): (1) vehicle (VHC, saline); (2) mix (400 mg/kg MLT and 10 mg/kg CBD; (3) $O_2/O_3$ (2.5 mL/kg $O_2/O_3$); (4) mix + $O_2/O_3$ (400 mg/kg MLT and 10 mg/kg CBD + 2.5 mL/kg $O_2/O_3$); (5) GEM (50 mg/kg GEM); (6) mix + $O_2/O_3$ + GEM (400 mg/kg MLT and 10 mg/kg CBD + 2.5 mL/kg $O_2/O_3$ + 50 mg/kg GEM). A total of 10 treatments were administered by intraperitoneal injection every 3 days for 30 days.

### 2.11 | In Vivo Data Analysis

Tumor volume was analyzed using the Vevo Lab software (Fujifilm Visualsonics). The volumes were measured by delineating the ROI (region of interest) for each axial slide using the Vevo LAB software.

### 2.12 | Statistical Analyses

Data are presented as the mean with a standard deviation of at least three independent experiments. Statistical analyses were performed using Welch's *t*-test, one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's multiple comparison test, and two-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 9.0.1(128) software (San Diego, CA, USA).

## 3 | Results

### 3.1 | The Effect of MLT on Human PDAC Cell Lines

The MTT assay revealed that doses up to 100 μg/mL of MLT did not affect PDAC cell viability, while higher doses reduced cell viability with half-maximal inhibitory concentration ($IC_{50}$) values of 594 and 579.5 μg/mL on PANC-1 and MIAPaCa-2 cell lines, respectively, after 72 h of incubation (Figure S1A,B). To determine if MLT interferes with PDAC cell proliferation, both the cell lines were treated with two sub-$IC_{50}$ (200 and 400 μg/mL) and near-$IC_{50}$ (600 μg/mL) doses of MLT, and cell proliferation was determined relative to the nonproliferative control (NPC) at 72 h after treatment. The data showed that all the doses of MLT significantly decreased the proliferation of the cell lines, relative to the untreated samples (Figure S1C). Moreover, using the same treatments followed by PI staining, the cells were analyzed to determine if the reduced cell proliferation was due to MLT-induced cell death. The results revealed an increased percentage of PI-positive cells with all the MLT doses (Figure S1D). Thus, MLT reduced cell proliferation by inducing cell death in both cell lines 72 h after treatment. Since MLT can act in a receptor-dependent and receptor-independent manner, protein expression of the MLT receptors, MTNR1A and MTNR1B, and the implications of these receptors in MLT-induced inhibition of PDAC cell viability were investigated. The findings revealed that PANC-1 and MIAPaCa-2 cells expressed MLT receptors, with a marginally higher expression in MIAPaCa-2 cells than that in PANC-1 cells (Figure S2A). Further, PDAC cells were pretreated for 1 h with two different nontoxic doses of LUZ (Figure S2B), an MTNR1A and MTNR1B antagonist. The results showed that pretreatment did not influence the MLT-induced reduction in PDAC cell

viability (Figure S2C), suggesting that the MLT effect was receptor-independent.

### 3.2 | MLT Combined With CBD Induced Cytotoxic Effects in Human PDAC Cell Lines

The effect of CBD has been previously evaluated in PANC-1 and MIAPaCa-2 cells [17]. In this study, the effect of the CBD–MLT combination was assessed in both cell lines. Cells were treated with a combination of three doses each of MLT (100, 200, and 400 μg/mL) and three doses of CBD (1, 2, and 4 μg/mL) and analyzed 72 h after treatment. The findings revealed that the effects of MLT were increased with CBD 4 μg/mL, compared to those with MLT alone, based on the reduction in cell viability in both cell lines. Moreover, the combination of MLT and CBD resulted in significantly reduced cell viability, relative to that of untreated cells. Using the SynergyFinder software, synergism was evaluated and the heatmaps demonstrated synergistic effects in combination with CBD 4 μg/mL (Figure 1A,B).

The effects of CBD + MLT on cell proliferation and death were evaluated. CBD alone reduced cell proliferation and induced cell death in both cell lines (Figure S3A,B). PDAC cell lines were treated with the two effective combinations of CBD and MLT (CBD 4 μg/mL + MLT 200 μg/mL; CBD 4 μg/mL + MLT 400 μg/mL) and analyzed 72 h after treatment. Cell proliferation was significantly reduced with both treatments in both the cell lines, relative to that in the untreated samples. No significant differences were observed in comparing the effects among the two combinations (Figure 2A,C). A significant increase in the percentage of PI-positive cells was induced by combination treatment compared to that in the untreated samples, demonstrating the induction of cell death in both cell lines (Figure 2B,D). The results also evidenced that the CBD + MLT combinations were more effective than the individual compounds.

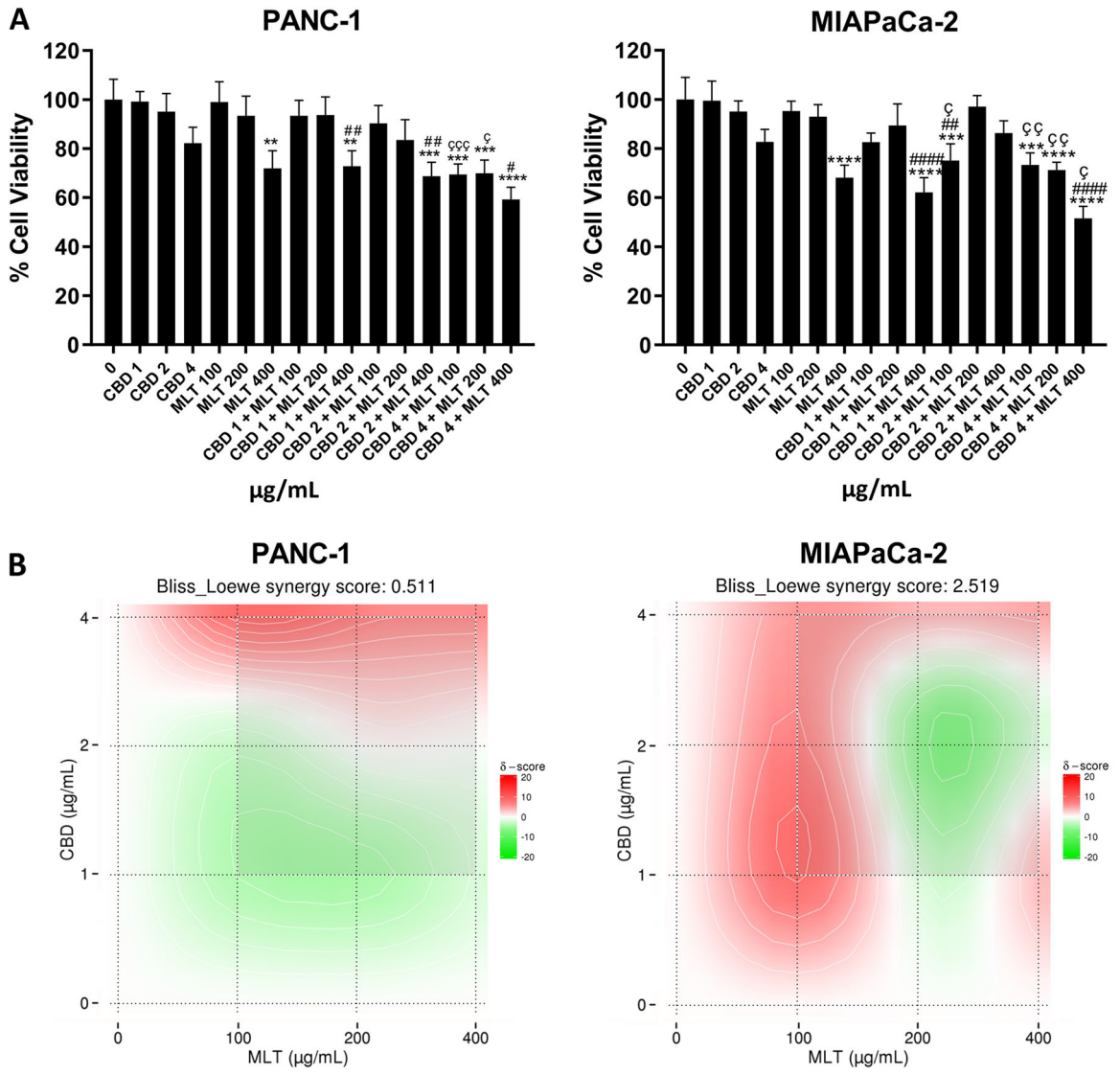



**FIGURE 1** | The effect of melatonin (MLT) + cannabidiol (CBD) in PANC-1 and MIAPaCa-2 cell lines. (A) Cell viability was determined using the MTT assay. Data are expressed as mean ± standard deviation (SD) of three separate experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ CBD + MLT versus CBD; $^{ç}p < 0.05$, $^{çç}p < 0.01$, $^{ççç}p < 0.001$ MLT + CBD versus MLT; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ treated versus untreated. (B) Drug interaction of MLT with CBD in PDAC cell lines was evaluated with SynergyFinder software using the Bliss–Loewe model. A synergy score larger than 10 is considered synergistic, a score from −10 to 10 is considered additive, and a score less than −10 is considered antagonistic.

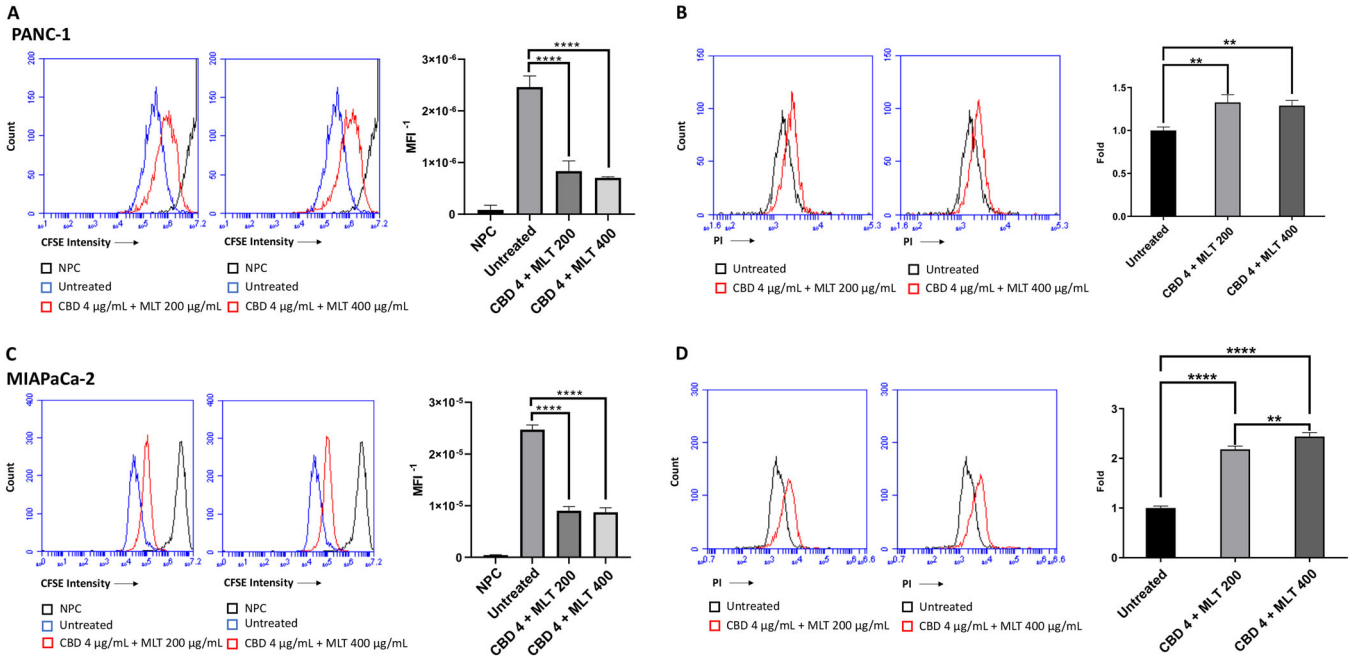



**FIGURE 2** | Effect of CBD + MLT combination on PDAC cell lines. (A, C) Inhibition of PDAC cell proliferation was evaluated using the CFSE dye after 72 h of incubation with two dose combinations of CBD + MLT. NPC, nonproliferative cells. Histograms represent one of the three replicates. Statistical analyses are calculated using the inverse of CFSE mean fluorescence intensity ($MFI^{-1}$). (B, D) CBD + MLT-induced cell death was evaluated using propidium iodide (PI) staining and flow cytometric analysis. The MFI values of treated cells were normalized to those of the untreated cells. $^{**}p < 0.01$ and $^{****}p < 0.0001$.

### 3.3 | $O_2/O_3$ Increased MLT and CBD Efficacy

The effect of MLT + CBD on the addition of $O_2/O_3$ was evaluated in PDAC cells. Cells were treated with CBD (2 and 4 µg/mL) in combination with MLT (200 and 400 µg/mL) alone or with the addition of $O_2/O_3$. The results showed that the effect of CBD + MLT significantly increased in the presence of $O_2/O_3$ in both the cell lines (Figure 3A,B) 72 h after treatment. A significant increase in PI fluorescence confirmed that the presence of $O_2/O_3$ improved cell death relative to the CBD + MLT treatment in both cell lines (Figure 3C,D).

### 3.4 | CBD + MLT + $O_2/O_3$ Increased the Cytotoxic Effect of GEM in Human PDAC Cell Lines

Since GEM is the predominantly used chemotherapeutic drug in PDAC, the effect of the combination of MLT + CBD + $O_2/O_3$ with GEM was evaluated. GEM (25 µg/mL) was combined with CBD (2 and 4 µg/mL), MLT (200 and 400 µg/mL), and $O_2/O_3$. The findings revealed that the efficacy of GEM was enhanced by the addition of all combinations (Figure 4).

### 3.5 | RAS Pathway Modulation in PANC-1 Cell Lines

To further elucidate the molecular mechanism of action of MLT + CBD + $O_2/O_3$ + GEM treatment, modulation in the levels of phosphorylated BRAF (pBRAF), CRAF (pCRAF), MEK1 (pMEK1), and total RAS protein were analyzed using the MILLIPLEX RAS-RAF Oncoprotein Magnetic Bead Panel 6-plex in PDAC cell lines. The cells were treated with CBD 4 µg/mL, MLT 200 µg/mL, $O_2/O_3$, and GEM. The results revealed that, in PANC-1 cells, all treatments, except GEM alone, induced a negative modulation of total RAS protein (Figure 5A), and all treatments, except $O_2/O_3$ alone, reduced pBRAF levels (Figure 5B). pCRAF levels were reduced by all treatments, compared to those in untreated cells, whereas pMEK1 levels were not significantly altered (Figure 5C,D). Similar results were obtained for MIAPaCa-2 cells (data not shown). In addition, the results showed highly significant decreases in total RAS and pBRAF levels using all treatments, compared to those using GEM alone (Figure 5A,B).

### 3.6 | Effect of MLT + CBD and $O_2/O_3$ in a PDAC Mouse Model

In vivo experiments were performed to further investigate the efficacy of these combinations in PDAC. Mice were subdivided into the following six groups: VHC (Group 1), MLT + CBD (Group 2), $O_2/O_3$ (Group 3), MLT + CBD + $O_2/O_3$ (Group 4), GEM (Group 5), and MLT + CBD + $O_2/O_3$ + GEM (Group 6) (Figure 6A,B).

During treatment, ultrasound and photoacoustic imaging were performed once a week to evaluate engraftment and development of the tumor mass. At the end point, the mice were euthanized, and macroscopic necroscopy was performed. All tumors were explanted, weighed, and photographed (Figure 7A).

The data showed no significant variation in tumor volume until Day 33 of treatment (Figure 7B). On Day 40, a significant

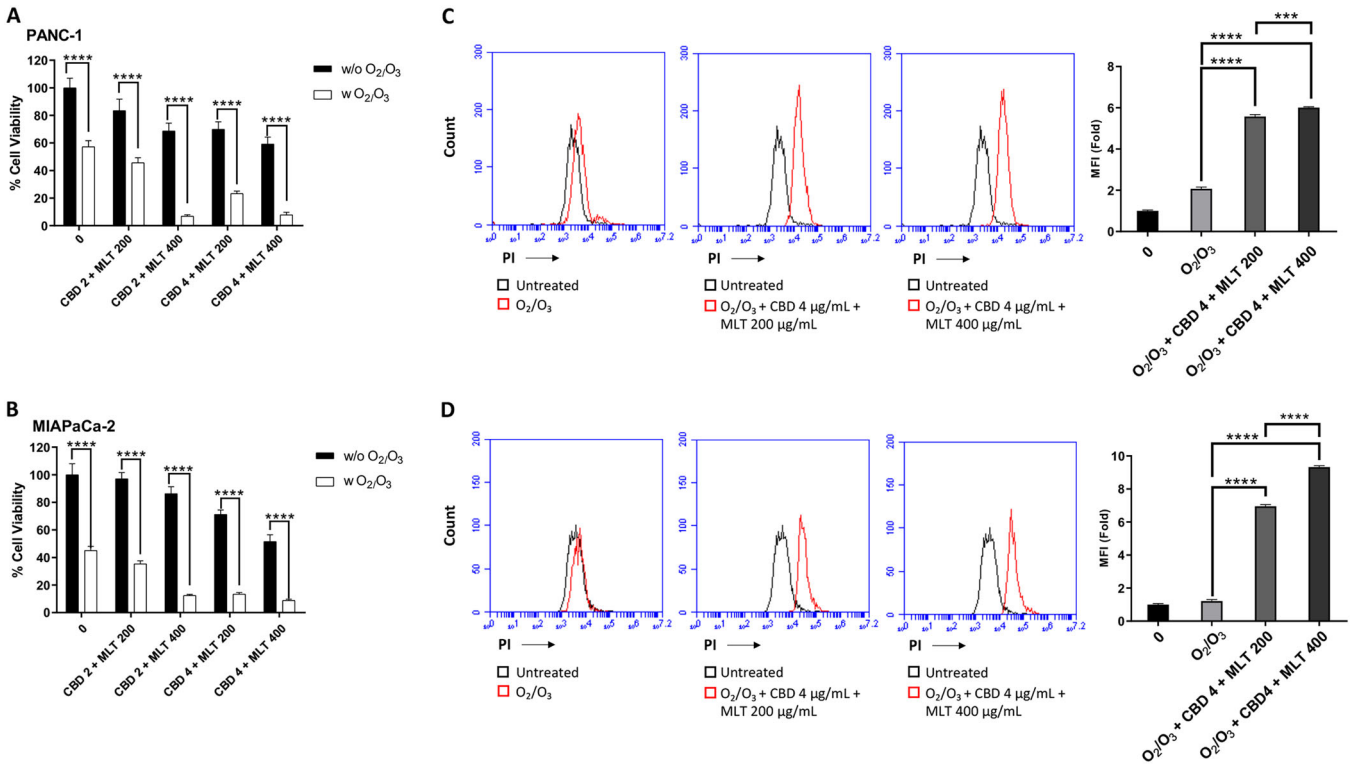



**FIGURE 3** | The effect of MLT + CBD + $O_2/O_3$ treatment in PDAC cell lines. (A, B) Cell viability was determined using the MTT assay 72 h after treatment. Data are expressed as the mean ± SD of three separate experiments. ****$p < 0.0001$. (C, D) To analyze cell death, flow cytometric analysis was performed after PI staining. The MFI values of treated cells were normalized to those of the untreated cells. Histograms represent one of the three replicates. ***$p < 0.001$ and ****$p < 0.0001$.

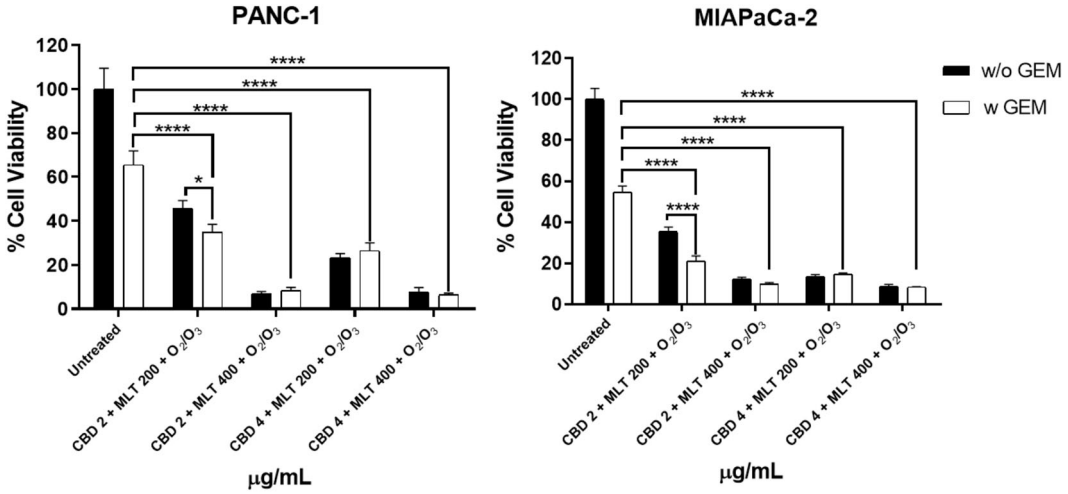



**FIGURE 4** | The effect of MLT + CBD + $O_2/O_3$ + GEM treatment on PANC-1 and MIAPaCa-2 cell lines 72 h after treatment. Cell viability was determined using the MTT assay. Data are expressed as the mean ± SD of the three replicates. *$p < 0.05$ and ****$p < 0.0001$.

reduction in tumor volume was observed in Group 5 compared to that in Group 1 and was more evident in Group 6 (Figure 7B). At the end point (Day 47), a significant reduction in tumor volumes was observed in all the treated groups with and without GEM, compared to that in the VHC group (Figure 7B). Moreover, if the reduction was not significant between Groups 5 and 6, it can be considered that the addition of CBD + MLT + $O_2/O_3$ marginally improved the reduction in tumor volume by GEM. Similar results were obtained by analyzing the explanted tumor mass, wherein tumor weight reduction was significant in all treated groups, particularly in Groups 5 and 6, compared to that in the VHC group (Figure 7A,C). A remarkable reduction in mouse weight was observed in Groups 5 and 6 after Day 33, whereas no such decrease was observed in Groups 2, 3, and 4 till the end point, suggesting that this effect was associated with the presence of GEM, whereas the other treatments did not induce animal toxicity (Figure 7D). In summary, all treatments reduced tumor growth. In particular, Groups 2, 3, and 4 showed approximately 50% reduction in tumor mass and volume,

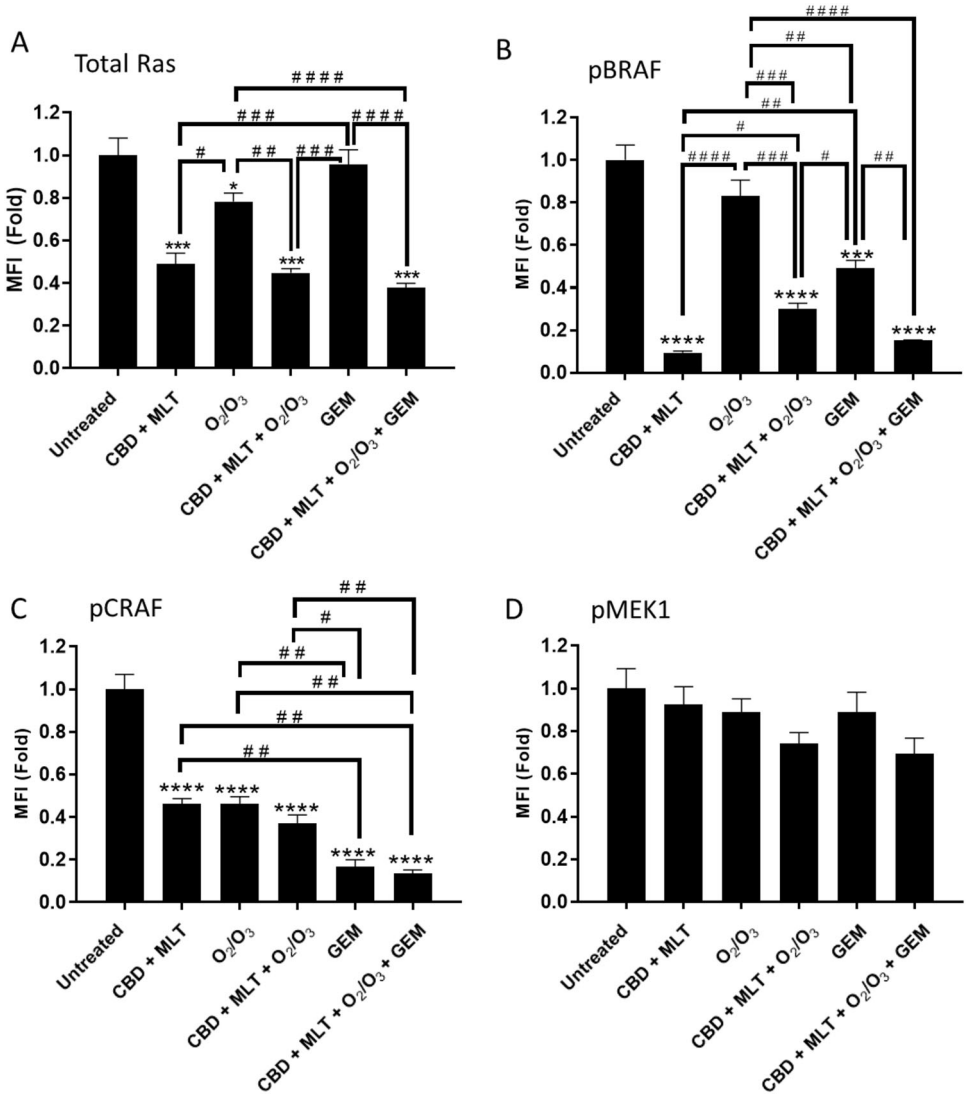
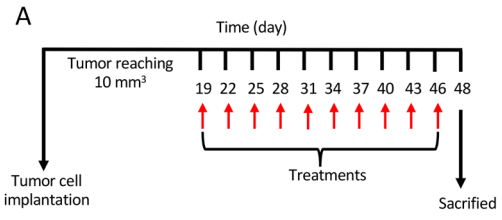



**FIGURE 5** | Alteretion of total RAS (A) pBRAF (B), pCRAF (C), and pMEK1 (D) protein levels in PANC-1 cells. These cells were treated with the various combinations for 48 h and the expressions of total RAS, pBRAF, pCRAF, and pMEK1 were evaluated using the Milliplex multiplex assay. The MFI was measured using the Luminex 200 system. Data are expressed as the mean ± SD of the three replicates. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ treated versus untreated cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.




B

| Groups | Number of animals | Treatment | Dose |
|---|---|---|---|
| 1 | 5 | Vhc (saline) | / |
| 2 | 5 | MLT + CBD | 400 mg/kg MLT<br>10 mg/kg CBD |
| 3 | 5 | $O_2/O_3$ | 2.5 ml/kg $O_2/O_3$ |
| 4 | 5 | MLT + CBD + $O_2/O_3$ | 400 mg/kg MLT<br>10 mg/kg CBD<br>2.5 ml/kg $O_2/O_3$ |
| 5 | 5 | GEM | 50 mg/kg GEM |
| 6 | 5 | MLT + CBD + $O_2/O_3$ + GEM | 400 mg/kg MLT<br>10 mg/kg CBD<br>2.5 ml/kg $O_2/O_3$<br>50 mg/kg GEM |

**FIGURE 6** | (A) Experimental design used for the treatment of mice inoculated with PANC-1 cells. When the tumor volume was approximately 10 $mm^3$, the animals were treated intraperitoneally with the respective treatments every 3 days for a total of 10 treatments. (B) Treatment of each animal group and dose received.

which did not influence mouse weight, suggesting the absence of toxicity, relative to Groups 5 and 6. On Day 40, the most significant reduction in tumor volume was observed in the CBD + MLT + $O_2/O_3$ + GEM group (Figure 7B). At the end point, the mice were euthanized, macroscopic necroscopy was performed, and the liver, brain, and heart of the animals were explanted and weighed; changes in the weights of these organs were not significant (Figure 8).

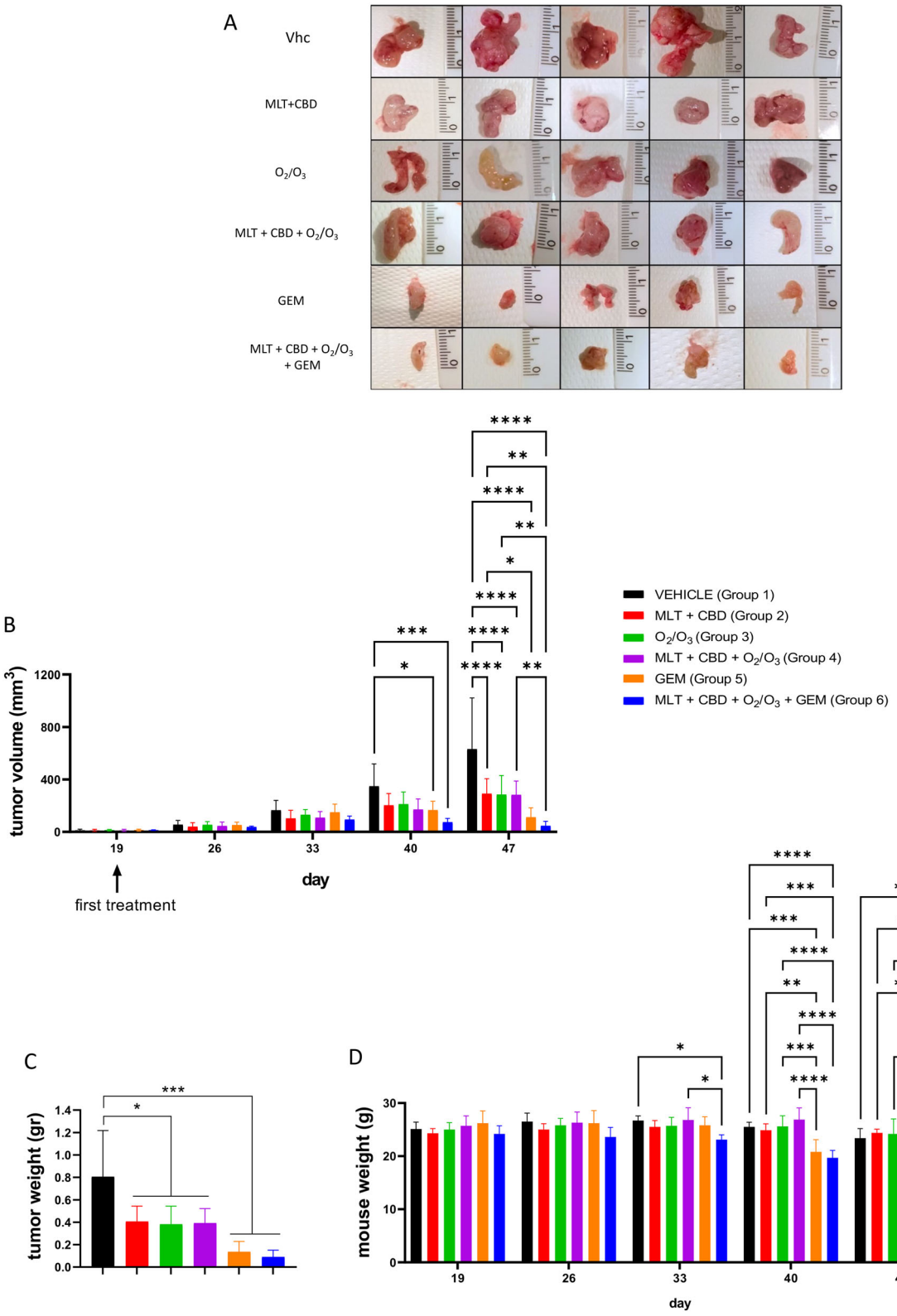



**FIGURE 7** | Effect of the study treatments on the PDAC mouse model. (A) Representative photographs of tumors at the end of treatments after the explantation. (B) Tumor volumes of PANC-1-inoculated mice, treated as described in Figure 6. Volumes were measured using ultrasound and photoacoustic imaging once a week. (C) Tumor weight at the end of treatments. (D) Body weight of the mice during the treatments. The values are expressed as the mean ± SD of five animals in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

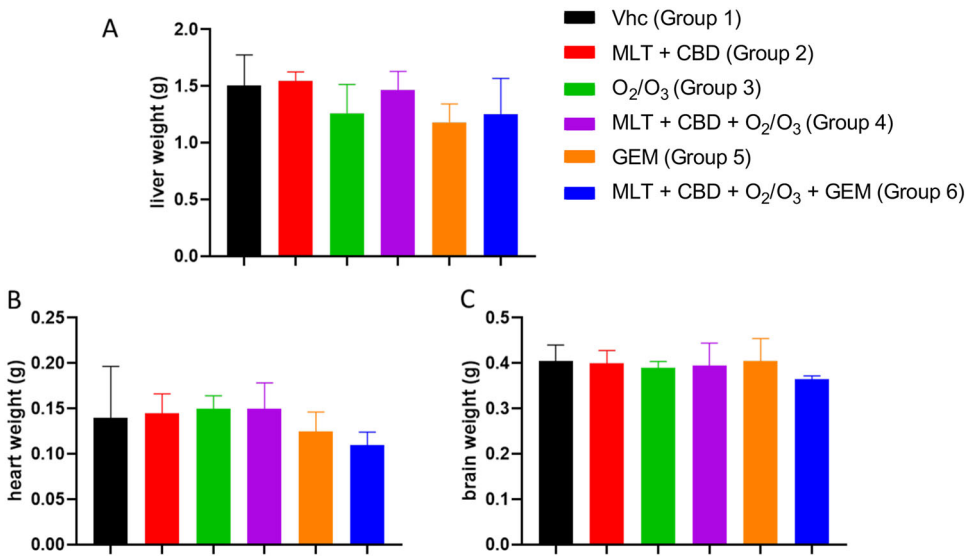



**FIGURE 8** | Effect of the study treatments on organ weight in PDAC mice. (A) Liver (five animals of each group), (B) heart (two animals of each group), and (C) brain (two animals of each group) weight. The differences were not statistically significant.

## 4 | Discussion

Despite advanced therapies, most of the PDAC cases are still incurable [1, 2]. In addition, tumor heterogeneity and metabolic changes are linked to low-grade clinical outcomes, which has highlighted the need to investigate the pathways regulating chemoresistance and develop new therapeutic strategies targeting specific genetic pathways [1, 2]. The standard first-line treatment for PDAC is GEM, but only with partial efficacy [2]. The efficacy of chemotherapeutic drugs, such as GEM, can be ameliorated by administering evidence-based integrative compounds [7, 8, 16, 17]. In our study, MLT reduced cancer cell viability and induced cell death in PANC-1 and MIAPaCa-2 cells, like the effects observed in SW-1990 and other PDAC lines (AsPc-1 and Panc-28), by inducing proapoptotic and pro-necrotic effects [7, 10]. MLT synergizes with sorafenib to suppress PDAC viability both in vitro and in vivo via promoting apoptosis by blocking the PDGFR-β/STAT3 signaling pathway [8]. MLT inhibits NF-κB by reducing IκBα phosphorylation and consequently the expression of NF-κB target genes in MiaPaCa-2, AsPc-1, and Panc-28 cells and enhances GEM cytotoxicity both in vitro and in vivo [7]. The effect of CBD on PDAC was investigated both in vitro and in vivo as a single compound and in combination with GEM. In vivo, the mice treated with the combination of CBD with GEM showed three times higher survival than that in mice treated with VHC or GEM alone [16]. Another in vitro study confirmed the antitumor effects of CBD combined with $O_2/O_3$ in PDAC cell lines [17]. Thus, CBD and $O_2/O_3$, alone or in combination, could induce cell death and enhance the efficacy of GEM [17]. Based on these data, the aim of this study was to evaluate the potential antitumor role of a novel combination therapy composed of MLT, CBD, and $O_2/O_3$. The in vitro results showed improved cytotoxicity on using CBD + MLT, compared to that with CBD or MLT alone, and this effect was significantly potentiated by $O_2/O_3$ addition. The efficacy was confirmed in in vivo model showing a significant decrease in tumor mass with the administration of MLT + CBD, $O_2/O_3$ alone, and the triple combination, without significant differences among the three groups. Nevertheless, on Day 40 of treatment, the triple combination combined with GEM showed the most significant difference, in tumor volume, relative to the VHC. More than 90% of PDAC cases harbor activated RAS (mainly KRAS) and RAS pathways [24]. Therefore, it is encouraging to impede RAS and/or downstream targets, such as BRAF, CRAF, and MEK1/2 in the MAPK pathway, for a better response to chemotherapy or to overcome resistance mechanisms. To our knowledge, the effect of MLT on RAS pathways is still unexplored, while those of CBD and $O_2/O_3$ have been previously evidenced, wherein mainly CBD reduces the levels of KRAS as well as some downstream signals at the transcriptional level [17]. In this study, it was demonstrated that CBD, MLT, and $O_2/O_3$, each with different efficacies, reduced total RAS, pBRAF, and pCRAF levels. The effect of the quadruple combination was observed on both pBRAF and pCRAF reduction, which could be related to the effects of MLT + CBD on pBRAF and GEM on pCRAF. Thus, the alteration of this signaling pathway could be one of the molecular mechanisms that induce in vitro and in vivo reduction of PDAC growth in these preclinical models.

In conclusion, these data highlight that MLT + CBD, $O_2/O_3$, and their triple combination exert anticancer effects, halving tumor volume without inducing animal toxicity. The quadruple combination showed the most significant reduction in tumor volume on Day 40, and its efficacy was maintained at the endpoint, although it was not significantly different from that of GEM alone. Further studies are required to confirm the responses observed in the in vivo model. A potential approach would be to use these integrative compounds with lower doses of GEM to achieve the same antitumor effects while reducing the toxicity of chemotherapeutic agents.

### Author Contributions

Conceptualization: Massimo Nabissi. Data curation: Laura Zeppa, Cristina Aguzzi, and Maria Beatrice Morelli. Formal analysis: Massimo Nabissi and Maria Beatrice Morelli. Funding acquisition: Massimo Nabissi and Margherita Luongo. Investigation: Cristina Aguzzi, Laura

Zeppa, and Oliviero Marinelli. Methodology: Cristina Aguzzi, Laura Zeppa, Maria Beatrice Morelli, and Massimo Nabissi. Project administration: Massimo Nabissi. Resources: Massimo Nabissi. Supervision: Maria Beatrice Morelli and Massimo Nabissi. Validation: Maria Beatrice Morelli, Massimo Nabissi, and Alessandro Fanelli. Visualization: Laura Zeppa, Cristina Aguzzi, and Maria Beatrice Morelli. Writing–original draft: Laura Zeppa, Cristina Aguzzi, and Massimo Nabissi. Writing–review and editing: Maria Beatrice Morelli, Consuelo Amantini, Martina Giangrossi, and Giorgio Santoni. The work reported in this paper was performed by the authors, unless otherwise specified.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## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that support the findings of this study are presented in the main text and Supplementary Information. The data are available from the corresponding author upon reasonable request.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section.