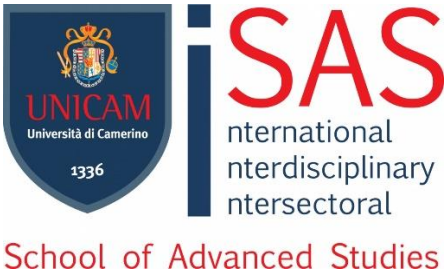

UNIVERSITÀ DEGLI STUDI DI CAMERINO

School of Advanced Studies

DOCTORAL COURSE IN

MOLECULAR BIOLOGY AND CELLULAR BIOTECHNOLOGY

LIFE AND HEALTH SCIENCES

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INTEGRATIVE THERAPIES IN HUMAN PANCREATIC DUCTAL ADENOCARCINOMA: A PRECLINICAL INVESTIGATION

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ABSTRACT

Over the last decades, the interest in additional therapeutic approaches to increase usual anticancer therapy efficacy, reducing side effects and improving patients' quality of life, is growing. An increasing number of cancer patients use, together with traditional anticancer therapies, herbal and dietary supplements. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and despite the therapeutic improvements, its prognosis is very poor. Firstly, it was evaluated the effect of Cannabigerol (CBG), a non-psychotropic cannabinoid from *cannabis Sativa* in two human PDAC cell lines, PANC-1 and MIAPaCa-2. CBG activity was investigated on cell viability, cell death and on EGFR-RAS-associated signaling. Moreover, CBG effect in combination with gemcitabine (GEM) and paclitaxel (PTX) was investigated. Data showed that CBG induced apoptosis and reduced EGFR/Akt/mTOR and Ras pathways, supporting the ability of cannabinoids in interfering with several pro-tumoral pathways. Moreover, GEM and PTX activity was increased by CBG addition. Subsequently, the effect of a combination of Cannabidiol (CBD), Melatonin (MLT) and Oxygen-Ozone (O_2/O_3) was analysed in *in vitro* and in xenograft mouse model of PDAC. CBD is the most studied non-psychotropic cannabinoid in preclinical and clinical studies for its anticancer properties. MLT effect is being assessed for preventing or treating chemotherapy and radiotherapy side effects and there are some evidences about its anticancer effect in several preclinical cancer models. O_2/O_3 therapy is considered an integrative opportunity for cancer patients for its effect in reducing pain, fatigue and musculoskeletal symptoms, but it is actually little investigated as direct anticancer molecule. Results evidenced that the different treatments inhibited PDAC cell lines viability, modulating also Ras signaling pathway, and inhibited PDAC growth in mice, alone and combined with GEM. Data suggest that these different treatments could be an interesting approach in supporting PDAC therapy

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1. Introduction

1.1. Integrative therapies

Cancer is one of the most widespread pathologies and it was the cause of 10 million deaths in the world in 2020. In 2023, 1.958.310 new cancer cases and 609.820 cancer deaths are expected in the United States and in the next 20 years, the World Health Organization (WHO) estimates an increase of cancer cases of approximately 70%. Hence nowadays, cancer treatment remains a challenge (Figure 1). Surgery, radiotherapy and systemic therapy such as chemotherapy, hormonal treatments and targeted therapies have different side effects and, in some cases, limited efficacy also for the therapeutic resistance (1,2).

Estimated New Cases

Males				Females		
Prostate	288,300	29%		Breast	297,790	31%
Lung & bronchus	117,550	12%		Lung & bronchus	120,790	13%
Colon & rectum	81,860	8%		Colon & rectum	71,160	8%
Urinary bladder	62,420	6%		Uterine corpus	66,200	7%
Melanoma of the skin	58,120	6%		Melanoma of the skin	39,490	4%
Kidney & renal pelvis	52,360	5%		Non-Hodgkin lymphoma	35,670	4%
Non-Hodgkin lymphoma	44,880	4%		Thyroid	31,180	3%
Oral cavity & pharynx	39,290	4%		Pancreas	30,920	3%
Leukemia	35,670	4%		Kidney & renal pelvis	29,440	3%
Pancreas	33,130	3%		Leukemia	23,940	3%
All Sites	**1,010,310**	**100%**		**All Sites**	**948,000**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	67,160	21%		Lung & bronchus	59,910	21%
Prostate	34,700	11%		Breast	43,170	15%
Colon & rectum	28,470	9%		Colon & rectum	24,080	8%
Pancreas	26,620	8%		Pancreas	23,930	8%
Liver & intrahepatic bile duct	19,000	6%		Ovary	13,270	5%
Leukemia	13,900	4%		Uterine corpus	13,030	5%
Esophagus	12,920	4%		Liver & intrahepatic bile duct	10,380	4%
Urinary bladder	12,160	4%		Leukemia	9,810	3%
Non-Hodgkin lymphoma	11,780	4%		Non-Hodgkin lymphoma	8,400	3%
Brain & other nervous system	11,020	3%		Brain & other nervous system	7,970	3%
All Sites	**322,080**	**100%**		**All Sites**	**287,740**	**100%**

Figure 1. New cancer cases and cancer related deaths based on sex estimated in the United States, 2023 (2).

For years, the term 'complementary' signified therapies that were added to conventional anticancer treatments, while 'alternative' signified therapies that were substituted for conventional anticancer

approaches. Together, these two terms, were known as Complementary and Alternative Medicine (CAM). Over the last decades, an increasing number of cancer patients use, together with traditional anticancer therapies, herbal and dietary supplements. The interest in additional therapeutic approaches is growing to increase usual anticancer therapy efficacy and survival, reducing side effects and improving the quality of life. The term "integrative oncology" is generally used to identify the use of complementary therapies combinations in addition to common therapies (3).

1.1.1 Integrative therapies in pancreatic cancer

Pancreatic cancer is the fourth cause of cancer-related death in the world and its incidence level is estimated to rise in the next years. Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer that involves the exocrine pancreas and it is able to metastasize to the liver and nearby organs. Rather than the current therapeutic improvements, PDAC-associated prognosis is very poor with a 5-year survival of about 6% of cases (4). Surgery and chemotherapy are the main therapeutical approaches for PDAC treatment. Surgical resection is applicable only in 20% of PDAC patients. The presence of major blood vessels, lymphatic system nodes and vital organs near the pancreas could hinder surgery, as could metastasis and patient's critical physical conditions. At the same time, when surgery is applicable, the morbidity remains of about 40%. Chemotherapy is used in presence of metastasis, or as neo-adjuvant (before-surgery) to make PDAC eligible for surgery, or as adjuvant (post-surgery) to reduce recurrence risk. Gemcitabine (GEM) is used alone, or in a combination with 5-fluorouracil (5-FU), leucovorin, irinotecan and oxaliplatin, called FOLFIRINOX, or in association with nab-paclitaxel (PTX) (albumin nanoparticle conjugate with PTX). Despite of the improvements in chemotherapy, the survival rate has only a little improvement, in the last 10 years. The interest in targeted therapy is growing, but few of these approaches showed enhancements in survival rate and in addition the immunotherapy is difficult to applied because PDAC is not "immunogenic" and the tumor micro-environment (TME) suppresses T-cells activity (4, 5). New therapeutic strategies are necessary to treat PDAC. There is a growing interest in several natural products and compounds derived from natural products have been shown to be effective in several types of cancers through *in vitro* and *in vivo* studies, in increasing conventional anticancer drugs efficacy. In particular, there are numerous studies about the use of natural compounds and their derivatives able to induce apoptosis and to inhibit metastasis, angiogenesis and drug resistance, in pancreatic cancer (6).

1.2 Cannabis and cannabinoids

Cannabis sativa L. is a highly variable plant species belonging to the Cannabaceae family. It originated in Western Asia and introduced in Western Medicine in the early 19th century (7). The different strains of *C. sativa* are characterized by different content of Δ^9-Tetrahydrocannabinol (Δ^9-THC) and Cannabidiol (CBD) (8). Cannabis has been used as food, source of fibers and medicine source (7). Regarding the use as "medical cannabis", it is administered for medical conditions such as nausea, anorexia, glaucoma, muscle spasm and pain management (8). There are oromucosal spray preparations based on cannabis extract (Nabiximols marketed as Sativex®) and drugs with synthetic Δ^9-THC (Dronabinol marketed as Marinol®) and Δ^9-THC analogue (Nabilone marketed as Cesamet®) used in therapy (8,9).

This plant has a large number of natural constituents, more than 545 known compounds, cannabinoids and non-cannabinoids compounds such as phenols, flavonoids, terpenes and alkaloids that make it a complex species. Cannabinoids produced by *C. sativa* are known as phytocannabinoids (PyCBs), while the generic term "cannabinoids" includes also cannabinoid receptors endogenous ligands (endocannabinoids) and synthetic cannabinoids. PyCBs are synthetized by the plant as acids and after a non-enzymatic decarboxylation, they are converted in their respective neutral form (Figure 2) (8,9). The first step is the synthesis catalysed by olivetolate geranyltransferase (GOT) of the precursor cannabigerolic acid (CBGA) from geranyl pyrophosphate and olivetolic acid (8).

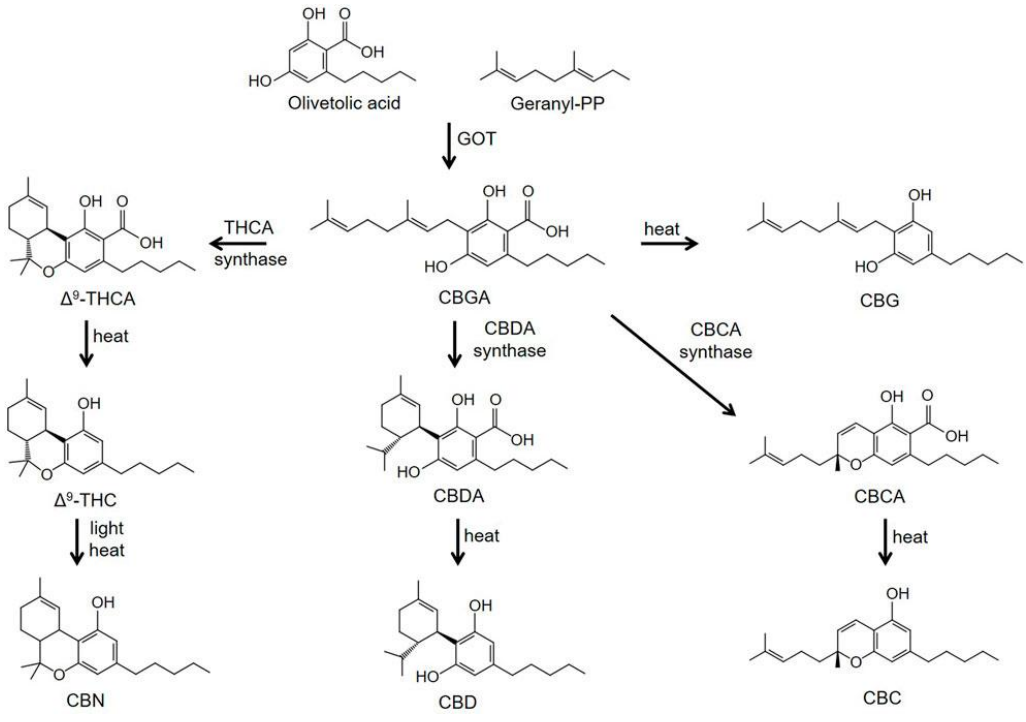


Figure 2. PyCBs biosynthesis. Olivetolate geranyltransferase (GOT); Cannabigerolic acid (CBGA); Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA); Δ^9-tetrahydrocannabinol (Δ^9-THC); Cannabinol (CBN); Cannabidioloic acid (CBDA); Cannabidiol (CBD); Cannabigerol (CBG); Cannabichromenic acid (CBCA); Cannabichromene (CBC) (8).

Δ^9-THC or THC is the main psychoactive molecule, while CBD is another important molecule with not hallucinogenic properties known for its pharmacological activities (7-9). Other PyCBs, known as minor, are identified in smaller amounts in *C. sativa* such as tetrahydrocannabivarin (THCV), cannabinol (CBN), cannabigerol (CBG), cannabidivarin (CBDV), cannabichromene (CBC) and others. PyCBs can interact with cannabinoid receptors and with non-cannabinoid receptors (7,8).

1.2.1 Endocannabinoid system

The observed clinical benefits of *C. sativa* could be explained by the activity of its components not only on a single target but also on a more complex system (10). The endocannabinoid system (ECS) is a complex ensemble of receptors, signaling molecules and biosynthetic and degrading enzymes (Figure 3) (10).

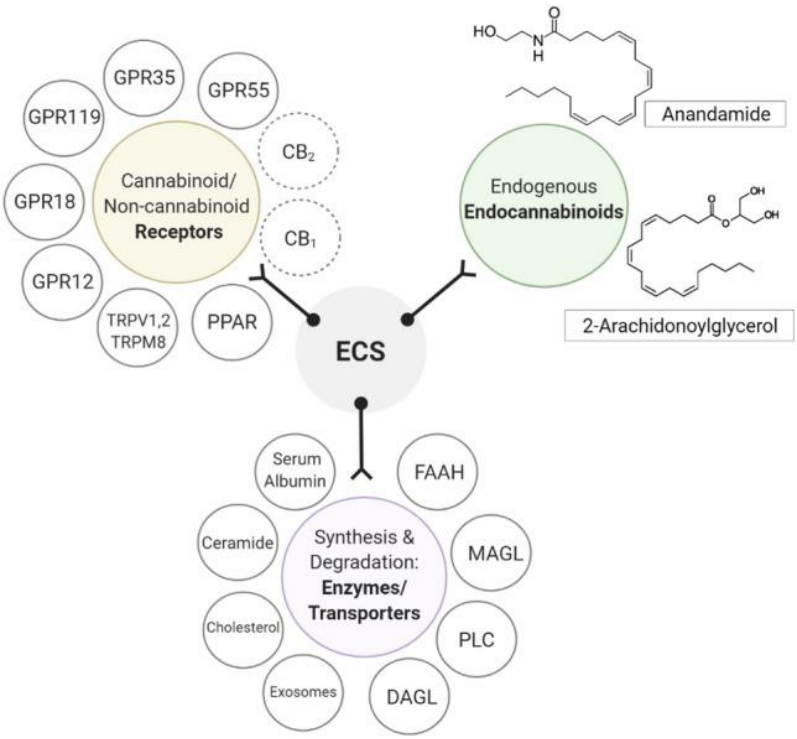


Figure 3. Components of ECS divided in receptors, endocannabinoids and enzymes/transporters. Cannabinoid receptor 1 and 2 (CB_1 and CB_2); G protein-coupled receptor (GPR12, GPR18, GPR119, GPR35, GPR55); Peroxisome-proliferator-activated receptors (PPAR); Transient receptor potential cation channel subfamily members (TRPV1, TRPV2, TRPM8); Fatty acid amide hydrolase (FAAH); Monoacylglycerol lipase (MAGL); Phospholipase C (PLC); Diacylglycerol lipase (DAGL) (11).

A first analysis defined the ECS composed by two G protein-coupled receptors (GPRs), Cannabinoid receptors type-1 and type-2 (CB_1R or CB_1 and CB_2R or CB_2), the endocannabinoids N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), and five enzymes involved in endocannabinoids biosynthesis and degradation (12). However, further studies demonstrated that the ECS is more complex and it was identified as "endocannabinoidome". The endocannabinoidome includes other novel mediators similar to the endocannabinoids, the transient receptors potential (TRPs) cation channels, other orphan GPRs (GPR55, GPR18, GPR119) and the peroxisome proliferator-activated nuclear receptors (PPARs) (12,13). PyCBs can activate or inhibit different receptors and enzymes of "endocannabinoidome" (Figure 4) (12).

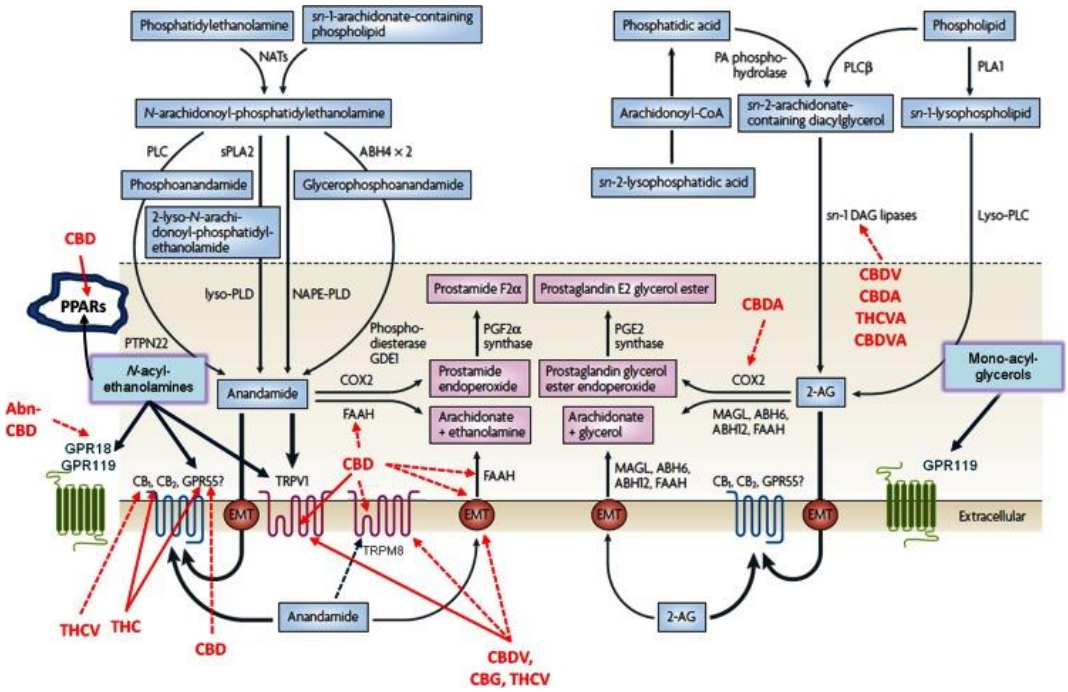


Figure 4. Overview of "endocannabinoidome" complexity and the interactions with PyCBs (12)

1.2.1.1 Cannabinoid receptors (CB_1 and CB_2)

CB_1 and CB_2 are two GPRs involved in PyCBs bioactivities. CB_1, encoded by the gene CNR1, is mainly expressed in the central nervous system (CNS), where it modulates different neurological activities and in small amount in peripheral tissues. CB_1 is coupled to Gi/o protein (in some conditions also with Gs and Gq). CB_2, encoded by the gene CNR2, is expressed in peripheral tissues, immune system (so it has immunomodulatory effect) and in small amount in the brain. CB_2 is coupled with Gi/q protein (14).

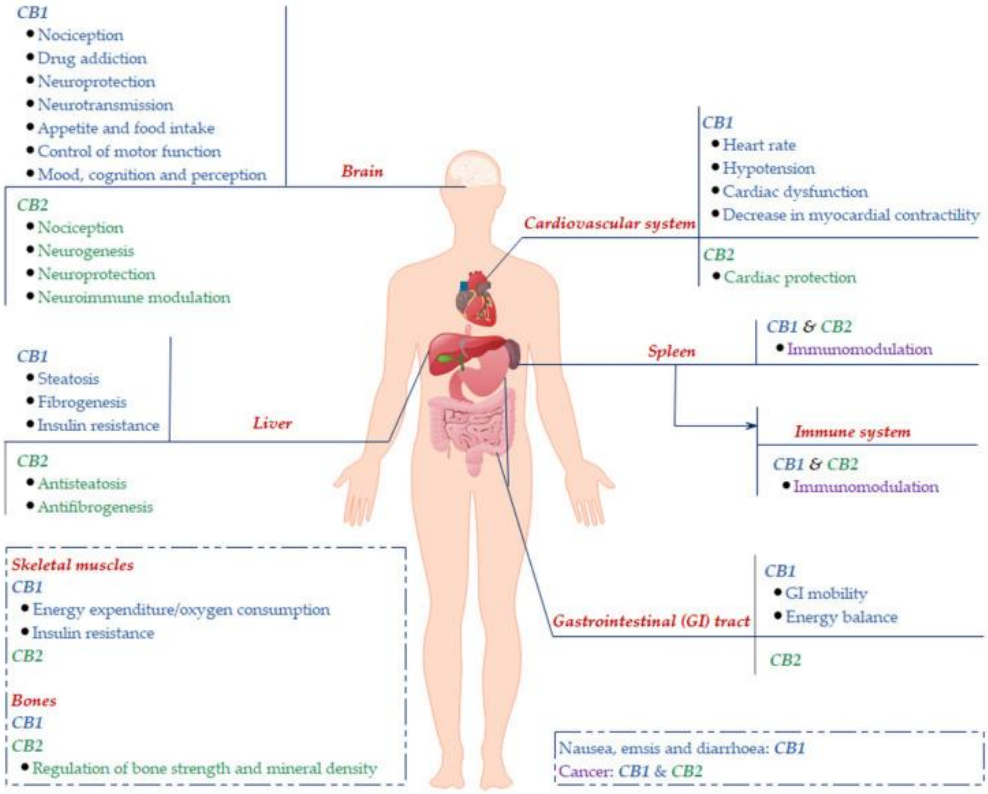


Figure 5. CB_1 and CB_2 expression and their involvement in physiological activities (14)

Previous studies demonstrated that among the high number of PyCBs found in *C. sativa*, only THC is able to bind with high affinity CB_1 and CB_2, with predominantly agonistic activity, while CBD has low affinity for both receptors (12, 14). THC psychotropic activity, appetite stimulation, analgesia and anti-emetic effect is due to its interaction as partial agonist of CB_1 (14).

1.2.1.2 Transient receptors potential (TRPs)

TRPs channels are a family of ion channels located in the plasma membrane and in cytosolic organelles involved in many physiological and pathological processes such as temperature sensation, cancer, pain and genetic disorders (15, 16). They are expressed in multiple tissues and in peripheral neurons where transmit chemical and physical stimuli as nerve impulses to the brain (17). These channels are structured to create a passage from a side of membrane to the other and they can be opened or closed based on the received stimuli. Generally, the ions passage is a transitory event and the TRP channels are involved in intracellular cation flux, mainly sodium and calcium, and in cellular depolarization (Figure 6) (16,17). THC and the other PyCBs interact with TRPs channels similarly to AEA (12). TRP channels modulated by PyCBs are the vanilloid TRP channels (TRPV1, TRPV2, TRPV3 and TRPV4), the melastatin TRP channel 8 (TRPM8) and the ankyrin TRP channel 1 (TRPA1). Also, endocannabinoids and synthetic cannabinoids can interact with the TRPs channels. Evidences reported that the main PyCBs interact as agonists of TRPV1, TRPV2, TRPV3, TRPV4 and TRPA1 with different affinity, and as antagonist of TRPM8. The potential mechanism of action of TRP channels is their desensitization upon their activation, which makes them refractory to other stimuli. This could explain the analgesic effect of TRP channels and the reduction of neuronal activity (15).

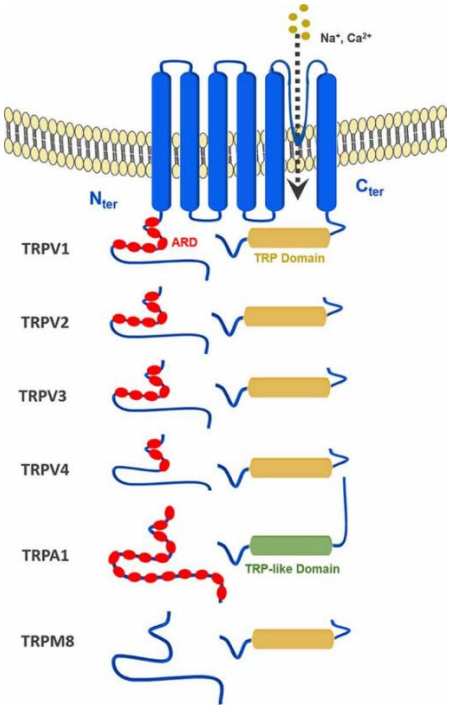


Figure 6. Structural representation of TRP channels involved in PyCBs activity (15)

1.2.1.3 Other receptors

In the last years, other receptors able to interact with cannabinoids were identified. They are GPR55, GPR18, GPR3, GPR6 and GPR12. GPR55 is a receptor expressed in the brain, peripheric nervous system and immune cells and it is associated with cannabinoids receptors activity; its endogenous ligand is the lysophosphatidylinositol (LPI). GPR18 has low sequence homology with CB_1, CB_2 and GPR55, and it is present in several tissues such as brain, lymphoid tissue, lungs, ovary and testis. GPR3, GPR6 and GPR12 have a 60% of homology with CB_1 and CB_2 and are expressed in the brain and reproductive system. The PPARγ is a receptor mainly expressed in adipose tissue and macrophages, and it is involved in glucose metabolism and lipids storage. Other receptors involved in cannabinoids activity are serotonin and adrenergic receptors, and ligand-gated ion channels (17,18).

1.2.2 Phytocannabinoids and endocannabinoid system anticancer effect

The ECS and its components are studied for their potential use in cancer therapy. The expression level of CB_1 and CB_2 is altered in several cancers and the role in cancer progression is controversial. Depending on cancer types, evidences showed that CB_1 and CB_2 could be positive or negative survival markers (19). The endocannabinoids, AEA and 2-AG, and the enzymes FAAH and MAGL, are produced in abnormal amount in some cancer tissues compared to normal ones. Furthermore, opposite results in the development of the pathology have been reported for these molecules, based on their presence in tumor tissues (19). However, the use of endocannabinoids and exogenous cannabinoids as potential anticancer molecules remains subject of interest. Several studies agree about their potential beneficial effects by inhibiting tumor growth and progression, even if their complex mechanism of action needs to be investigated to avoid interference with other concurrent therapies and disease progression. Several evidences showed that cannabinoids can induce apoptosis and inhibit cancer proliferation, angiogenesis and metastasis, but on the other side there are also reports in which they could promote tumorigenesis (10). Regarding endocannabinoids and FAAH and MAGL enzymes, their action reduced cancer proliferation, invasion and migration. For examples, evidences in brain tumors showed that AEA and 2-AG inhibited cancer proliferation (19). THC and CBD are the mostly investigated PyCBs. Different studies reported their potentiality to enhance cytotoxicity acting in synergism with specific chemotherapeutic drugs, in different model of cancer, both *in vitro* than *in vivo*. In 2017, a phase II randomized placebo-controlled clinical trial showed the potential efficacy of cannabinoids as adjuvant to chemotherapy. 12 patients were randomized with a combination of THC and CBD plus dose-intensive of temozolomide (TMZ), while 9 patients were treated with

placebo plus TMZ. Results showed an increase of one year survival rate and the median survival in the cannabinoids group (10).

1.2.3 Cannabidiol (CBD)

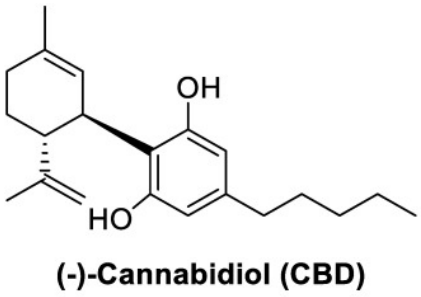


Figure 7. CBD chemical structure (17)

CBD (Figure 7) is the second major PyCBs obtained for the first time in 1940 from *C. sativa*. It has not psychotropic activity and does not induce the effects of a drug of abuse. CBD is a hydrophobic molecule, so its absorption happens in the intestine, then it reaches the bloodstream and it is absorbed by the adipose tissues and organs. After some evidence reported in patients treated daily with doses from 10 to 400 mg, CBD has proven to have a good safety profile and in recent years the interest in its pharmacological use has been growing. CBD is effective in pain, inflammation, epilepsy and anxiety conditions and in some neuropsychiatric and neurodegenerative disease (17). Its mechanism of action remains complex. It has low affinity for CB_1 and CB_2 receptors, but it can modulate some of their activities acting as a non-competitive negative allosteric modulator, reducing the potency and effectiveness of THC agonists and other cannabinoid receptors. CBD interacts with ionotropic channels, nuclear and metabotropic receptors, and enzymes. The Food and Drug Administration approved the use of CBD (Epidiolex) for the treatment of medically refractory seizures in Dravet syndrome and Lennox-Gastaud syndrome, while a combination of CBD and THC 1:1 plant-derived (Sativex) is used for pain relief, spasticity in multiple sclerosis and Tourette syndrome (17).

1.2.3.1 CBD molecular targets

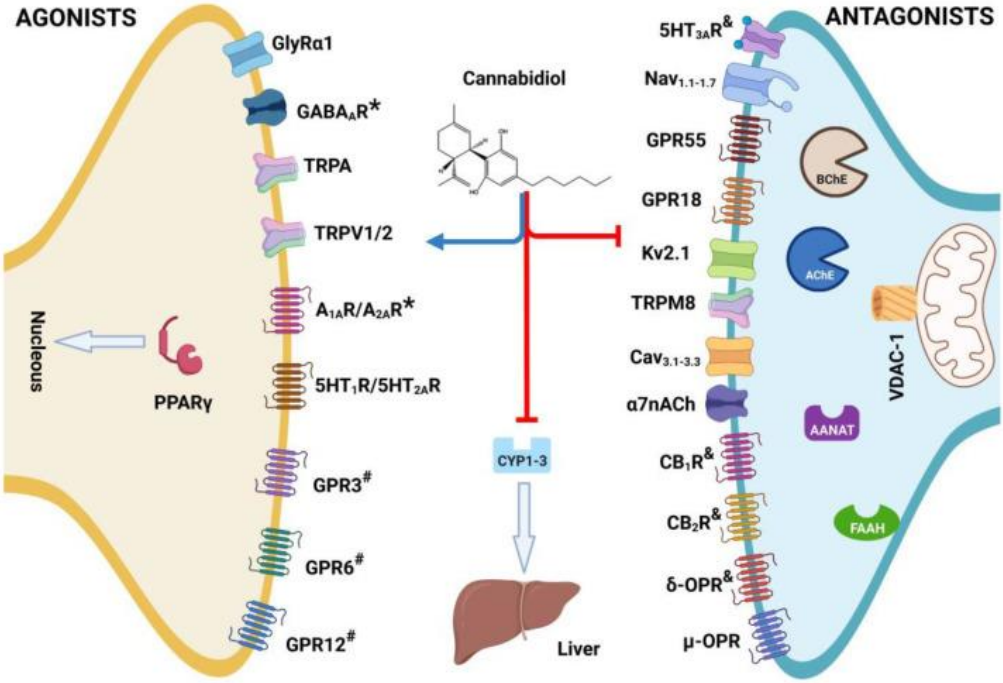


Figure 8. CBD different molecular targets. Glycine receptor (GlyR); 5-HT$_{1–7}$ serotonin receptor (5-HT$_{1–7}$R); Adenosine receptors (A $_{1–3}$R); Voltage-gated potassium channel (Kv2.1); Voltage-gated sodium channel (Nav); Nicotinic acetylcholine receptors (7-nAChR); Opioid receptor delta (δ-OPR); Opioid receptor mu (μ-OPR) (17).

CBD is a weak ligand of CB_1 and CB_2 receptors, in particular it is a weak CB_1 antagonist and a CB_2 inverse-antagonist (18). Then, CBD is a TRPV1, TRPV2, TRPV3, TRPV4 and TRPA1 agonist and TRPM8 antagonist (17, 20). Moreover, CBD interacts with some ligand-gated ion channels. Glycine receptors (GlyRs), ligand-gated chloride ion channels involved in fast inhibitory neurotransmission, are activated by high CBD concentration, while CBD low micromolar concentrations acts as its positive allosteric modulator (17). CBD is also a positive allosteric modulator of GABA-A receptors, ligand-gated ion channels that reduce neuronal activity and it is a negative allosteric modulator of the ionotropic serotonin receptor 5-HT_3 and of the nicotinic receptor (nAChR) α7 (17). Other inhibitory effects are exerted also with voltage-gated sodium channel (VGSC or Nav), with T-type voltage-gated calcium channels (VGCCs) and with voltage-dependent anion-selective channel protein 1 (VDAC1), a mitochondrial channel that controls cellular energy and metabolism (17). In addition, CBD interacts as serotonin receptor 5-HT_{1A}R agonist and as 5-HT_{2A}R partial antagonist, and it activates adenosine receptors A_{1A}R and

$A_{2A}R$. Other receptors of interest are the opioid receptors (OPRs); indeed, CBD is a positive allosteric modulator of δ-OPR and reduces μ-OPR activity (17). CBD is GPR55 and GPR18 antagonist, while is an inverse agonist of GPR3, GPR6 and GPR12 and an agonist of PPARγ (17).

1.2.3.2 CBD toxicity

Generally, CBD has low toxicity and a good safety profile. Until now, there are not be reported evidences about the risk of physical depends and potential abuse. It can interact with co-administered drugs and data showed that it can reduce THC side effects. Cautions should be observed in patients with hepatic impairment or administrated with drugs that can alter CYP3A4 or CYP2C19, causing CBD decrease or increase in plasma. CBD could act also as CYP1A1 inducer and as P-glycoprotein-mediated drug transporter, affecting the plasmatic concentration of drugs (18).

1.2.3.3 CBD therapeutic evidences

CBD is considered a promising molecule for its pharmacological effects. CBD induces anticancer, analgesic, neuroprotective, antiemetic, anti-inflammatory, anticonvulsant and antispasmodic activity (18). Many of these effects are associated with the different interactions with receptors. For example, CBD anticonvulsant effect could be associated with its interaction with TRPs, GABA-A receptor and VDAC1 (involved in human epilepsy) (17). Other evidences showed that the interaction with GlyRs could be responsible of anti-inflammatory and neuropathic pain, while neuroprotective effect was observed in multiple sclerosis and Alzheimer's disease rat models, in which CBD prevents neurodegeneration for the interaction with TRPs and PPARγ (17).

1.2.3.3.1 CBD anticancer activity

CBD is considered a molecule of interest in cancer therapy. Firstly, some clinical trials showed that CBD, alone or in combination with THC, is able to reduce cancer pain and chemotherapy-associated side effects. Moreover, the interest in CBD use is also related to the direct anticancer effect not only for relieving pain (21). Further, several preclinical *in vitro* and *in vivo* studies demonstrated CBD anticancer efficacy in different kind of cancers.

1.2.3.3.1.1 Lung Cancer

CBD anticancer effect was evaluated both in non-small cell lung cancer (NSCLC) and in small cell lung cancer (SCLC) (21). CBD decreased cell viability and induced apoptosis in two NSCLC (A549 and H1299) and in one SCLC (H69) cell lines (22). In another preclinical study, CBD induced apoptosis in cisplatin resistant NSCLC cells and reduced tumor progression and metastasis in cisplatin resistant mouse model, by increasing ROS production and reducing Nrf2 activity; these effects were associated with a TRPV2-dependet mechanism (23). Then, CBD, alone or in combination with THC, also affected the TME by decreasing cell density of normal fibroblasts (NFs) and cancer-associated fibroblasts (CAFs) isolated from lung adenocarcinoma patient. Then, cannabinoids decreased the effects of NF or CAF conditioned medium in A549 cells such as increasing cell density, inhibiting cadherin-1 (CDH1) gene expression and enhancing cadherin-2 (CDH2) and vimentin (VIM) expression levels (24).

1.2.3.3.1.2 Breast Cancer

CBD exercises its anticancer effect in estrogenic positive-receptor type and in triple-negative type breast cancers (TNBC) (21). CBD reduced viability of epidermal growth factor receptor (EGFR) positive TNBC cells and EGF-induced proliferation, migration and invasiveness by decreasing the activation of EGFR, Extracellular signal-regulated kinase (ERK), AKT and Nuclear factor kappa B (NF-kB) signaling pathways and the secretion of Matrix Metallopeptidase 2 and 9 (MMP-2 and MMP-9). Similar results were confirmed in mouse models. CBD also showed the ability to reduce the recruitment of tumor-associated macrophages (TAMs) in primary tumor stroma and in lung metastasis (25). In another study, CBD increased the efficacy of doxorubicin in reducing MDA-MB-231 cells viability, through induction of G_1 phase cell cycle arrest and reduced migration. Thus, CBD, alone or with doxorubicin, more significantly reduced the expression of markers of inflammation and metastasis, and increased apoptosis compared to the single chemotherapy. The combination also decreased the tumor volume, the expression of inflammatory proteins and the presence of metastasis in mouse models (26).

1.2.3.3.1.3 Glioblastoma

In U87MG glioma cell line and MCZ primary glioblastoma cells, CBD induced TRPV2 activation, by increasing also its expression. CBD, by triggering a TRPV2-dependet Ca^{+2} influx, increased the uptake of chemotherapeutic drugs and synergized with them by promoting a greater

cytotoxic effect. A deletion of the TRPV2 pore domain inhibited the CBD effects (27). In another *in vitro* study, CBD always reduced glioma cancer stem cell (GSCs) viability in TRPV2-dependet manner. In addition, CBD induced autophagy in GSCs, the expression of differentiation stem cell markers and increased their sensitiveness to carmustine. Then, it was demonstrated that the Acute myeloid leukemia (Aml-1) transcription factor, upregulated during GSCs differentiation, is able to bind the TRPV2 promoter and the CBD treatment enhances the Aml-1 expression level (28). Moreover, lethal autophagy and mitochondrial disfunction, in human glioma cell lines, were induced also by CBD interaction with TRPV4, whose expression is correlated with aggressiveness and poor prognosis in glioma patients. CBD also synergized with TMZ in patient-derived neurospheres and in glioma *in vivo* mouse model (29). Interesting results were also observed by combining CBD and THC. In fact, the combination improves TMZ and radiotherapy efficacy in glioma animal models. These evidences were the basis of a phase Ib clinical trial, in which the activity of nabiximol and TMZ was evaluated in recurrent glioblastoma multiforme patients. Results showed an increase in overall survival at 12 months in the group treated with nabiximol compared to placebo group, 83% and 44% respectively, but no differences were observed in progression free survival. Despite of the promising results, this first clinical trial remains small with risk of bias, so further studies are necessary (30).

1.2.3.3.1.4 Prostate cancer

CBD reduced prostate cancer cell (PC3) viability and inhibited the release of exomes and of macrovesicles associated with the activation of chemo-resistance and pro-oncogenic factors (31). CBD and CBG reduced cancer cell viability both in non-hormone refractory prostate cancer (non-HRPC) and hormone refractory prostate cancer (HRPC) cells, suggesting that their activity was hormone-independent, while the induction of apoptosis was more relevant in HRPC cells. Moreover, data showed that the two molecules induced divergent metabolic effects in cancer cells depending of cell phenotype. CBD also induced mitochondrial dysfunctions that modulated oncogenic pathways and induced autophagy. CBD and CBG administration reduced tumor burden with a good tolerability for the animals and the combined therapy was effective also in enzalutamide-resistant mouse models (32).

1.2.3.3.1.5 Colorectal cancer

CBD, via GPR55, receptor involved in cancer aggressiveness, inhibited HCT116 cancer cell adhesion and migration, while GPR55 knockdown reverted this effect. CBD and GPR55

antagonist were also able to maintain the integrity of endothelial cell monolayer, while the endogenous ligand of GPR55, LPI, reduced this integrity. CBD reduced also HCT116 cells migration in liver, after intrasplenic inoculation (33). Oxaliplatin-resistant cell lines, DLD-1 R and colo205 R, were treated with CBD alone or in combination with oxaliplatin, and cell proliferation reduction was observed. Further investigations showed that the combination between CBD and oxaliplatin reduced phospho-nitric oxide synthase (NOS3) levels and nitric oxide (NO) production both associated with cancer cells resistance; on the other hand, the combined treatment induced reactive oxygen species (ROS) production, by reducing superoxide dismutase 2 (SOD2) levels. Results were also confirmed in resistant mouse model, where CBD and oxaliplatin reduced tumor size and weight, and induced autophagy (34).

1.2.3.3.1.6 Myeloma multiple

CBD in combination with THC induced CB_2-indipendent cytotoxicity, cell cycle arrest and autophagic cell death in U266 and RPMI cells. The combination also inhibited immune-proteasome β5i subunit similar to Carfilzomib effect. CBD and THC synergized with Carfilzomib by inducing cancer cells cytotoxicity and by inhibiting CXCR4 and CD147 protein expression associated with chemotactic activity (35). The CBD cytotoxicity in multiple myeloma cell lines was TRPV2-dependent, indeed TRPV2 transfection increased cells sensitiveness to CBD administration. CBD combined with Bortezomib increased its cytotoxicity and TRPV2 expression triggered the synergistic efficacy of CBD and Bortezomib in reducing cancer cells viability, proliferation and cell cycle arrest. The combined therapy also induced necrotic cell death and ROS production (36).

1.2.3.3.1.7 Leukemia

CBD reduced cell viability in different leukemic cell lines. Data showed the suppression of T-cell acute lymphoblastic leukemia (T-ALL) migration after CBD administration and an induction of apoptotic and necrotic cell death. CBD caused a decrease of mitochondrial transmembrane potential (ΔΨm) and cytochrome C release, with an increase of cytoplasmatic Ca^{+2}. These effects were associated with direct interaction between CBD and mitochondria (37). Similar results, about the effectiveness of CBD in modulating mitochondrial activity was observed also in *in vitro* models of chronic myeloid leukemia (CML). CBD, by binding TRPV2 receptor, reduced K562, KU812 and MOLM-6 CML cell lines viability and increased intracellular levels of Ca^{+2} and ROS.

CBD synergized with Imatinib in inhibiting cell growth and it is effective also in Imatinib-resistant K562 (38).

1.2.3.3.1.8 Endometrial and ovarian cancer

CBD reduced Ishikawa and Hec50Co cells viability in a dose-dependent manner and induced chromatin condensation and nuclear fragmentation as apoptotic signals. CBD efficacy was TRPV1-depent in Ishikawa cells, but not in Hec50c0 (39). CBD was investigated also as TRPV2 ligand and data showed a decrease of cancer cell viability in Ishikawa, MFE-280, HEC-1a, PCEM002, PCEM004a and PCEM004b, an induction of cell death in MFE-280, HEC-1, primary PCEM002 and in Ishikawa, while a partial cell cycle arrest and autophagy was observed in CBD-treated PCEM004a and PCEM004b. The efficacy of chemotherapeutic drugs was increased by CBD (40). Moreover, CBD administered in solution or incapsulated into nanoparticles induced cytotoxicity and apoptosis in ovarian cancer cells. CBD effect was also evaluated in SKOV-3 cells implanted in chorioallantoic membrane and a tumor growth reduction was observed after CBD nanoparticles treatment (41).

1.2.3.3.1.9 Pancreatic cancer

Several studies were performed to evaluate the effects of Cannabis, its derivatives and the ECS in pancreatic cancer. Some evidences showed a potential correlation among the expression levels of endocannabinoid enzymes and cannabinoid receptors, and PDAC patients' prognosis. Indeed, in a study performed in tissue samples of PDAC patients, only mRNA expression of CB_1, and not of CB_2, was associated with shorter survival. Further, low expression of FAAH and MAGL enzymes were associated with shorter survival (42). Synthetic cannabinoids WIN-55,212-2 (CB_1/CB_2 receptor agonist), ACEA (CB_1 receptor-selective agonist), JWH-015 (CB_2 receptor-selective agonist), AM251 and SR141716A (CB1 receptor-selective antagonists) and AM630 (CB_2 receptor-selective antagonist) were analysed in MIAPaCA-2 cells. Results showed AM251 was the most active compound in reducing cell viability, activating caspase-3/7 and in inducing changes in transcriptional factors and in 5-FU efficacy (43). Arachidonylcyclopropamide (ACPA) and GW405833 (GW), other synthetic cannabinoids, were tested in PANC-1 cells, analysing proliferation inhibition and the proteomic profile. The two molecules regulated energetic metabolism and cell growth proteins (44). ACPA and GW increased AMP-activated protein kinase (AMPK) activation for the increase of AMP/ATP caused by ROS production. AMP/ATP increase was correlated with an impairment of glycolytic pathway and the increase of NADPH with Krebs

cycle inhibition. So, the cannabinoids could induce autophagy and cell growth inhibition, altering cancer cell metabolism (45). THC induced cytotoxicity and apoptosis in PANC-1 and MIAPaCa-2 and its efficacy was reverted in combination with CB_2-selective antagonist SR144528. THC, CB_2-selective agonist JWH-133 and WIN-55,212-2, reduced tumoral growth in *in vivo* models and tumoral spread in proximal tissues and distal organs (46). The role of synthetic cannabinoids was analysed also in combination with GEM in six different PDAC cell lines. Results showed that cannabinoids synergistically with GEM inhibited cancer cells proliferation, also in GEM-resistant cells. GEM induced cannabinoid receptors expression, cannabinoid-induced endoplasmic reticulum (ER) stress and the cannabinoid-induced autophagy by a ROS-dependent mechanism. The combination between GEM and SR141716A decreased mice tumoral mass more than the single treatment (47). CBD effect was studied *in vitro* and *in vivo*. Six PDAC cell lines and one model of murine pancreatic stellate cells were treated with THC and CBD, alone or in combination, and results showed a reduction of cell proliferation. The reduction of CBD and THC efficacy in a knockout model of serine/threonine-protein kinase (PAK1), a protein involved in the stimulation of cancer cell proliferation and survival, underlined a relationship between cannabinoids and PAK1 related pathways. These results were confirmed *in vivo*, in which the effect of a CBD:THC (1:1) oil was not effective in PAK1 knockout mouse model. At the same time, PAK1 reduction is correlated with the reduction of programmed death-ligand 1 (PD-L1) expression in pancreatic cancer cells, and also CBD and/or THC reduced PD-L1 expression (48). GRP55 expression was demonstrated important for PDAC development and progression, and its effect on cancer cell growth depends on MAPK/ERK signaling pathway regulation. CBD was used as GPR55 antagonist and results showed that pancreatic cancer cells growth is reduced and MAPK/ERK pathway is inhibited. CBD also potentiated GEM effect, indeed the combination strongly reduced PDAC growth, *in vivo* and in animal models. (49). Cytokines-induced killer (CIK) cells, cells with activity similar to T and natural killer cells, express CB_2 receptor and this expression is induced by IL-2. Low doses of CBD increased the cytotoxicity of CIK cells against PANC-1, reducing significantly cancer cells viability (50). Then, the anticancer effect of a THC and CBD (1:6) formulation was evaluated in Capan-2 derived xenograft mouse model and data showed apoptosis induction and cancer cell proliferation inhibition (51). Another study also showed that CBD is effective not only combined with chemotherapeutic drugs, but also administered as a mixture of oxygen/ozone (O_2/O_3). This combination, reduced cancer cell viability, induced necrotic cell death and regulated PDAC associated genes (52).

1.2.4 Cannabigerol (CBG)

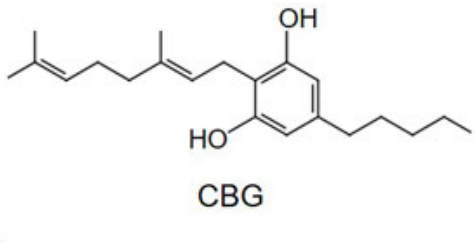


Figure 9. CBG chemical structure (8,53)

CBG (Figure 9) is a minor PyCBs obtained from *C. sativa*. CBG is a weak partial agonist of CB_1 and CB_2 receptors, activates TRPV1, TRPV2, TRPV3, TRPV4 and TRPA1 and antagonizes TRPM8. CBG is an antagonist also of $5\text{-}HT_{1A}$ receptor and an agonist of α-2 adrenoceptor and PPARγ (8,53).

1.2.4.1 CBG therapeutic effect

CBG is known for its anti-inflammatory and anti-bacterial activity (8,53**).** Some evidences demonstrated that CBG has anti-inflammatory properties and reduces peripheral pain. This anti-inflammatory effect could be related with CB_2, TRP channels and PPARγ biding. Some preclinical evidences showed CBG activity in reducing inflammatory bowel diseases such as Crohn's and ulcerative colitis (8). Mice model of colitis treated with CBG showed a reduction of inflammatory markers, NO and oxidative stress in intestinal epithelial cells (8,53). The reduction of inflammation and oxidative stress can protect also from neurodegenerative diseases. VCE-003, a synthetic CBG analogue, inhibited antigen-induced-T-cells proliferation, the expression of surface activation markers, and reduced inducible NOS (iNOS) enzymes in microglia and in animal model of multiple sclerosis. In mice, multiple sclerosis is attenuated for VCE-003 interaction with CB_2 and PPARγ (8). Also, in a mouse model of Huntington's disease, CBG reduced pro-inflammatory cytokines and TNF-α (8).

1.2.4.2 CBG anticancer activity

There are few studies about the effects in cancer of CBG used alone or in combination with other PyCBs. One of the first study used CBG in the treatment of *in vitro* and *in vivo* mouse models of colorectal carcinoma as TRPM8 antagonist. CBG reduced cancer cell viability in time and serum protein concentration-dependent way and its effect was reduced in TRPM8-silenced cells. CBG also induced apoptosis and ROS induction in CaCo-2 cells. In xenograft models, CBG reduced tumor growth and it had a chemopreventive effect in mice treated with the colon cancer inducer, azoxymethane (AOM) (54). In cholangiocarcinoma cells, CBG reduced cancer cells proliferation and migration, and induced apoptosis (55). A study performed to evaluate the role of TME in tumor progression showed that CBG, contained in a *Cannabis* extract, reduced colony-stimulating factor 1 (CSF-1) secretion from B16F10 murine melanoma cells. Bone-marrow-derived cells (BMDCs) isolated from bones of healthy mice were treated with conditioned media of B16F10 cells treated with CBG. The percentage of monocytic myeloid-derived suppressor cells (MO-MDSCs), derived from sorted BMDCs, was reduced after the treatment with CBG conditioned media. Tumor volume in mice injected with B16F10 cells showed a decrease after CBG administration and also tumor-associated macrophages (TAMs) decreased their frequency in the myeloid subpopulation. Finally, CBG effect was evaluated in combination with an inhibitor of the immune checkpoint PDL-1. Both the molecules promoted tumor volume reduction but the effect was increased by the co-administration of both molecules. Also, cytotoxic T-cells infiltration was more evident with the combined treatment (56). Human and rat mesothelioma cells were treated with CBG and CBD. CBG reduced proliferation, migration and invasion, and induced G_0/G_1 cell cycle arrest and apoptosis. The administration in mesothelioma cells with CBG and CBD caused an up-regulation of cannabinoid-related genes and other pathways involved in cell cycle regulation, apoptosis, intracellular calcium levels and inflammatory and immune response. Despite that, CBG and CBD did not increase the survival of mesothelioma rat models (57). In breast cancer cells, CBG was used in combination with THC, CBD and CBN. This combination reduced breast cancer cell viability with a limited cytotoxicity for normal breast cells, and exerted cell cycle arrest in G_2 phase, apoptosis, lipid droplets accumulation and increased lysosome size (58). Moreover, CBG exercised cytotoxic effect in human glioblastoma cells and GSCs, alone or in combination with CBD or THC. The most effective combination resulted in CBG plus CBD. At the same time, CBG and CBD combination plus TMZ increased apoptosis and reduced cancer cell invasion, while no additive effects were observed if CBG and CBD were administrated with THC (59).

1.3 Melatonin (MLT)

Melatonin (MLT) or N-acetyl-5-methoxy tryptamine is an indole hormone produced from serotonin by pineal gland. Its production and concentration in the pineal gland are based on inputs from brain circadian centres. MLT activity regulates sleep promotion, other circadian functions, immune system and the modulation of pituitary and adrenal hormones (60). Sleep is a physiological and necessary process checked by circadian rhythm. The circadian rhythm is controlled by natural and artificial light, that involves neural, hormonal and genetic elements, known as endogenous oscillators. The suprachiasmatic nucleus (SCN) in the hypothalamus is the main central oscillator and it receives inputs from retina; peripheral oscillators are controlled by the SCN. A central role is played by the cyclical expression of clock genes locate in the 20.000 neurons that composed the SCN (Figure 10) (60).

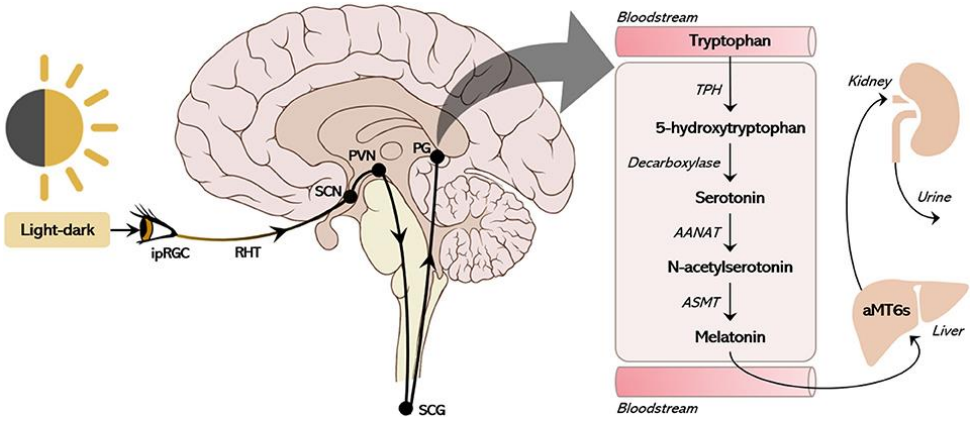


Figure 10. Production of MLT (61)

Environmental light hits and modifies melanopsin structure in intrinsically photosensitive retinal ganglion cells (ipRGCs) and the information reaches the SCN via retinohypothalamic tract (RHT). SCN via GABAergic projection inhibits the hypothalamic paraventricular nucleus (PVN) in presence of light, while in light absence, the inhibitory stimulation is blocked and PVN activates the ganglion cervical nuclei (SCG) and the noradrenergic neurotransmission that innervates the pineal gland. Through this pathway light information reaches pineal gland and regulates MLT production and release (Figure 10) (60, 61).

Endogenous MLT is synthetized in the pinealocytes and in other tissues from tryptophan and serotonin (Figure 11) (60). After the synthesis, MLT is released in the cerebrospinal fluid and

bloodstream and, through the first-pass metabolism in the liver, it is converted and excreted from urine (60, 61).

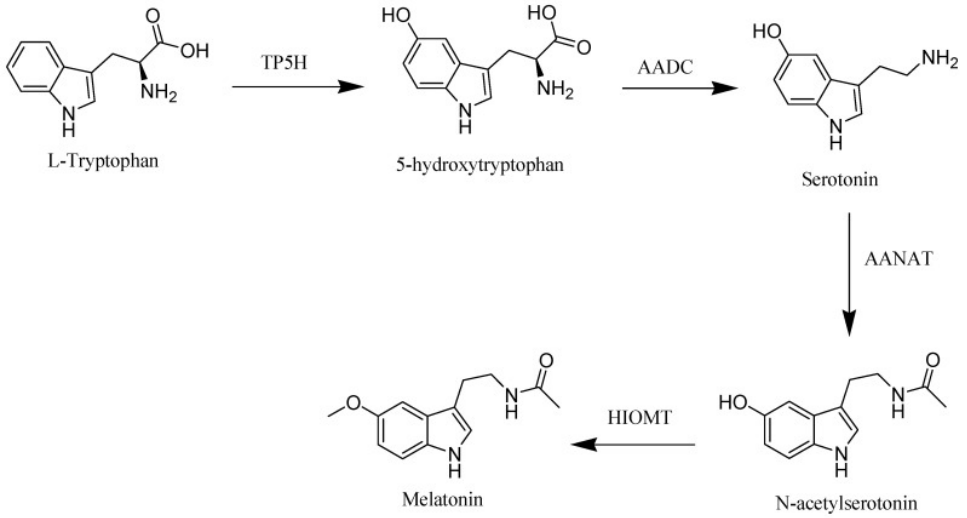


Figure 11. Biosynthetic pathway of MLT. Tryptophan by tryptophan-5-hydroxylase (TP5H) and aromatic acid decarboxylase (AADC) enzymes activity form serotonin. Serotonin by arylalkylamine-N-acetyltransferase (AA-NAT) and acetylserotonin-O-methyltransferase (ASMT or hydroxyindole-O-methyltransferase or HIOMT) form MLT. Finally, MLT is catabolized by cytochrome P450 enzymes (CYP1A2) in 6-hydroxymelatonin (60, 62).

MLT synthesis is limited by arylalkylamine-N-acetyltransferase (AA-NAT) enzyme indeed the adrenergic stimulation of pinealocytes increases cytoplasmic cAMP and cAMP-dependent protein kinase A (PKA) that activates a signal cascade to AA-NAT production (Figure 11) (60).
MLT release is strictly related to light presence. Exposure to high wavelength light suppress MLT production and release, as happens during the day. During the night, light absence activates MLT biosynthesis and its release. Abnormal time of exposure to high wavelength light, such as nocturnal artificial light, causes alteration in MLT production and alteration in circadian rhythm production (60). Endogenous MLT release can be modulated also from different variabilities, for examples hormones and other metabolic products, sex- and age-based differences (60). MLT is produced also in the major systems of the body not only in the pineal gland, for example in the retina, gastrointestinal tract an innate immune system (60, 63).

1.3.1 MLT molecular targets

MLT can interact with MT1 (Mel1a) and MT2 (Mel1b) GPRs expressed in central nervous system and in peripheral tissues, with MT3, a cytosolic enzyme quinone reductase 2 (GR2), and with nuclear receptors such as retinoid orphan receptors ROR/RZRα and ROR/RZRβ. Moreover, MLT does not bind directly the orphan receptor GPR50, considered the mammalian orthologue of Mel1c, a MLT receptor found in non-mammalian vertebrates, but GPR50 is a regulatory protein associated with the other MLT receptors (Figure 12) (63,64).

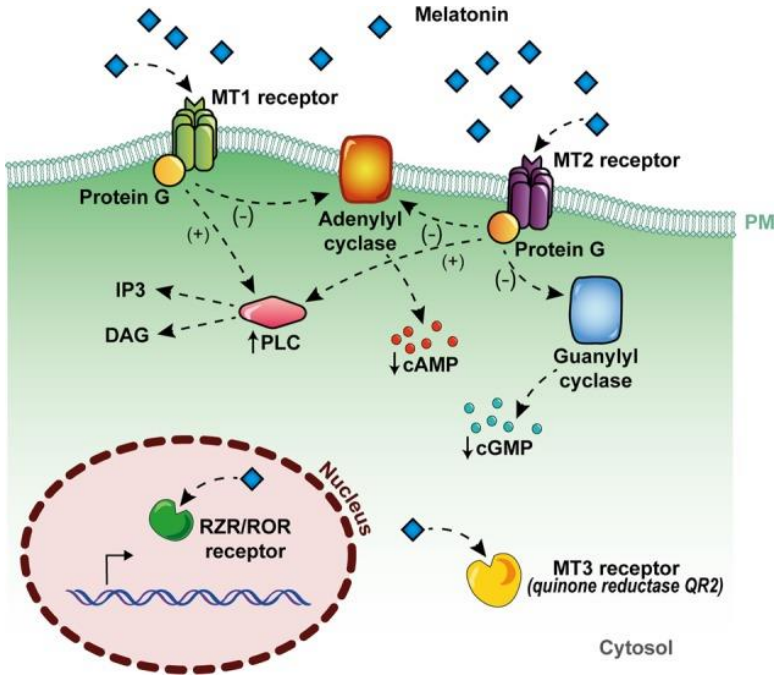


Figure 12. MLT receptors (63).

MT1 and MT2 are two $G_{i/o,}$ and, in smaller quantity $G_{q/11}$, protein-coupled receptors. Their activation decreases cAMP levels, PKA signaling and cAMP-responsive element binding (CREB) phosphorylation; further, their activation modulates also phospholipase C (PLC), diacylglycerol (DAG) and inositol trisphosphate (IP_3) that increase intracellular Ca^{2+} levels. The interaction with ligands induces also mitogen-activated protein kinase 1/2 (MEK 1/2) and ERK 1/2 phosphorylation. Unlike MT1, MT2 activation inhibits also cGMP formation (Figure 13) (64, 65).

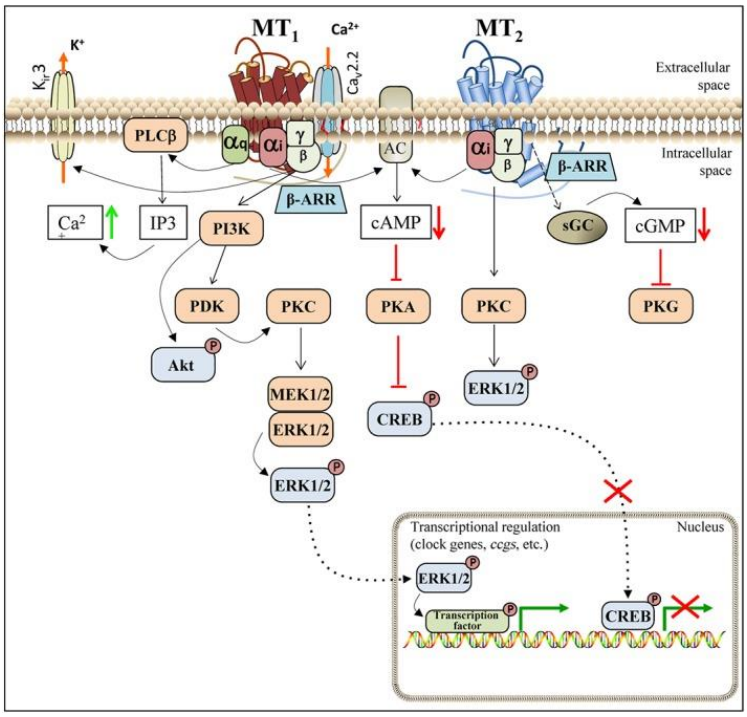


Figure 13. MLT receptors signaling pathway (64).

MT1 and MT2 receptors can form homo- or hetero-oligomers between themselves and other G protein-coupled proteins, and MLT can change its responsiveness based on the presence of oligomerization. For example, MT1/MT2 heteromers are located in retinal photoreceptor cells and improves retinal light sensitiveness during night and PKC. MLT receptors form oligomers also with GPR50 and in this case MT1 G_i activity is lost. MT1 or MT2/GPR50 hetero-oligomers are present in the pineal gland and they could be involved in physiological MLT function. Finally, MLT induces G_q protein activity when MLT receptor makes heteromers with 5-HT receptors, but this activity is not observed when MT2 is expressed alone (Figure 14) (64, 65).

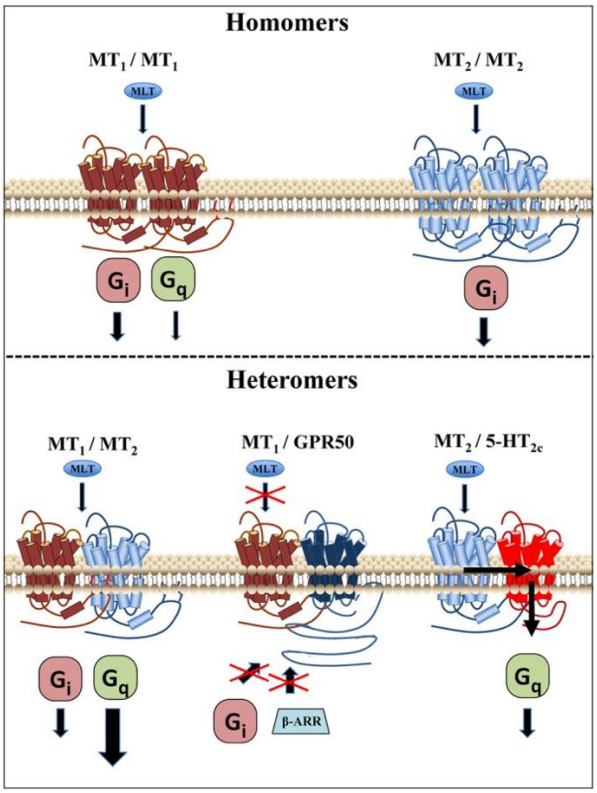


Figure 14. Homo- and hetero-oligomers of MLT receptors. MT1/MT1 homomers trigger predominantly G_i activation over G_q, while MT2/MT2 activates G_i signaling pathway. MT1/MT2 heteromers activates G_q protein over G_i, MT1/GPR50 loss the ability to bind MLT; MT2/5-HT2c activated G_q protein (64).

1.3.2 MLT physiological effect

Studies demonstrated MLT implication in early fetal development by influencing placenta, glia, neuronal development and fetal biological clock synchronization (66). MLT regulates also the timing of central biological clock located in the hypothalamus as an endogenous synchronizer of body rhythms (66). Other important MLT functions involve the regulation of blood pressure and autonomic cardiovascular circulation, immune system and retinal functions and the antioxidant activity based on its interaction with MT3 receptor. Some studies evidenced also MLT involvement in energy consumption and body mass regulation, and in promotion of osteoblast differentiation and bone formation. MLT is also involved in down-regulation of gonadotropin-regulation hormone (GnRH) gene expression and it controls the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The influence of photoperiod on LH release and the consequent seasonal variations in reproduction was observed mainly in the animals (66).

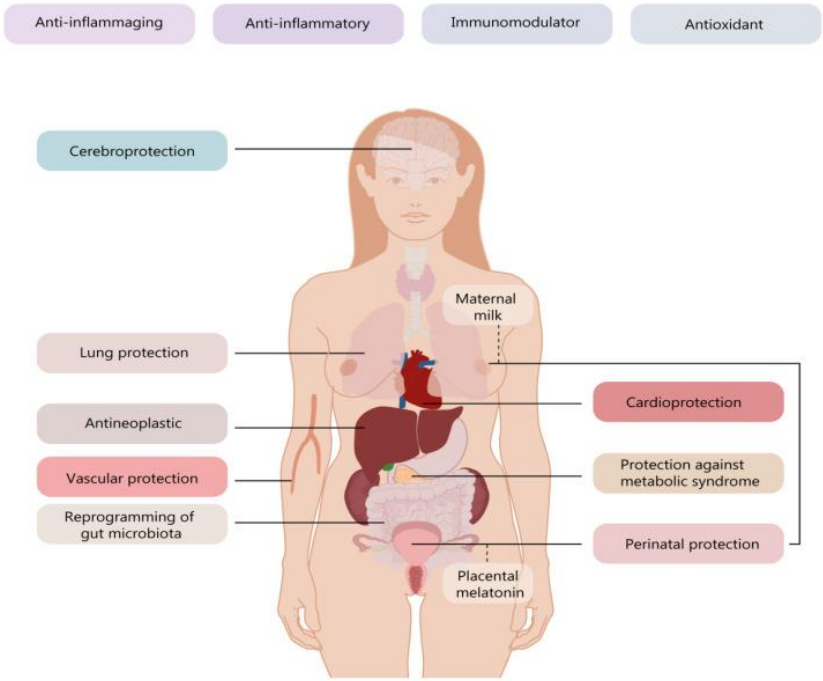


Figure 15. MLT action in different organs (67)

1.3.3 MLT therapeutic effect

The main use of MLT is to treat sleep disorder such as problems to fall or to stay asleep, but there are other therapeutic implications (60). Evidences reported MLT effects in tumors, cardiovascular disease and psychiatric disorders. MLT anti-oxidant role can be associated to its oncostatic effect and a decrease of MLT levels was observed in patients with cardiovascular disease. Alterations in nocturnal MLT secretion was observed in depressive disorder, bipolar disorders, schizophrenia and autism spectrum disorders. Clinical therapeutic benefits and improvements were found in the concomitant increased of MLT and the use of antidepressants (66).

1.3.3.1 MLT anti-inflammatory and anti-oxidant activity

MLT is known for its activity as antioxidant, immune regulator and pro- and anti-inflammatory molecule. Evidences demonstrated that MLT anti-oxidant properties are implicated in brain protection and gastrointestinal protection from ulcerations increasing the bicarbonate release in duodenal mucosa (66). Mitochondria are the main sites of MLT synthesis and mitochondria are also source of free radicals. In particular, MLT structure composed by an electron-rich aromatic indole ring makes it an electron donor able to inactivate different types of free radicals, exercising

a direct anti-oxidant action. Further, the interaction with MT1 and MT2 increases the activity of anti-oxidant enzymes and also the MT3 receptor is a detoxifying enzyme able to reduce quinones (Figure 16) (63).

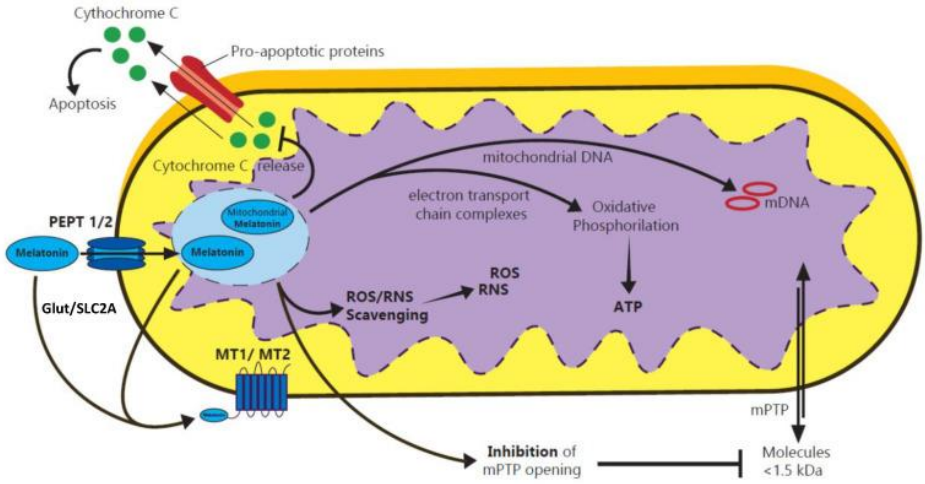


Figure 16. MLT role in mitochondria. MLT is transported or synthetized in the mitochondria. Here, MLT scavenges free-radicals and interrupts electron-transport chain. MLT interacts also with MT1 and MT2 receptors and blocks cytochrome c release in the membrane (67).

MLT has direct and indirect antioxidant effects. It can scavenge free radical, but also induce endogenous antioxidant enzymes production and their stimulation. In particular, MLT antioxidant effect is related with the chemical structure of its metabolites that makes it a cascade of antioxidant molecules. It interacts also with non-radical oxidants such as hydrogen peroxide, singlet oxygen and peroxynitrite, and inhibits metal-induced oxidation (Figure 17) (67). Further, it protects mitochondria against oxidation, regulating mitochondrial membrane potential, facilitating electron flux within the cell (67).

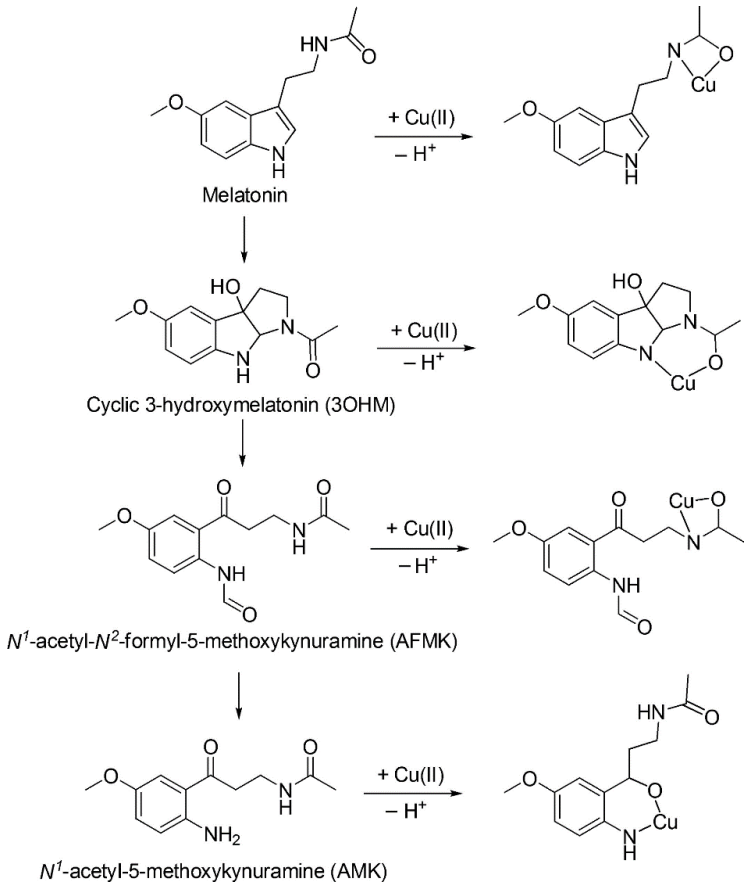


Figure 17. MLT and its metabolites. Free radical scavenging cascade in vertical and metal-chelating cascade in horizontal (68)

MLT acts on immune response and in particular as immune stimulatory molecule inducing T helper response. The immune stimulatory behaviour is also associated with a pro-inflammatory activity, even if other evidences showed that MLT is an anti-inflammatory agent. The pro-inflammatory effect was observed mainly in immune cells collected from blood, in which there was an increase of pro-inflammatory cytokines. One study observed that in monocytes, MLT increased ROS production associated with inflammatory response and immune activation (67, 69). On the other side, MLT activity is also addressed to an anti-inflammatory response, especially when it is strong. MLT anti-inflammatory action is performed in different ways, for example by promoting antioxidant processes and reducing ROS and NO formation protecting mitochondria from damages. Further, MLT inhibits inflammasome NLRP3 (nucleotide-binding oligomerization domain, leucine-rich-containing family, pyrin domain-containing-3) and it down-regulates pro-inflammatory cytokines, even if in some experimental cases MLT protective effect is followed by the upregulation of IL-6, IL-10 and TNF-α. Other evidences showed that MLT inhibits cyclooxygenase (COX) and iNOS, limiting excessive production of leukotrienes, prostanoids and

NO (67, 69). MLT demonstrated protective effect also in low-grade inflammation considered a common featured of ageing. This effect protects brain that is subjected to inflammation for aging and neurodegenerative disease (69).

1.3.3.2 MLT anticancer effect

In addition to antioxidant, anti-inflammatory and immune regulatory action, numerous studies also demonstrated that MLT could exert anticancer effect (69). *In vitro* and *in vivo* in mice evidences showed MLT activity in several kind of cancers, alone or in combination with chemotherapeutic drugs.

1.3.3.2.1 Lung cancer

In a study performed in A549, PC9 and LLC lung cancer cells, MLT inhibited the Warburg effect, by influencing mitochondria energy metabolism, and reduced cancer cell proliferation and promoted apoptosis. Data showed that MLT improved mitochondrial activity so MLT-treated cells showed an increase of ATP production. The expression of Sirt3 and pyruvate dehydrogenase (PDH), metabolic enzymes located in mitochondria, was up-regulated by MLT and their inhibitors reduced MLT anticancer effect and MLT-dependent ROS production (70). In another study, MLT influenced migration and invasiveness of lung cancer. Epithelial–mesenchymal transition (EMT) phenotype of A459 and CL1-5 cells was evaluated after MLT treatment and data showed a shift from mesenchymal to epithelial phenotype. MLT activity was associated with the inhibition of PLC, p38/ERK and β-catenin pathway and through this mechanism, suppressed also Twist expression. This activity was reduced by MT1 silencing. In mouse models MLT reduced cancer progression and metastasis to liver in a dose-dependent manner (71).

1.3.3.2.2 Breast cancer

MLT effect was evaluated in three human epidermal growth factor receptor 2 $(HER2)^{+}$ breast cancer cell lines. MLT increased cancer cell death and modulated gene and protein expression of HER-targeted therapy. MLT attenuated EMT, KRAS, NF-kB and IL-6-JAK-STAT3 gene expression, and decreased phospho-AKT, -ERK and NF-kB. MLT reduced also the HER2 protein stability for endocytosis and lysosomal degradation and, by interacting with HER inhibitor Neratinib, synergized with it increasing apoptosis. Also, in HCC1954 xenograft mice, this combination reduced most significantly tumor growth (72). MLT pre-treatment sensitized also

MCF-7 cells to apoptosis and, combined with docetaxel, cancer cell proliferation reduction was more evident than with single administration (73).

1.3.3.2.3 Glioblastoma

The administration of 1 mM of MLT reduced glioblastoma cells migration and invasion after 24 h of treatment, and reduced MMP-2 and Vascular endothelial growth factor (VEGF) expression. In hypoxic conditions, thanks to MLT, Hypoxia-inducible factor 1 α (HIF-1α) expression was reduced such as invasion and migration (74). In three glioblastoma-initiating cell (GICs) lines, MLT reduced cancer cell growth and GICs stem-like properties, reducing self-renewal ability and formation of neurospheres (75).

1.3.3.2.4 Prostate cancer

Men with low levels of MLT are more prone to develop prostate cancer. Data showed that male C57BL/6 mice with an altered light exposure developed a more evident tumor mass compared to mice exposed to normal light cycle. MLT increased enzalutamide effectiveness and reduced lipid accumulation, that is associated with prostate cancer development. MLT treatment induced the reduction of lipids accumulation by up-regulating carboxylesterase 1 (CES1) in pancreatic cancer. Indeed, CES1 knockdown hampered MLT effect on lipid accumulation and, in addition, CES1 down-regulation was associated with poor prognosis in prostate cancer. In mouse model, MLT reduced tumor growth in presence of CES1 and increase the sensitiveness to enzalutamide (76).

1.3.3.2.5 Colorectal cancer

MLT and 5-FU synergized, increasing the reduction of cancer cell viability, inhibiting invasiveness and metastasis in human colon cancer cells. Protein expression of MMP-9 and N-cadherin was reduced, while E-cadherin level was increased after administration of the combined treatment. The combination reduced the phosphorylation of p85, 110ß, pyruvate dehydrogenase kinase 1 (PDK1) and AKT proteins without modulating total Akt and suppressed NF-kB/iNOS signaling pathway. Synergistic effects were also observed in mice, in which a more significant reduction of tumor mass and volume was found (77). Co-treatment with MLT and 5-FU inhibited cell proliferation and the expression of stem cell markers in colon cancer stem cells, and also reduced tumor volume in mice, even if the overexpression of the gene associated with the cellular prion protein considered a marker for colorectal adenoma-to-carcinoma transition, attenuated this efficacy (78).

1.3.3.2.6 Leukemia

MLT, at pharmacological concentrations, reduced cell viability of FLT3-ITD mutated mouse (BaF3/ITD) and human (MV4-11 and MOLM13) leukemia cells with a greater effectiveness than in wild type leukemia cells. As reported in literature, MLT is known for its anti-oxidant properties, but in cancer cells and at high doses, it could increase ROS production and oxidative stress. MLT, through ROS increasing and glutathione decreasing, induced apoptosis in FLT3-ITD leukemia cells and increased sorafenib efficacy. In mice, MLT and sorafenib depleted leukemia cells in peripheral blood and the survival rate of the combination group resulted more prolonged than the group with single compounds (79). MLT effect was also evaluated in mixed lineage leukemia (MLL-r) rearranged cells. Data showed that MLT inhibited the proliferation of MLL-r cells more than non-MLL-r cells. It induced apoptosis and down-regulated hTERT expression by suppressing the transcriptional factor on its promoter region, RBFOX3. Similar results were obtained in primary acute lymphoblastic leukemia blasts from patients and in mice treated with MLT, that showed reduction of body and spleen weight (80).

1.3.3.2.7 Endometrial cancer

A first study was performed in two endometrial cancer cell lines, SNG-II and Ishikawa, estrogen-receptor negative and estrogen-receptor positive respectively. Data showed that MLT reduced Ishikawa number and the effect was inhibited by 17β-estradiol administration and by the melatonin receptor antagonist, luzindole (81). In another study, MLT role was evaluated in relation with estrogen/ubiquitin C (UBC)/succinate dehydrogenase (SDH) B axis. MLT reverted estrogen effect by decreasing SDHB expression in Ishikawa and RL95-2 cells. Mice treated with estrogen and MLT displayed higher tumor volume reduction compared with the administration of estrogen alone. Finally, MT1B expression and activation was associated with MLT inhibitory role in estrogen-succinate metabolism and its overexpression increased sensitivity to MLT (82).

1.3.3.2.8 Pancreatic cancer

From literature, there are many evidences about the use of MLT in pancreatic cancer treatment. MLT reduced cancer cell viability, growth and colony formation, and induced apoptosis and/or necrosis in PANC-1, SW-1990, MIAPaCa-2 and Panc-28 cells in dose-dependent manner, while it exercised minimal toxicity in nontumorigenic pancreatic ductal epithelial cells (83-85). In some evidences MLT activity is associated with the interaction with MEL1A/B receptors, while in others, its activity appeared independent of the receptors (83, 85). MLT activity modulates different molecular pathways. For example, it inhibited the angiogenesis by reducing proliferation

and migration of human umbilical vein endothelial (HUVEC) cells, stimulated by the co-culture with PANC-1. MLT reduced also VEGF release and its expression in PANC-1 (86). MLT decreased nuclear levels of NF-kB/p65, p-NF-kB/p65 and NF-kB downstream genes in MIAPaCa-2 and AsPc-1 cells, reverting the effect of GEM to induce NF-kB and its associated genes (85). In a study published in 2007, MLT was administered during PDAC induction and/or postinduction phase with N-nitrosobis (2-oxopropyl)amine (BOP) in animal models. BOP induced oxidative stress and antioxidant enzymes reduction; this effect was reverted by MLT both during the induction and post-induction phase. At the same time, MLT also reduced the number of animals with tumor and its administration during BOP-induction phase eliminated the presence of moderately differentiated adenocarcinoma, while tissue atypical hyperplasia remained (87). In another correlated study, MLT increased lypoperoxides (LPO) efficacy if administrated during the induction phase of carcinogenesis and in general, it reduced pancreatic tumor and prevented animals' death. Finally, evidences showed that the co-administration of MLT and celecoxib, during the post-induction phase, increased animals' survival and reduced LPO levels (88). The combination between MLT and GEM had higher efficacy than the single molecules in SW-1990, MIAPaCa-2 and AsPc-1 cells, and in mice (84, 85). AR42J cells treated with MLT plus 5-FU, or doxorubicin, or cisplatin showed a major decrease of cell viability, a major apoptosis induction and a more evident mitochondrial membrane depolarization (89).

1.4 Ozone therapy (O_2/O_3)

Ozone (O_3 or O_2/O_3) is an inorganic cyclic molecule composed by three atoms of oxygen and it is present in a gaseous form in the atmosphere but it can be also produced applying a high-voltage electrical discharge to the oxygen (O_2) molecule. In 1916, O_3 was used for its antimicrobial properties in wound healing and in the 19th century, it was introduced in medical therapy after the development of the O_3 generator. The O_3 generator creates a mixture with specific concentrations of O_2/O_3 in order to reduce the potential cytotoxicity caused by O_3 induced oxidative stress (90). Medical O_3 generators produces the gaseous mixture from pure O_2 subjected to a high-voltage gradient (5-13 mV) (90).

1.4.1 O_2/O_3 mechanism of action

O_3 mechanism of action is still object of study. O_3 is an oxidizing molecule and, in human fluids and tissues, it can react with water and polyunsaturated fatty-acids (PUFAs) and produces hydroperoxides such as hydrogen peroxide (H_2O_2) and lipid ozonation products (LOPs) such as

malonyl dialdehyde (MDA), 4-hydroxynonenal (4-HNE) and lipoperoxides (90, 91). Hence, O_3 is a ROS producer. ROS have a high degree of reactivity and their effect is involved in the cellular defence and immune response; indeed, they can damage bacterial cell membrane. ROS are early short-acting messengers and LOPs are late and long-acting messengers of O_3 response (92). In physiological conditions, human tissues suppress ROS action with the production of endogenous radical scavengers. A small and repeated oxidative stress can activate the nuclear factor-erythroid 2-related factor 2 (Nrf2), which is involved in the activation of antioxidant response element (ARE) sequence in the DNA. ARE sequence regulates the transcription of antioxidant enzymes. If O_3 induces a mild oxidative stress, stimulates Nrf2 and ARE activation, and consequently the cellular antioxidant activity. This mechanism of action could explain O_3 use for stress response created by chronic inflammation (90). Based on different state of cell oxidation or on its amount, O_2/O_3 can regulate pro-inflammatory and anti-inflammatory effects associated with prostaglandin formation or with the activation of the Nrf2 (92). O_3 can exercise a potential cytotoxic effect in cancer cells. Cancer cells have an overload antioxidant system for the major presence of intracellular ROS rather than normal cells, so they cannot increase their antioxidant production. Further, ROS can react also with cytoplasmic molecules such as NADPH, but in cancer cells NADPH is present at low levels, so the reactive molecules induce acute oxidative stress and cellular damage. This could suggest a possible explanation for O_3 doses which are safety for normal cells, but toxic for cancer ones (Figure 18) (91).

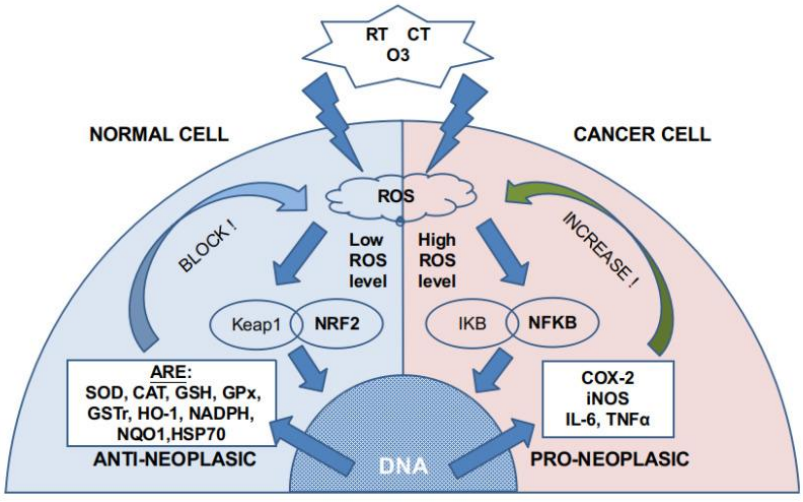


Figure 18. O_3 potential mechanism of action in normal and cancer cells (91).

1.4.2 O_2/O_3 therapy toxicity

At high concentrations, O_3 can induce toxic effect such as pain at the site of injection of insufflation, haemolysis in case of anaemia, allergic reactions and transient oxidative stress. An acute exposure to O_3 can damage glial cells for the increase of VEGF overexpression (93).

1.4.3 O_2/O_3 therapeutic effect

Today, O_3 is mainly known as bactericidal and virucidal, inflammatory modulator and circulatory stimulator. Applications in medical fields are related with wound healing, ischemic disorders, infections and chronic inflammation (90). About its use as adjuvant in cancer treatment, there are some preclinical evidences about O_3 potentiality to induce cancer cell damage, but other investigations are necessary.

1.4.3.1 O_2/O_3 anti-inflammatory effect

As previously described, low doses of O_3 could be use in chronic inflammations for its mechanism of action and clinicians usually integrate O_3 with common anti-inflammatory drugs. O_3 effects were also observed in regulation of pain for inflammatory process, in fact the increase of anti-oxidant molecules after O_3 administration could stimulate anti-nociceptive pathways. The oxidative stress after O_3 administration, if correctly balance, reverts chronic oxidative stress typical of chronic pathologies (92).

1.4.3.2 O_2/O_3 anticancer effect

For their accelerated metabolism, cancer cells may require higher O_2 levels, but for their abnormal structure and their unfunctional blood network, they are often in hypoxic conditions. Hypoxia induces oxidative stress and activates genes involved in cell survival, metabolism and angiogenesis, adapting cancer cells to this condition. Preclinical results showed that O_3 can improve blood circulation and cancer tissues oxygenation, induce oxidative stress, activate the immune response, inhibit growth and metastasis and induce tumor necrosis. O_3 was observed also to improve chemotherapy, increasing TME permeability and facilitating drugs penetration within the tumoral mass, and to enhance cancer cells radiosensitivity (93). Preclinical i*n vitro* and *in vivo* studies showed that O_3 promotes cytotoxic effect in cancer cells, but to date, there are few papers about its use in cancer patients (91). In a 1980 study, O_3 induced DNA damages as X-rays

irradiation and had additive effect if combined with X-rays in cancer cell culture. This evidence was later confirmed in HeLa cells. Subsequently, O_3 cytotoxic effect was demonstrated in breast, lung, uterus, ovarian and colon cancer cells, but in other evidences O_3 did not affect cancer cells. At the same time, it was reported that O_3 was not injurious for normal cells and that it increased radiations and 5-FU effect (91). For example, in a first study performed in glioma cells, O_3 administration did not inhibit cancer cells growth, neither alone nor in combination with 5-FU. In another study, rat C6 glioma cells were treated with O_3 and data reported that O_3 was more effective at low dose than at high dose (93). SK-N-SH and SK-N-DZ neuroblastoma cells were treated with O_2/O_3 and their growth was reduced in a dose-dependent manner. O_2/O_3 increased ROS production in both cell lines and induced cell cycle block in G_2/M, inhibiting the association between cyclin B1 and cyclin dependent kinase 1 (Cdk1) proteins in SK-N-SH, while it increased sub-G_1 phase and apoptosis rate in SK-N-DZ. In SK-N-SH, O_2/O_3 potentiated cisplatin and etoposide effect but not GEM; in SK-N-DZ, O_2/O_3 exerted cytotoxic effect but not enhance chemotherapeutic drugs activity (94). O_2/O_3 reduced liver cancer cells viability and this effect was reverted by N-acetylcysteine (NAC) administration. Positive effects were also observed in the reduction of migration and in G_2/M cell cycle arrest. O_2/O_3 increased ROS and inactivated NF-kB and PI3K/Akt pathway (95). In pancreatic cancer cells, O_2/O_3 was administered alone or in combination with CBD, GEM and PTX. O_2/O_3 reduced cancer cell viability inducing necrosis and this effect was potentiated when combined with CBD. The efficacy of chemotherapeutic drug was implemented by O_2/O_3. Moreover, O_2/O_3 down-regulated cell cycle and Ras-associated genes, NF-kB, REL, ras homolog family member A (RHOA) and PI3K/AKT pathway genes, while increased breast cancer type 2 susceptibility protein (BRCA2) and tumor protein 53 (TP53) gene expression (52). About *in vivo* application, rabbits inoculated with VX2 head neck cancer cells were divided in three groups and daily insufflated intraperitoneally with O_2 and O_2/O_3. Results showed a significant increase of survival in O_2/O_3 treated group than in O_2 alone and control group. In particular, six of seven O_2/O_3 treated surviving animals showed full tumor regression and absence of lung metastasis. Further investigations showed also that O_2/O_3 rabbits were resistant to the development of another tumoral mass after VX2 cells reimplantation. In O_2/O_3 treated immunosuppressed rabbits this resistance was not observed, suggesting that the O_2/O_3 inhibition of cancer development was associated with immune response activity (96).

About clinical application, there were reported only few case reports. In one case glioblastoma, after the relapsing, was re-resected and then patients were treated with O_3 intratumorally (with the use of a catheter) and standard chemotherapy. There was observed an increase of survival from 11.9 months to 30.5. In another case report, a patient was treated with O_3 and chemotherapy, after the first surgical resection, and he is still alive and without recurrence (91).

1.5 Pancreatic cancer

1.5.1 Pancreas anatomy

The pancreas is involved in protein and carbohydrates digestion and glucose homeostasis. The major area (80%) is the exocrine pancreas composed by acinar and ductal cells, involved in digestive enzymes production. The other part, the endocrine pancreas, is formed by four types of clustered cells called Islets of Langerhans that secrets hormones into the blood stream and regulates glucose homeostasis. Based on the different cellular characteristics, there are different malignancies that can be developed in the pancreas (97).

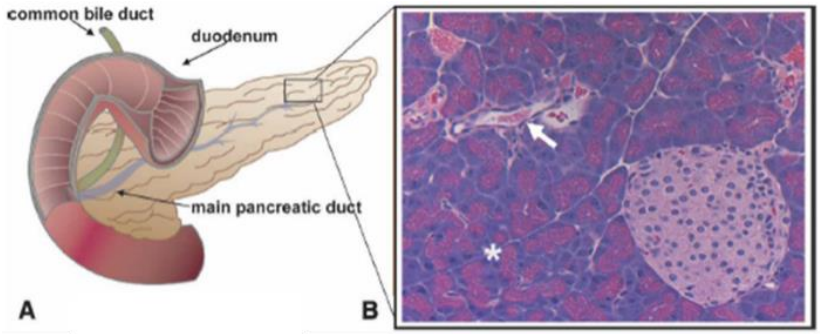


Figure 19. A) Pancreas anatomy and its position next to the common bile duct and duodenum. B) Pancreas histology: Islet of Langerhans in the lower right; the asterisk identifies the acini; the arrow indicates the duct (97).

1.5.2 Epidemiology

In 2020, pancreatic cancer substituted breast cancer as the 3rd cause of cancer-related death in the United States (Figure 20) (98). In the last two decades, it was observed an increase of pancreatic cancer diagnosis and the age-adjusted incidence rates of pancreatic cancer are rising as a result of key risk factors increase (99).

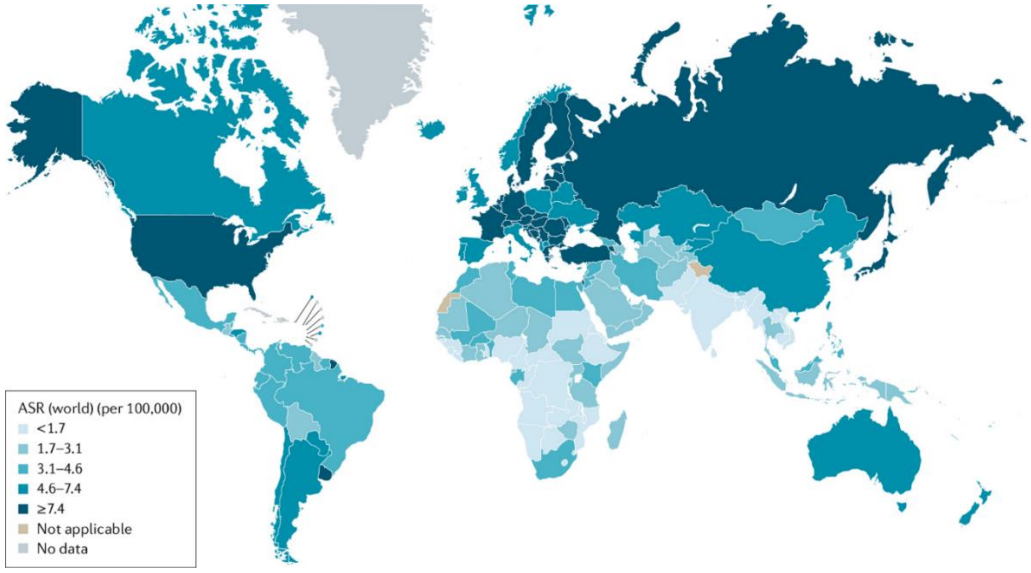


Figure 20. Age-standardized incidence rates (ASR) of pancreatic cancer incidence across the globe in 2020 (99)

For the late diagnosis pancreatic cancer survival-rate is low, the 5-years overall survival increased only from <5% in the 1990s to 9% in 2019 in USA and Europe. Only about 20% of patients presenting a surgically resectable disease and among them, 5-year survival rate is 15–25% (99).

1.5.3 Pancreatic ductal adenocarcinoma (PDAC)

Pancreatic ductal adenocarcinoma (PDAC), which derives its name from the histological similarity with ductal cells, is the most common type of pancreatic neoplasm and represents more than 85% of pancreatic tumors (97). It can metastasize to the liver and lymph nodes and it is not associated with specific symptoms, so it is difficult to diagnose at early stage and often, it had already metastasized at the moment of discovery (100). About the factors associated with an increased risk of developing PDAC, there are advanced age, smoking and some pathological conditions such as long-standing chronic inflammations, diabetes and obesity. A familiar predisposition was estimated in the 10% of PDAC cases and this is often associated with germline mutations of tumor suppressor genes INK4A, BRCA1, BRCA2, LKB1, the DNA mismatch repair gene MLH1 and the cationic trypsinogen gene serine protease 1 (PRSS1) (pancreatitis predisposition gene), and further poly-cancer syndromes such as Peutz-Jeghers syndrome, hereditary pancreatitis and familial atypical multiple mole melanoma could be include among risk factors. The germline mutations were mostly correlated with the malignant progression of precursor lesions rather than the cancer development (97, 101).

1.5.3.1 PDAC morphological characteristics

In the majority of cases, PDAC is defined "not otherwise specified", but some histopathological variants were identified. Adenosquamous carcinoma is an uncommon variant of PDAC with a mixture of glandular and squamous differentiation, while other carcinomas with acinar differentiation are pancreatoblastomas, acinar cell carcinomas and carcinomas with mixed histology (101).

PDAC usually arises in the head of pancreas and then it can infiltrate near tissues such as spleen, lymphatics and peritoneal cavity; metastasis can reach lungs and liver. PDAC is characterized also by a dense stroma called desmoplasia, formed by fibroblasts and inflammatory cells. PDAC development can start from some precursor lesions such as pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN). MCN are epithelial cystic lesions mucin-producing, characterized by an ovarian stroma type with variable degree of dysplasia and focal regions of invasion. IPMNs is similar to PanIN but with larger cystic structures. Morphological changes in normal ducts observed in PanINs are correlated with graded stages of increasing dysplastic growth: from I to III grade increases the architectural disorganization and nuclear atypia, until the high-grade PanIN develops PDAC. During the transition from PanIN to PDAC, it was observed an increased number of genetic alterations (Figure 21) (97).

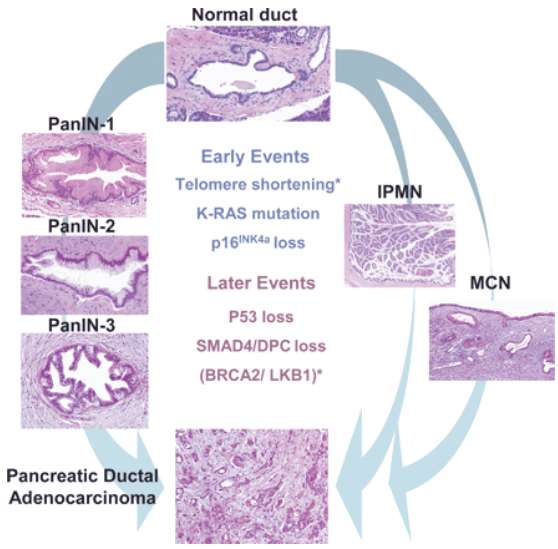


Figure 21. PDAC progression form precursor lesions and genetic events, divided into early or late in PDAC progression (97).

In early-stage precancerous lesions, the first mutations were already observed. The major genetic mutations regard oncogenes activation and tumor suppressors inactivation (97, 102).

1.5.3.2 Genetic mutations

PDAC is known for the mutation of KRAS oncogene and the inactivation of TP53, SMAD4, BRCA2 and CDKN2A that suppress tumor growth; further, there are also chromosomal loss, gene amplifications and telomere shortening which cause chromosomal instability. KRAS mutations and telomere shortening are in low-grade pancreatic intraepithelial neoplasia, while TP53, SMAD4 and BRCA2 genes abnormalities were observed in advance pancreatic intraepithelial neoplasia and invasive carcinomas (101, 103).

1.5.3.2.1 KRAS

KRAS gene encodes a small GTPase and mediates cell proliferation, differentiation and survival. K-Ras is a GTP-binding protein member of the RAS family. In physiological conditions, K-Ras can be in the inactive GDP-bound state or in the active GTP-bound state. A guanine nucleotide exchange factor (GEFs e.g., Sos1) mediate the transition between the two states and a GTPase-activating protein (GAPs e.g., NF1) induced K-Ras ability to hydrolyse GTP. Quiescent cells present a GDP-bound K-Ras, after growth factors stimulation, GEFs catalyses K-Ras shift from GDP to GTP state. In some solid cancers, KRAS mutations inhibit its GTPase activity, inhibiting GTP hydrolysis and sustaining the active GTP-bound state. In this way, there is a continuous stimulation of pathways involved in tumor development (97, 100). KRAS point mutation can interest the substitution of glycine with aspartate, or valine, or arginine in G12, G13 or Q61. These mutations are detected in about 30% of early neoplasms and in about 100% of advanced PDAC. All the substitutions interest the catalytic domain of GTPase. G12 mutation (G12D or G12V) is present in the 82% of PDAC cases, Q16 in 14% and G13 in <1%. According to the type of *KRAS* mutation, the tumorigenesis is promoted in different ways (97, 100).

KRAS can be activated by the Epidermal Growth Factor (EGF) that binds EGFR. This binding induces receptors dimerization in plasma membrane and the phosphorylation of the transforming protein SHC, which creates a complex with SOS and GRB2 facilitating the binding of GEFs with KRAS, which results in the KRAS GTP-activate form. EGFR activation can also promote the phosphorylation of PIP2 in PIP3, which stimulates AKT via PDK1 and inhibits the inactivation

of the pathway through PTEN. Mutation at EGFR levels were not identified in PDAC, even if its high expression was associated with patient's poor prognosis (Figure 22) (104).

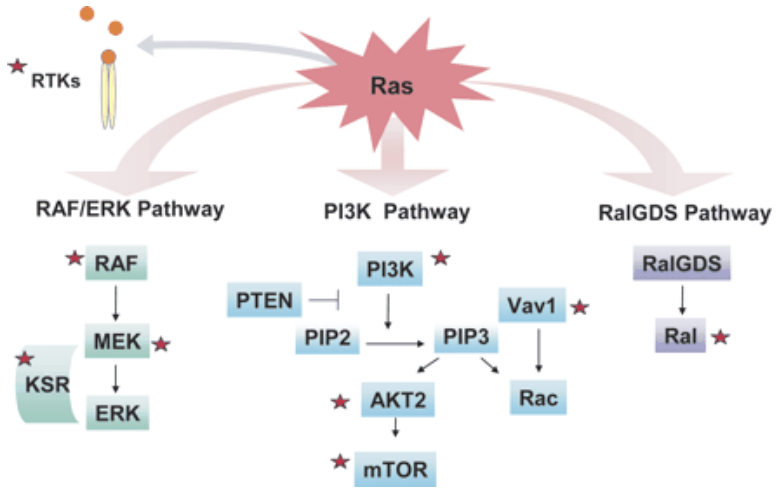


Figure 22. Three main signaling cascades implicated in PDAC progression and maintenance: RAF/ERK pathway, PI3K/Akt pathway, and Ral GDS pathway (97)

KRAS activate three main effector pathways: Raf/MEK/ERK, PI3K/PTEN/AKT and Ral guanine nucleotide exchange factor (Figure 22) (104).

1.5.3.2.1.1 Raf/MEK/ERK signaling pathway

KRAS activated engages RAF. RAF is a kinase composed by A-Raf, B-Raf and C-Raf; all are KRAS effectors. Activated RAF phosphorylates the two kinases MEK1 and MEK2, that then activate the serine-threonine kinases ERK1 and ERK2 (104). The oncogenic mutation BRAF V600E was identified in about the 3% of PDAC cases and, in mice, it was demonstrated that its expression induces multifocal PanIN lesions formation (104).

1.5.3.2.1.2 PI3K/PTEN/AKT signaling pathway

PI3K is composed by different catalytic subunits: I, II and III classes. Class I are p110 subunits (α, β, γ and Δ) and they are encoded by PIK3CA, PIK3CB, PIK3CG and PIK3CD genes. PI3KCA and PI3KCD mutations are identified in PDAC. Activated PI3K p110 α, p110 β and p110 Δ bind p85 regulatory subunit; PI3K p110 γ binds p101 and p87 subunits. At this point, PI3K activated

induces the PIP2 activation in PIP3, that engages AKT and PDK1. AKT is activated by phosphorylation. AKT in turn activates mTOR signaling pathway and RAC, member of GTPases RHO family. PDK1 activates AKT and PKA, PKC and p70S6K implicated in cancer progression. The activation of p110 alpha subunit was considered an important effector of oncogenic KRAS in PDAC and several mice models demonstrated that the loss of the tumor suppressor PTEN, that inhibits AKT activation dephosphorylating PIP3 to PIP2, is another element involved in PDAC progression (104).

1.5.3.2.1.3 Ral guanine nucleotide exchange factor signaling

Ral guanine nucleotide exchange factors (RalGEFs) activate RalA and RalB small GTPase that are up-regulated in PDAC. Compared to the other two main pathways, RalGEF signaling in PDAC progression is less known (104).

1.5.3.2.2 Tumor-suppressor genes

Tumor-suppressor genes alterations are closely involved in PDAC development. CDK2A and TP53 maintain the genome integrity, preventing the accumulation of mutations and the promotion of tumor development. CDKN2A gene is mutated in approximately 95% of tumors. CDKN2A is responsible for encoding a protein that prevents cells from entering the S-phase of cell cycle, known as p16/INK4A. CDKN2A inactivation was observed in advanced PanINs before PDAC development. p16 induces senescence after K-Ras activation, indeed CDKN2A inactivation follows KRAS mutation. CDKN2A locus encodes also for ARF/p14 protein involved in tumor growth inhibition or apoptosis, but this mechanism seems not to be involved in PDAC progression since it induces MDMD2-depent p53 proteolysis but p53 is frequently inactivated in PDAC. TP53 mutations contribute to PDAC metastatic nature and has complex role since it is a tumor suppressor but also an enhancer of metastasis. TP53 mutation appears in the later-stage PanINs with a high grade of dysplasia. p53 protein blocks cell cycle and actives DNA repair enzymes; when the DNA damage is irreparable p53 induces senescence or apoptosis. Cancer cells with chromosomal aberrations could benefit from p53 loss to grow and survival (97, 100).

There are other tumor-suppressor genes mutated in minor percentage in PDAC. LKB1 gene loss for a germline mutation is associated with Peutz-Jeghers syndrome in which patients develop benign polyps in gastrointestinal tract and an increased risk to develop other malignancies such

as PDAC. LKB1 encodes for a serine/threonine kinase that regulates cell polarity and metabolism, and regulates also mTOR pathway trough AMPK regulation (97, 100).

The transforming growth factor-β (TGF-β) signaling pathway can induce apoptosis and/or promote invasion and metastasis. Its role in human malignancies can be both growth-inhibitor and growth-promoter depending on the cell types and the context. For example, in some epithelial cells and tissues, TGF-β exerts a growth inhibitory, pro-apoptotic and telomerase repression activity; at the same time in epithelial cancer cells, TGF- β inhibits cancer initiation. Alterations in this signaling pathway is associated with ovarian, intestinal and pancreatic tumorigenesis. TGF-β antiproliferative effect is associated with the activity of transcriptional co-activator SMAD4/DCP4. SMAD4 alterations are observed in later-stage of PanINs and it is considered a PDAC progression allele (97, 100). TGF-β promotes also proliferation and EMT in different cancer types and through this process carcinomas loss their differentiation and develop an aggressive and invasive phenotype. So, in cancers, TGF- β has a dual role both inhibiting tumor initiation and promoting high-grade development of manifested cancer. In PDAC, SMAD4 alterations can modulate cancer interaction with the microenvironment and inhibit TGF-β-induced cell cycle arrest and cell migration, but not influence EMT (97).

Telomere erosion is considered an early event in PDAC pathogenesis. This genetic alteration results in p53-induced senescence in normal cells, but an inactivated p53 causes tumor cells proliferation. p53 loss can be preceded by an important telomere shortening. Cellular immortalization is promoted by telomerase reactivation in invasive PDAC after telomere shortening and dysfunction, which leads to carcinogenesis for cancer chromosomal rearrangements (97, 100).

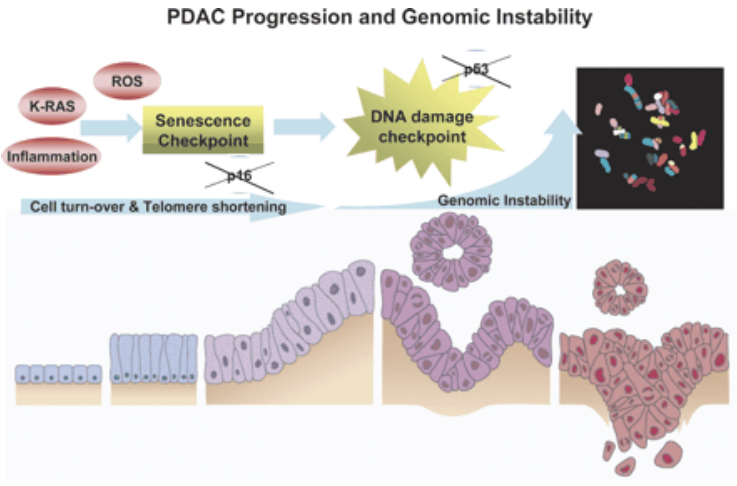


Figure 23. PDAC progression and genomic instability (97)

1.5.3.3 PDAC current therapies

As previously reported, today, the only effective way to treat pancreatic cancer is surgery resection. Unfortunately, only a 20 % of patients present a resectable pancreatic cancer form. Further, after the successful resection, over the 80 % of patients develop recurrence and metastasis (100). Generally, chemotherapy is used as primary therapeutical approach both with a resectable and unresectable form, and also surgical resection is followed by adjuvant chemotherapy and in presence of metastasis. Finding optimal drug doses and combinations to administer was a strategy to improve the effectiveness of current therapies and reduce side effects, for example GEM and erlotinib (EGFR inhibitor) combination had a small effect on extending survival by 12 days (100). Despite of this, prognosis remains poor also for the chemoresistance develops by several patients. Among the major problems associated with pancreatic cancer treatment there are the dense desmoplasia, the hypoperfusion and the immunosuppressive microenvironment, indeed another therapeutic approach is also to exploit the pancreatic cancer microenvironment in order to activate drugs (105). TME is rich in hyaluronic acid that creates a physical barrier for drugs, but hyaluronidases could be used to improve drug delivery. Moreover, drugs could be activated by the hypoxic environment such as the evofosfamide, that releases its active form in hypoxia. Targeting only KRAS as a therapeutic target was not very successful, indeed none of its direct inhibitors were used for clinical application, but KRAS regulates other signaling pathways that could be potential targets (102). About immunotherapy, one approach was to stimulate the immune system against pancreatic cancer cells. GVAX vaccine is composed by engineered pancreatic cells that express granulocyte-macrophage colony-stimulating factor. They can recruit dendritic cells able to phagocyte tumor cells and then they present the antigens to T-cells. In pancreatic cancer this technology needs to be improved. The immune checkpoints are considered a way to stimulate the immune system against cancer cells, but in pancreatic cancer the single use of immune checkpoints inhibitors was not successful (100).

2. Aim of the Project

PDAC is one of the most aggressive malignancies characterized by a late and difficult diagnosis and by resistance to current therapies. Up to now, not significant improvements were obtained in the treatment of PDAC patients and the life expectation remains short. The use of molecules as integrative therapy could be a promising approach to increase the efficacy of conventional chemotherapeutic drugs and to reduce drug resistance and side effects. Thus, the aim of the project was to investigate the effects of molecules that could be employed as integrative therapy in order to evaluate and clarify their activity in preclinical models of PDAC. The minor PyCBs, CBG was studied in two human PDAC cancer cell lines, evaluating the molecular mechanisms involved in its activity. Additionally, it was evaluated the biological effect of a combination composed by CBD, MLT and O_2/O_3, *in vitro* and *in vivo* human models of PDAC. All the different treatments were combined with the main chemotherapeutic drugs used in PDAC treatment.

3. Materials and Methods

3.1 Cell lines

Human PANC-1 and MIAPaCa-2 PDAC cell lines (Sigma Aldrich, Milan, Italy) and normal fibroblast (NHFA12) cell line (IFOM, Rome, Italy) were cultured in DMEM high glucose medium (EuroClone, Milan, Italy) supplemented with 10% of fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg streptomycin and 1 mM sodium pyruvate. Epithelial cell line derived from the human normal colon (NCM460D; Cytosens, Milan, Italy) was cultured in RPMI1640 supplemented with 10% of FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg streptomycin. Cell lines were maintained at 37 °C with 5% CO_2 and 95% of humidity. NHFA12 and NCM460D were used as normal cells for viability assay.

3.2 Reagents

Pharmaceutical grade Cannabidiol (CBD) and Cannabigerol (CBG) crystals were purchased (Cayman Chemical, Ellsworth, MI, USA) and solubilized in ethanol (Et-OH) 70% at 15.7 mg/mL (50 mM). Luzindole was purchased (Sigma Aldrich) and solubilized in dimethyl sulfoxide (DMSO) at 14.6 mg/mL (50 mM). PTX (6 mg/mL) and GEM (50 mg/mL) supplied by Sigma Aldrich were dissolved in water. Aliquots were done and stored at -20 °C and each aliquot was used one time. Melatonin (MLT, Cayman Chemical) was solubilized in Et-OH 70% at 40 mg/mL and prepared fresh each time.

3.3 O_2/O_3 treatment

Cell lines seeded on 96-well plate or 12-well plate 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 a E100 Ozonline machine (Eco3 s.r.l., Torino, Italy). Then plates were placed back in the incubator at normoxia condition (37 °C with 5% CO_2 and 95% humidity) and incubated for 72 h, before performing the experiments.

3.4 Cell viability assay

3×10^4 cells/mL were seeded in 96-well plates in a final volume of 100 µL/well. After one day of incubation, treatments were added and six replicates were used for each treatment. All experiments were repeated three times. After 72 h, cell viability was investigated 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 hours the supernatant was removed and the pellet of salt crystals was solubilized with 100 µL/well of DMSO. The absorbance of the sample against a background control was measured at 570 nm using an ELISA reader microliter plate (BioTek Instruments, Winooski, VT, USA).

3.5 Cell death assay

Annexin V-FITC and Propidium Iodide (PI) staining followed by FACS analysis was used to evaluate cell death. 3×10^4 cells/mL were seeded in 12-well plates and after one day of incubation treatments were added. Cells were stained with Annexin V-FITC (5 µL) or PI (PI 20 µg/mL) for 10 minutes at room temperature and then washed with binding buffer (10 mM N-(2-Hydroxyethyl) piperazine-N0-2-ethanesulfonic acid [HEPES]/sodium hydroxide, pH 7.4, 140 µM NaCl, 2.5 µM CaCl2). The percentage of Annexin V-FITC or PI positive cells were analysed by FACScan flow cytometer using the CellQuest software (BD Biosciences, Rome, Italy) or with BD Accuri C6 plus flow cytometer using the BD Accuri C6 plus software (BD Biosciences).

3.6 Cell proliferation assay

CellTraceTM Cell Proliferation kit (Thermo Fisher Scientific, Rome, Italy) was used to analyse the proliferation of PDAC cells in response to stimulation by treatments. Cells were labelled with 1-2 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 20 min at 37°C, then seeded 3 x 10^4 cells/mL and cultured for 72 h in culture medium containing specific treatment. At the end of the treatment florescence was analysed by FACS. All experiments were repeated three times.

3.7 Western Blot analysis

Lysates were obtained with lysis buffer (composed by TRIS 1M pH 7.4, NaCl 1M, EGTA 10 mM, NaF 100 mM, Deoxycholate 2%, EDTA 100 mM, TritonX-100 10%, Glycerol, SDS 10%, $Na_2P_2O_7$ 1M, Na_3VO_4 100 mM, PMSF 100 mM, Cocktail of enzyme inhibitors). Lysates were

separated on a SDS polyacrylamide gel and transferred onto Hybond-C extra membranes (GE Healthcare, Chicago, IL, USA) using Bio-Rad system. Non-specific binding sites were blocked with 5% low-fat dry milk or 5% BSA in PBS containing 0.1% Tween 20 for 1 h at room temperature. The following antibodies were used: mouse anti-mTOR (1:1000, GeneTex, Alton Pkwy Irvine, CA, USA), mouse anti-EGFR (1:5000, GeneTex), rabbit anti-pAkt (1:1000, Cell Signaling, Danvers, MA, USA), rabbit anti-Akt (1:1000, Cell Signaling), rabbit anti-LC3 (1:1000, Cell Signaling), rabbit anti-Caspase-3 (1:1000, Cell Signaling), mouse pan-RAS (1:500, Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti-MEL1A/B-R (MTNR1A/B) (1:1000, Dallas, TX, USA), mouse β-actin (1:500, Santa Cruz Biotechnology), mouse anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH, 1:1000, Santa Cruz Biotechnology). Abs were incubated overnight or 1 hour according to manufacturer's protocol and then incubated with their respective HRP-conjugated anti-rabbit or anti-mouse (1:2000, Cell Signaling) Abs for 1 hour. Peroxidase activity was visualized with the LiteAblot®PLUS or TURBO (Euro-Clone, Milan, Italy) kit and densitometric analysis was carried out by a Chemidoc using the Quantity One software version 4.6 (Bio-Rad, Milan, Italy).

3.8 Acridine Orange Staining

To detect acidic vesicular organelles, the vital staining of cells with acridine orange (AO, Sigma-Aldrich) was performed. $3x10^4$ cells/mL were seeded in 12-well plates and treated with CBG at 11.08 and 12.66 μg/mL for 48 h. Then, cells were stained with 1 μg/mL AO, washed in PBS, immobilized on slides using the cytospin centrifuge and analysed with C2 Plus confocal laser scanning microscope (Nikon Instruments, Florence, Italy). Optimized emission detection bandwidth was configured by Zeiss Zen control software. Images were processed using NIS Element Imaging Software (Nikon Instruments, Florence, Italy). Cytoplasm and nuclei of AO-stained cells fluoresced bright green, whereas the acidic autophagic vacuoles fluoresced bright red.

3.9 Drug interaction

Drugs interaction was evaluated with SynergyFinder, using the Bliss model [106]. Bliss independence reference model was used for multiplicative effect of single drugs as if they acted independently. Bliss synergy score larger than 10 is considered synergistic, from -10 to 10 is considered additive and less than -10 antagonistic.

3.10 Milliplex multiplex assay

The levels of TOTAL Ras, pBRAF, pCRAF, pMEK1 in treated cells were measured using 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 analysed using Luminex® 200 instrument with xPONENT® software (Luminex Corporation, Austin, TX, USA).

3.11 Treatment on orthotopic pancreatic tumor mice model

Orthotopic pancreatic tumor mice model derived from the inoculation of PANC-1 tumor cells on athymic nude mice. Female Athymic Nude-Foxn1nu mice, 5 weeks old, were supplied by Envigo RMS SARL (Gannat, France). All the procedures involving the animals were conducted according to the national and international laws on experimental animal (d.l. 4 March 2014, n° 26, implementation of directive n. 2010/63/UE) and to the approved experimental protocol procedure (Authorization n° 844/2021-PR). Animal were maintained at a temperature of 22 ± 2 °C under a daily 12 h photoperiod in ventilated cabinet at the Animal Facilities of University of Florence (CESAL), Florence, Italy. Following a period of acclimation (13 days), 40 animals were anesthetized with isoflurane (induction at 4% and maintenance at 2%) and inoculated with PANC-1 tumor cells ($1x10^6$ cells in 20 µL of PBS) that were orthotopically injected into the tail of the pancreas exploiting the echo-guided procedure. Tumor size was determined by ultrasound imaging analysis and when it reached a volume of approximately 10 mm^3 (19 days after inoculation), 30 out of 40 animals were divided into 6 groups (n=5 per group): 1) vehicle (Vhc) (received saline), 2) Mix (received 400 mg/kg MLT & 10 mg/kg CBD, 3) Ozone (received O_2/O_3 2.5 ml/kg), 4) Mix + Ozone (received 400 mg/kg MLT & 10 mg/kg CBD + 2.5 ml/kg O_2/O_3), 5) Gemcitabine (received 50 mg/kg GEM), 6) Mix + Ozone + Gemcitabine (received 400mg/kg MLT & 10 mg/kg CBD + 2.5 ml/kg O_2/O_3 + 50 mg/kg GEM). Treatments were administered by i.p. injection every 3 days for 30 days, for a total of 10 treatments.

3.12 Experimental Design

From the beginning of the treatments to the following 4 weeks, ultrasound (US) and photoacoustic (PA) imaging was performed once a week to evaluate the engraftment and development of tumor masse PA and US imaging were performed with Vevo LAZR-X system (Visualsonics Fujifilm, Amsterdam, The Netherlands). For volumetrics analysis, axial 3D scans of the tumor masses were performed in B-Mode imaging by using a 55-MHz transducer. For PA imaging Vevo Optical Fiber

(Silica fiber, Narrow) were placed on the Vevo Fiber Jacket positioned on the transducer. Moreover, the body weight of the animals was measured once a week. At the end-point mice were sacrificed and macroscopic necroscopy was performed. Moreover, all the tumors were explanted, weighted along with liver, brain and heart (for brain and heart, of 2 out of 5 animals of each group).

3.13 *In vivo* data analysis

Tumor volumes were analyzed by using Vevo Lab software (Fujifilm Visualsonics). The volumes were measured delineating the ROI (Region of Interest) for every axial slide using Vevo LAB software.

3.14 Statistical analysis

Data represented the mean with standard deviation (SD) of at least 3 independent experiments. The statistical analysis was determined by Welch's t-test, One-way ANOVA followed by Dunnett's or Tukey's multicomparison test and by Two-Way ANOVA followed Tukey's multicomparison test, using GraphPad Prism 9.0.1(128) software (San Diego, CA, USA).

4. Results

4.1 CBG inhibits cell growth of PDAC cell lines

PANC-1 and MIAPaCa-2 PDAC cell lines, and immortalized NCM460D and NHFA-12 cell lines were treated with different doses of CBG (up to 31.648 µg/mL) as single administration and cell viability was determined by MTT assay at 72 h post-treatment. Results showed that CBG reduces cell viability in both cell lines with an IC_{50} of 15.64 ± 0.83 µg/mL for PANC-1 and 13.77 ± 0,72 µg/mL for MIAPaCa-2 (Figure 24A), while lower cytotoxicity was observed in normal human NCM460D and NHFA12 cell lines (Figure 24B). For the next experiments, two doses of CBG (11.08 and 12.66 µg/mL) have been selected, doses with lower growth inhibitory effect for studying molecular and biological mechanisms in PDAC cell lines and with no cytotoxic effects on normal cells. CBG was always used as single ad-ministration.

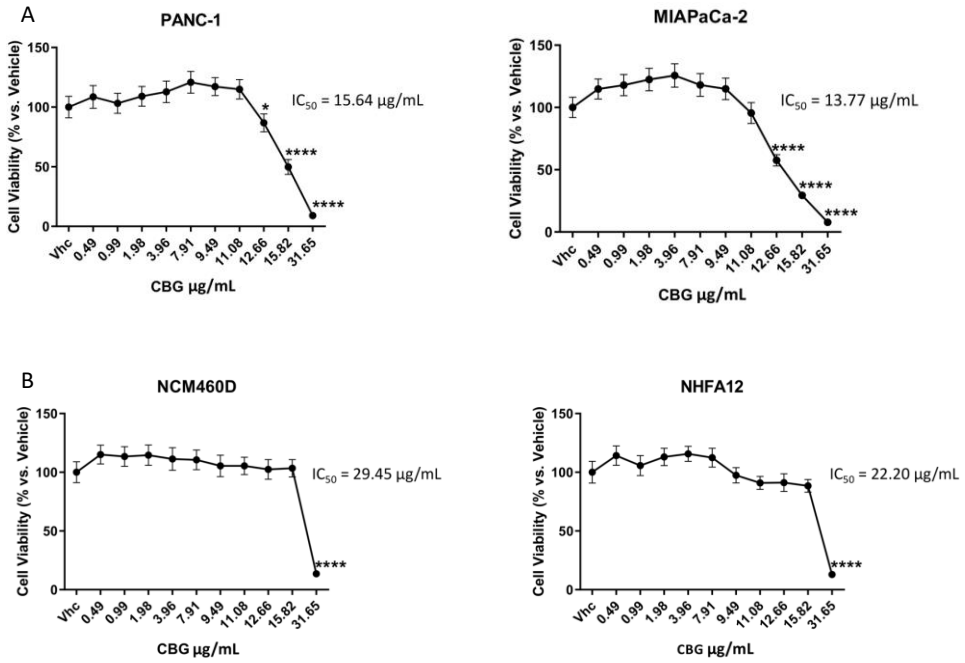


*Figure 24. CBG reduces cell viability in PDAC cell lines. Cell viability was determined by MTT assay. PANC-1 and MIAPaCa-2 cells were treated with vehicle (Vhc; Et-OH 70%) or different concentrations of CBG and cell viability was evaluated at 72 h post-treatment. Data shown are the mean ± SD of three separate experiments. *p<0.05, ****p<0.0001 vs Vhc.*

4.2 CBG induces autophagy by inhibition of EGFR and Akt/mTOR pathway in PDAC cell lines

EGFR and Akt/mTOR protein levels were evaluated by Western blot in PDAC cell lines at 48 h post-treatment with CBG 11.08 and 12.66 µg/mL. Immunoblots evidenced a decrease of EGFR expression in PANC-1 with the CBG highest dose, while for MIAPaCa-2 the reduction was significant with both doses (Figure 25A). Similar results were obtained for mTOR protein expression, that was reduced especially after the treatment with the highest dose of CBG (Figure 25A). Then, total Akt and its phosphorylated form were investigated. Data showed for PANC-1 cells a slight modulation of total Akt protein with CBG at dose of 11.06 µg/ml and a reduction of phospho-Akt (pAkt) levels after the administration of both CBG doses (Figure 25B). In MIAPaCa-2 cells, the highest dose of CBG induced a marked reduction of total Akt and a significant decrease of its phosphorylation (Figure 25B).

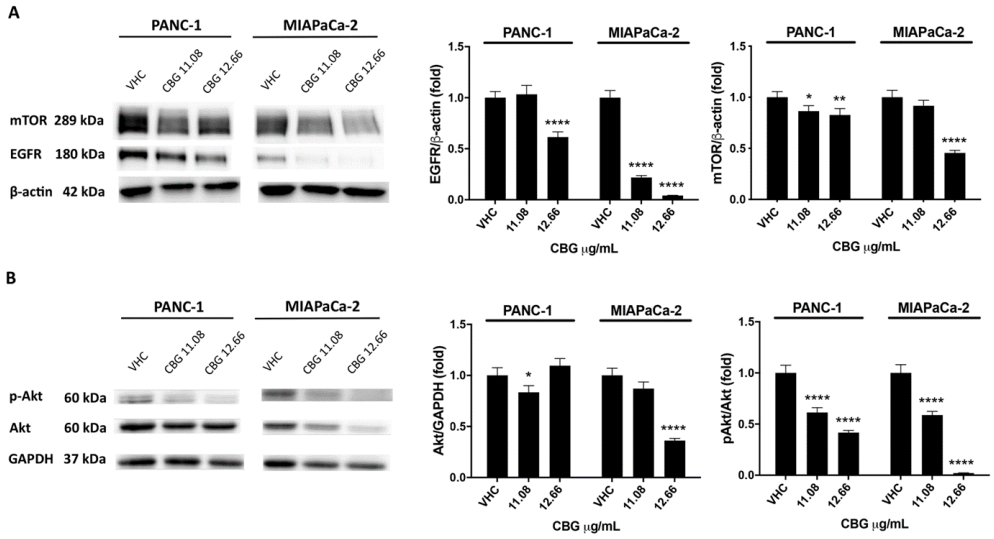


*Figure 25. CBG treatment reduces EGFR and Akt/mTOR protein expression in PDAC cell lines. PDAC cell lines were treated with CBG, and the expression levels of mTOR and EGFR (A), Akt, and pAkt (B) were evaluated by Western blot at 48 h post-treatment. EGFR, Akt, and mTOR densitometric values were normalized to β-actin or GAPDH, which were used as loading controls; pAkt densitometric values were normalized to Akt. Images are representative of one of three separate experiments. Data are expressed as mean ± SD of three separate experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ treated cells vs. VHC.*

Since CBG reduced the Akt/mTOR signaling pathway, also autophagy induction was evaluated. It was observed the conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) to LC3-II form at 48 h post-treatment with CBG (11.08 and 12.66 µg/mL). CBG induced in both cell lines a significant increase of LC3-II lipidated form suggesting the activation of the early steps of autophagy; MIAPaCa-2 cells showed the highest levels of LC3-II at the highest dose of CBG (Figure 26A).

To confirm the induction of autophagy, acridine orange dye (AO) analysis, which accumulates in acidic spaces and emits bright red fluorescence related to the degree of the acidity and the volume of acidic vesicular organelles (AVOs), was applied. AVOs are indicative of autophagy, therefore it was evaluated AVOs under confocal fluorescence micro-scope. As shown (Figure 26B), an increase in the level of red fluorescent signals reflecting an AVO formation was observed in CBG-treated cell lines, 48 h post-treatment.

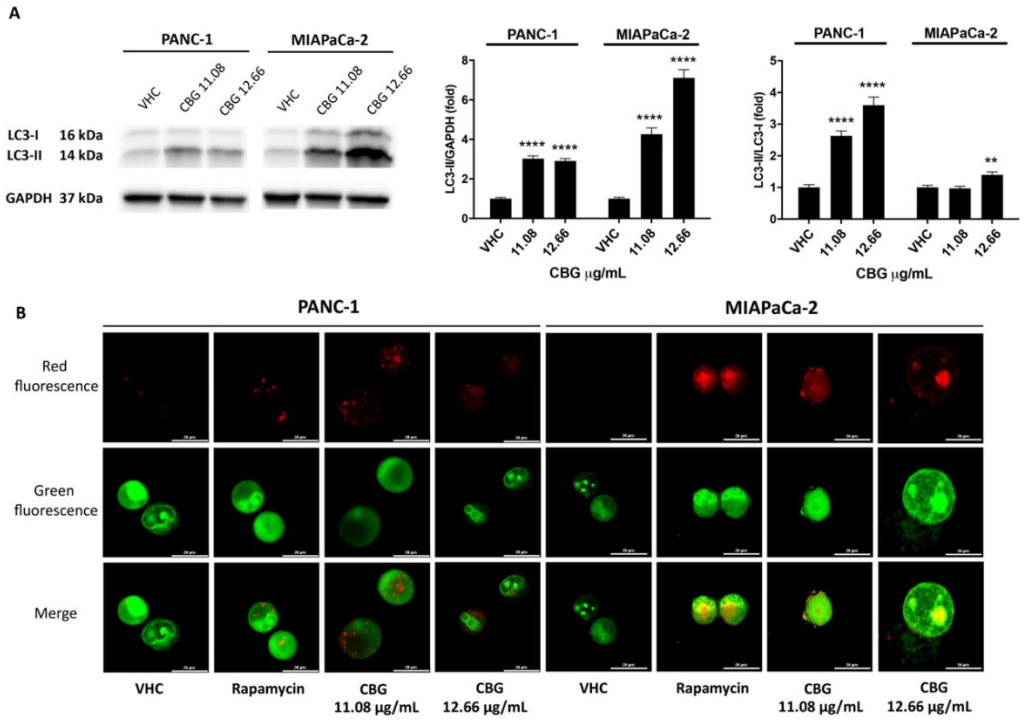


*Figure 26. CBG stimulates autophagy in PDAC cell lines. PDAC cell lines were treated with CBG and analysed at 48 post-treatment h. (A) The expression of LC3-I and LC3-II was assessed by Western blot. Densitometric values of LC3-II were normalized to GAPDH used as loading control. Densitometric values of LC3-II were also normalized to LC3-I. Images are representative of one of three separate experiments and data are expressed as the mean ± SD of three separate experiments. **p<0.01, ****p<0.0001 CBG- vs VHC-treated cells. (B) Fluorescent microscope was used to visualize AVOs (red*

fluorescence) as well as the cytoplasm and nucleus (green fluorescence) after the vital staining of the cells with AO. Representative images of PANC-1 and MIAPaCa-2 cells stained with AO. Rapamycin was used as positive control of autophagic induction.

4.3 CBG reduces RAS downstream pathway in PDAC cell lines

To further investigate the CBG-induced effect in inhibiting EGFR pathway, the expression of total RAS (Pan-RAS) was assessed by Western blot analysis. As evidenced in Figure 27A, Pan-RAS levels were statistically reduced at 48 hr post-CBG treatment. Due to the relevance of the RAS/RAF/MEK pathway in PDAC, it was measured the signaling proteins downstream of RAS in the lysate of PDAC cells treated with CBG at the most effective dose of 12.66 μg/mL. By MILLIPLEX® RAS-RAF Oncoprotein Magnetic Bead Panel 6-plex, it was evaluated changes in phosphorylated BRAF (Ser446, pBRAF), CRAF (Ser338, pCRAF), and MEK1 (Ser217/Ser221, pMEK1), as well as the relative total protein levels (Figure 27B). The results confirmed that CBG treatment reduced total Pan-RAS levels in both cell lines. It also promotes the downregulation of BRAF, CRAF and MEK1 phosphorylation in PANC-1, and of BRAF in the MIAPaCa-2 cell line. Summarizing, these data further confirm that CBG was able to interfere with the EGFR-RAS pathways by reducing also downstream effectors often aberrantly activated in PDAC.

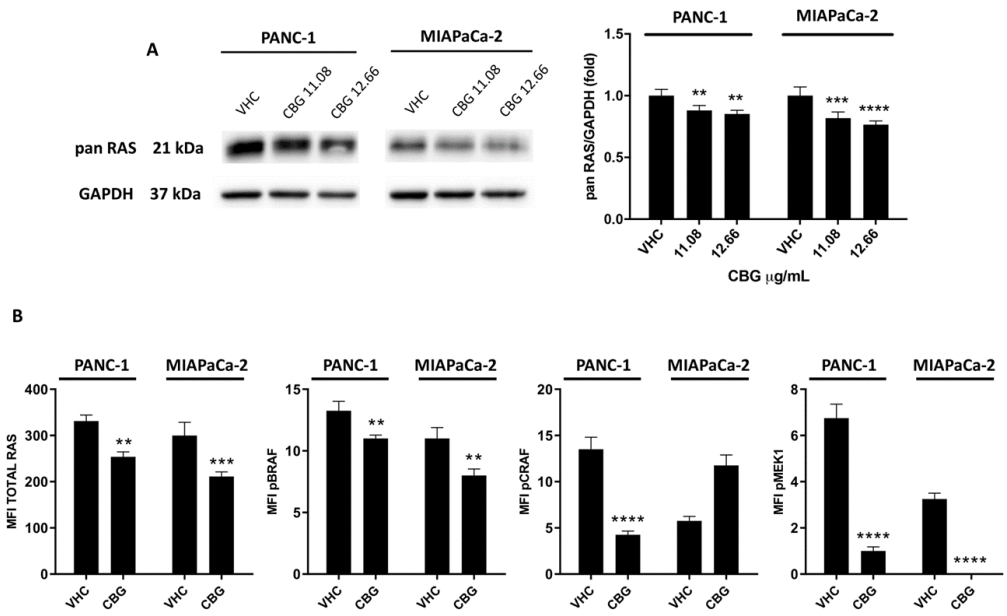


*Figure 27. CBG reduces the total Pan RAS expression and the phosphorylated forms of BRAF, CRAF and MEK1 in PDAC cell lines. (A) PDAC cell lines were treated with CBG, and the expression of pan RAS was determined with Western blot at 48 h post-treatment. The densitometric values were normalized to GAPDH used as loading control. Images are representative of one of three separate experiments and data are expressed as the mean ± SD of three separate experiments. **p<0.01, ***p<0.001, ****p<0.0001 treated cells vs VHC. (B) PDAC cell lines were treated with CBG 12.66 µg/mL for 48 h and the expression of RAS, pBRAF, pCRAF, pMEK1 was evaluated by Milliplex multiplex assay and the Median Fluorescence Intensity (MFI) was measured with the Luminex ® system. Data shown are expressed as the mean ± SD of three separate experiments. **p <0.01, ***p<0.001, ****p<0.0001 treated cells vs VHC*

4.4 CBG induces apoptosis in PDAC cell lines

To further investigate whether reduction of cell viability and activation of autophagy was associated with cell death, PANC-1 and MIAPaCa-2 cells were treated with CBG 11.08 and 12.66 µg/mL and Annexin V-FITC staining analysis were performed 48 h post-treatment. Results showed that CBG increases Annexin V^+ cells in a dose-dependent mode (Figure 28A), suggesting the induction of apoptotic cell death. The activation of ProCaspase-3 (ProCasp-3) and the presence of Caspase-3 (Casp-3) cleaved form was investigated by Western blot analysis. Blots showed an increase in Casp-3/ProCasp-3 ratio after 48 h post-treatment with CBG 12.66 µg/mL, in both cell lines (Figure 28B).

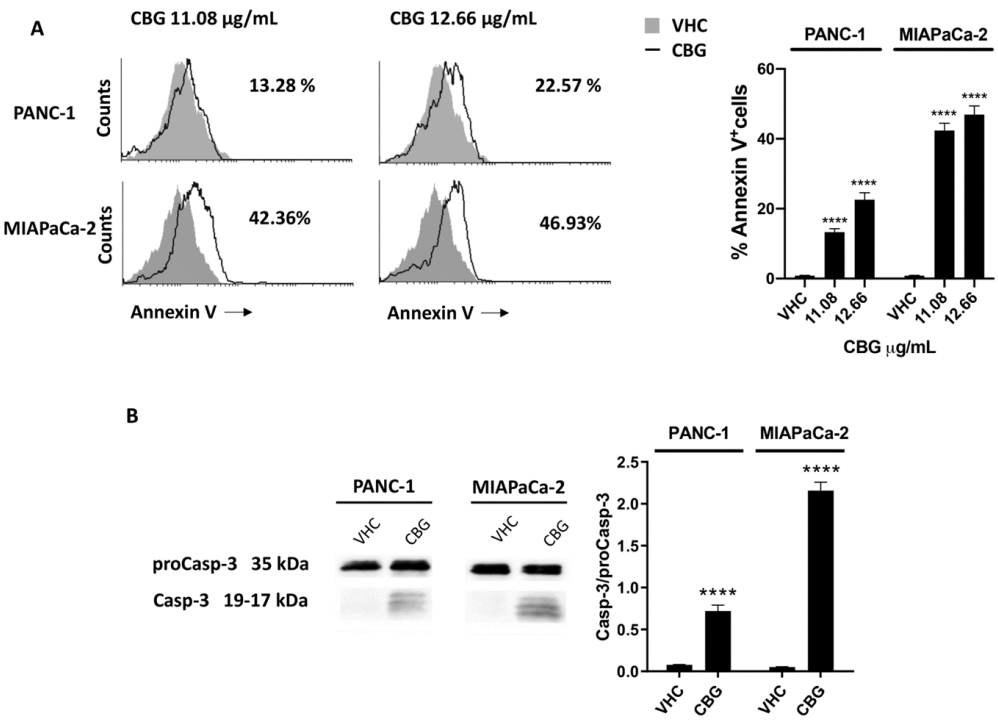


*Figure 28. CBG induced apoptotic cell death in PDAC cell lines. PDAC cell lines were treated with CBG and analysed at 48 h post-treatment. (A) Flow cytometric analysis was performed after Annexin V staining. Images are representative of one of three separate experiments and histograms are the mean ± SD of three separate experiments. The percentage refers to Annexin V+ cells of CBG- respect to VHC-treated cells. (B) The expression of Casp-3 was determined by Western blot. Casp-3 densitometric values were normalized to pro-Casp-3. Image is representative of one of three separate experiments and bars represent the mean ± SD of three separate experiments. ****p<0.0001 treated cells vs VHC.*

4.5 The combination of CBG with PTX or GEM induces higher cytotoxicity compared to administration of single drugs in PDAC cell lines

To support the potential use of CBG as integrative therapy in PDAC, it was evaluated the combination effects of CBG with PTX and GEM, by MTT assay. Both cell lines were treated with CBG 7.91, 12.66 and 15.82 µg/mL in combination with three cytotoxic doses of PTX (1.5, 3 and 6 µg/mL) or GEM (25, 50 and 100 µg/mL) and cell viability was evaluated by MTT assay at 72 h post-treatment. Results showed that the combinations with the two higher doses of CBG resulted in greater cytotoxicity when compared to PTX or GEM alone, in both cell lines (Figure 29A, B). Then drugs interaction was evaluated with SynergyFinder, using the Bliss model. It was obtained

a Bliss synergy score of 4.40 for PTX and CBG, and 10.87 for GEM and CBG, on PANC-1 cell line, indicating an additive and synergistic effect, respectively. In MIAPaCa-2 cell line, the Bliss synergy score was 17.19 for PTX and CBG and 14.05 for GEM and CBG combination, suggesting a synergistic effect (Figure 29C, D).

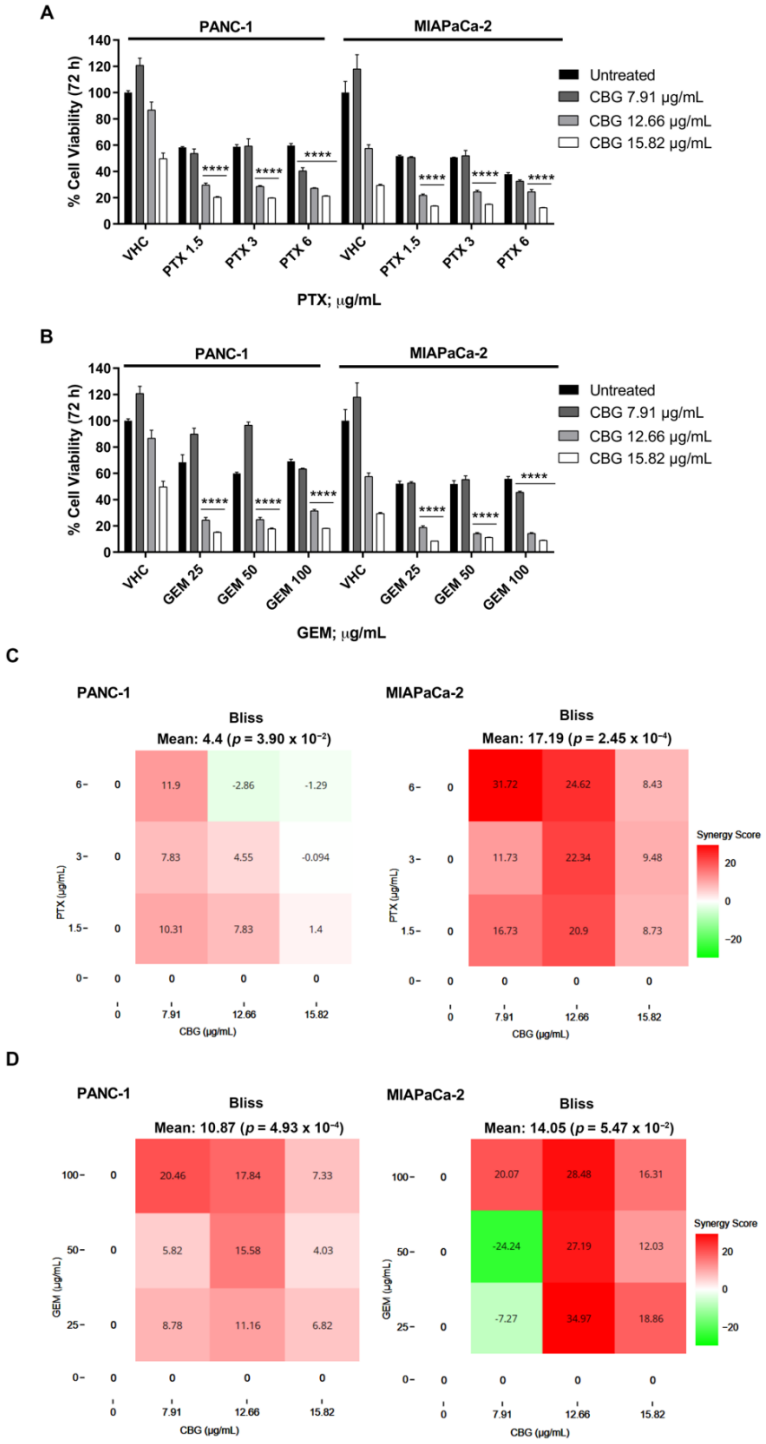


*Figure 29. CBG increased the cytotoxic effect of chemotherapeutic drugs in PDAC cell lines. (A, B) Cell viability was determined in PDAC cell lines by MTT assay. Cells were treated for 72 h with CBG alone or in combination with different doses of GEM and PTX. Data shown are ex-pressed as mean ± SD of three separate experiments. ****p < 0.0001 CBG combined to chemotherapeutic drug vs chemotherapeutic drug alone. (C, D) Drug interaction of CBG with PTX and GEM in PDAC cell lines. Effect of single and combined treatments with CBG (7.91, 12.66, 15.82 µg/mL), PTX (1.5, 3, 6 µg/mL), GEM (25, 50, 100 µg/mL) on cell vi-ability of PDAC cell lines. Drugs interaction was evaluated with SynergyFinder software, using the Bliss model. Bliss synergy score larger than 10 is considered synergistic, from -10 to 10 is considered additive and less than -10 antagonistic.*

4.6 MLT effect in human PDAC cell lines

MLT effect was evaluated in PANC-1 and MIAPaCa-2 cell lines. Results showed that MLT after 72 h of incubation reduced cell viability in a dose-dependent manner, with an IC_{50} of 594 µg/mL on PANC-1 and of 579.5 µg/mL on MIAPaCa-2 cell lines (Figure 30).

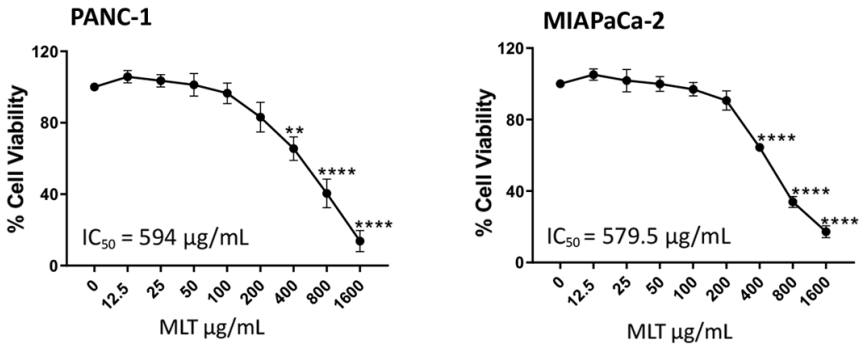


*Figure 30. MLT reduced cells viability in PDAC cell lines. PANC-1 and MIAPaCa-2 cells were treated with MLT and analysed 72h post-treatment. Cell viability was determined by MTT assay. Data shown are expressed as the mean ± SD of three separate experiments. **p < 0.01, ****p < 0.0001 treated cells vs untreated cells.*

Then, it was investigated if MLT interfered with PDAC cells proliferation by exploiting CFSE dye. PANC-1 and MIAPaCa-2 were treated with a sub-IC_{50} dose (400 µg/mL) and a close IC_{50} dose (600 µg/mL) of MLT, and cell proliferation was compared with the non-proliferative control (NPC) sample at 72 h post-treatment. Data showed that MLT significantly decreased PDAC cell

lines proliferation in a dose dependent manner, compared to untreated samples (Figure 31A, C). Moreover, the increased number of PI positive cells showed that the two doses of MLT induced cell death in both cell lines after 72 h of incubation (Figure 31B, D). So, MLT administration was able to reduce PDAC cells proliferation and to induce cell death.

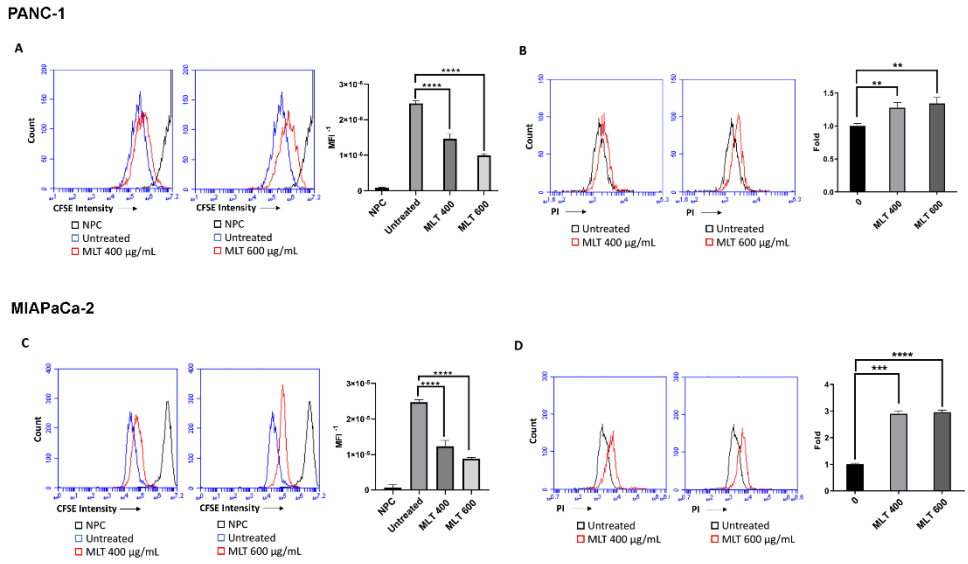


*Figure 31. (A, C) Inhibition of PDAC cells proliferation was evaluated with CFSE dye treated with two doses of MLT. NPC, non-proliferative cells. Histograms are representative of one of three separate experiments. Statistical analysis is calculated using the inverse of CFSE MFI values (MFI^{-1}). (B, D) Flow cytometric analysis was performed by PI staining to analyse MLT induced cell death. MFI values of treated cells were normalized to those of the untreated cells. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.*

4.7 MLT receptors expression in PDAC cell lines

Since MLT can act in a receptor-dependent and -independent manner, it was investigated the protein expression of MLT receptors MEL1A and MEL1B (MTNR1A/B), and the implication of these receptors in MLT-induced inhibition of PDAC cell viability. Data showed that PANC-1 and MIAPaCa-2 expressed MLT receptors, and their expression was slightly higher in MIAPaCa-2 compared to PANC-1 (Figure 32A). Cancer cells were pre-treated for 1 h with 2.9 ug/mL of

luzindole (LUZ), a MLT receptors antagonist. Results showed that the pre-treatment did not influence MLT-induced reduction of PDAC cells viability (Figure 32B), suggesting that MLT effect is receptor-independent in these PANC-1 and MIAPaCa-2 cells.

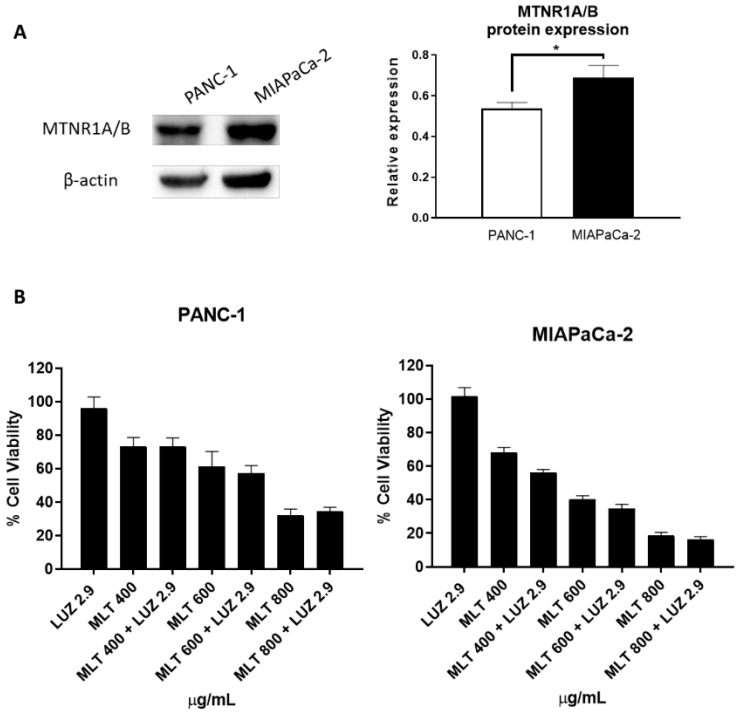


*Figure 32. (A) MTNR1A/B receptors protein expression in PANC-1 and MIAPaCa-2 cell lines. MTNR1A/B densitometric values were normalized to β-Actin used as loading control. Blots are representative of one of three separate experiments. *p < 0.05 (B) MLT effect in combination with LUZ in PDAC cell lines after 72 h post-treatment. Cell viability was determined by MTT assay. Data shown are expressed as the mean ± SD of three separate experiments. Not statistically significant differences for MLT vs MLT + LUZ.*

4.8 MLT combines with CBD induces cytotoxic effect in human PDAC cell lines

The cytotoxic effect of CBD was previously evaluated in PDAC cell lines (52), herein, the effect of the combination between CBD and MLT in PANC-1 and MIAPaCa-2 cell lines was evaluated. Cells were treated with the combination of two doses of MLT (200 and 400 μg/mL) and two doses of CBD (2 and 4 μg/mL), and analysed at 72 h post-treatments. Results showed that the effect of the two MLT doses was increased by CBD 4 μg/mL, compared to MLT or CBD (4 μg/mL) alone, as observed in the reduction of cell viability in both cell lines (Figure 33). Moreover, MLT and

CBD combinations resulted in a statistically significant reduction of cell viability compared to the untreated cell lines (Figure 33).

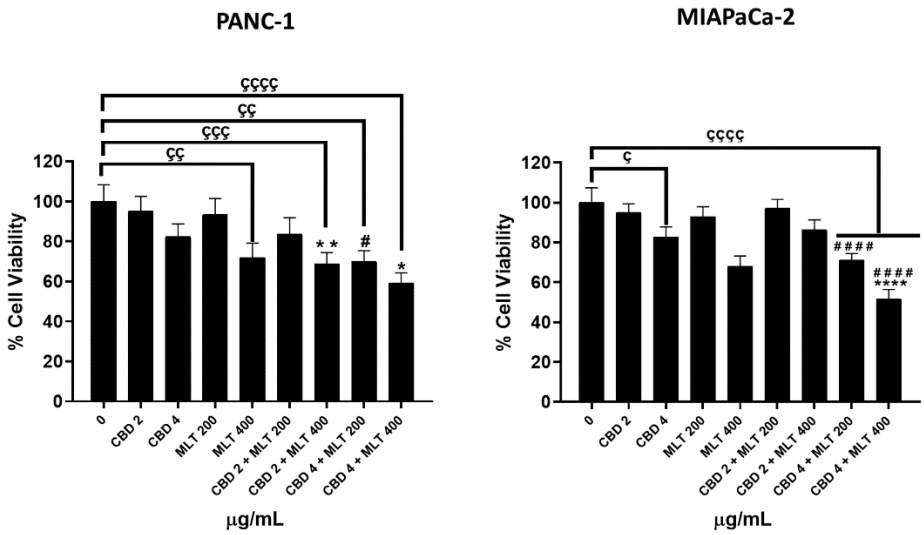


Figure 33. MLT + CBD reduced PANC-1 and MIAPaCa-2 cells viability. Cell viability was determined by MTT assay. Data shown are expressed as the mean ± SD of three separate experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ CBD + MLT vs MLT; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ CBD + MLT vs CBD.

Then, it was evaluated the effect of CBD + MLT combination on cell proliferation and death. PDAC cell lines were treated with the two effective combinations determined by MTT assay (CBD 4 µg/mL + MLT 200 µg/mL; CBD 4 µg/mL + MLT 400 µg/mL) and the tests were performed after 72 h post-treatment. In both cell lines, it was observed a significant proliferation reduction with the two combinations compared to the untreated samples. No significative differences were observed from the comparison between the two combinations (Figure 34A, C). A significant increase of PI positive cells induced by the combinations compared to untreated sample evidenced an induction of cell death in both cell lines. In line with previous results, the difference between the two combinations was not significant (Figure 34B, D).

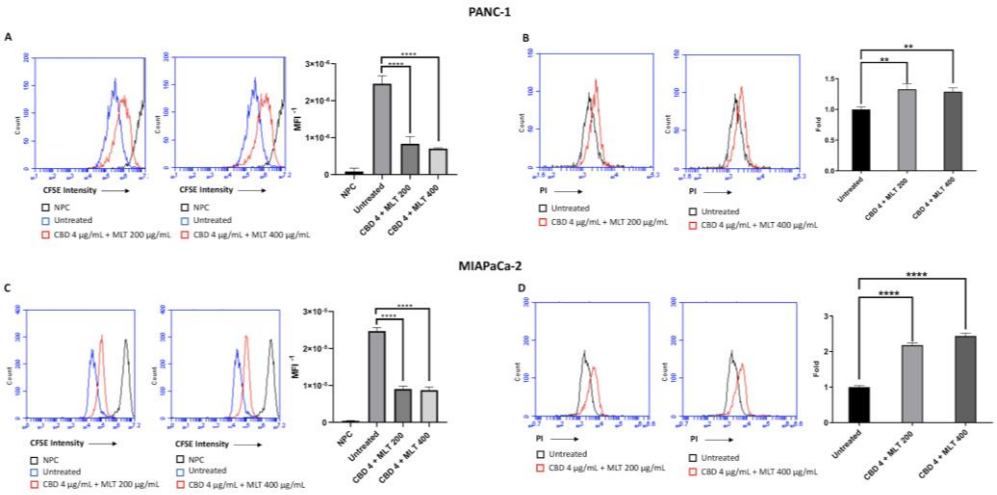


*Figure 34. Effect of CBD + MLT combination on PDAC cell lines. (A, C) Inhibition of PDAC cells proliferation was evaluated with CFSE dye after 72 h of incubation with two combinations of CBD + MLT. NPC, non-proliferative cells. Histograms are representative of one of three separate experiments. Statistical analysis is calculated using CFSE MFI^{-1}. (B, D) CBD + MLT induced cell death was evaluated by PI staining and flow cytometric analysis. MFI values of treated cells were normalized to those of the untreated cells. **$p < 0.01$, ****$p < 0.0001$.*

4.9 O_2/O_3 increases MLT and CBD efficacy

The effect of MLT + CBD with the addition of O_2/O_3 was evaluated on PANC-1 and MIAPaCa-2 cells. Cells were treated with four combinations of CBD (2 and 4 µg/mL) and MLT (200 and 400 µg/mL) alone or with O_2/O_3. Results showed that CBD + MLT effect was significantly increased by the addition of O_2/O_3 in both cell lines (Figure 35) at 72h post-treatments.

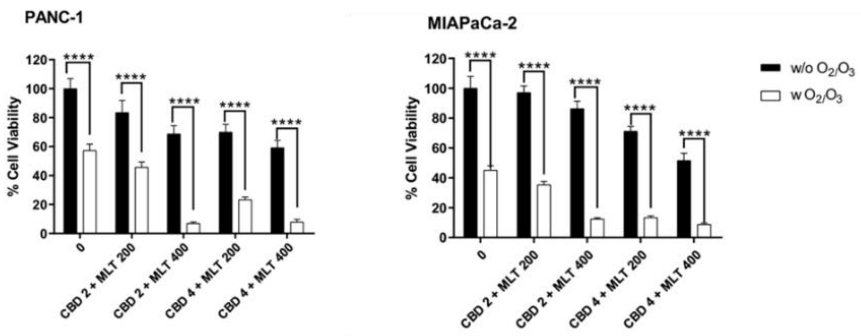


*Figure 35. MLT + CBD + O_2/O_3 treatment in PDAC cell lines. Cell viability was determined by MTT assay after 72 h post treatment. Data shown are expressed as the mean ± SD of three separate experiments. ****p < 0.0001.*

Then, a significant shift of PI signal observed by cytofluorimetric analysis confirmed that O_2/O_3 improved the cell death respect to CBD + MLT (Figure 36).

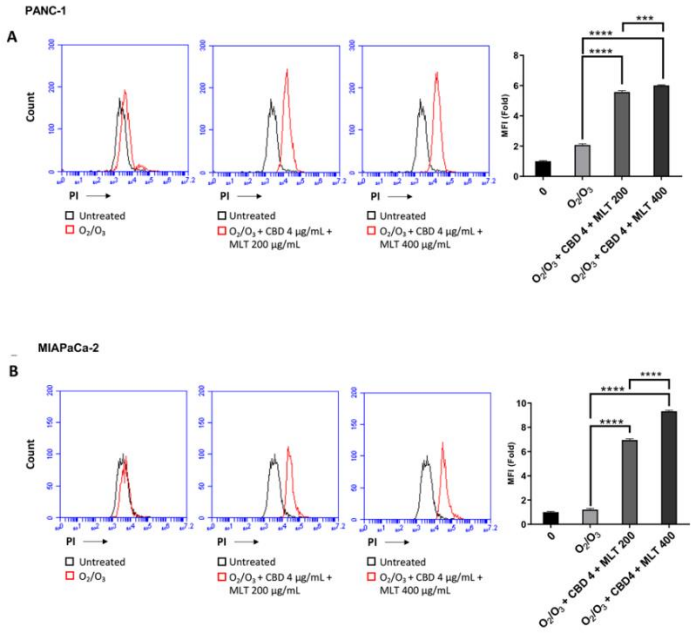


*Figure 36. MLT + CBD + O_2/O_3 induced PANC-1 and MIAPaCa-2 cell death. To analyse cell death flow cytometric analysis was performed after PI staining. The mean fluorescence intensity (MFI) values of treated cells were normalized to those of the untreated cells. Histograms are representative of one of three separate experiments. ****p < 0.0001.*

4.10 CBD + MLT + O_2/O_3 increases the cytotoxic effect of GEM in human PDAC cell lines

Lastly, the effect of the combination of MLT + CBD + O_2/O_3 with GEM was analysed, the main chemotherapeutic drug used in PDAC therapy. GEM (25 µg/mL), was combined CBD (2 and 4 µg/mL), MLT (200 and 400 µg/mL) and O_2/O_3. Results showed that the efficacy of GEM was implemented by the addition of all combinations and CBD 2 µg/mL + MLT 200 µg/mL + O_2/O_3 effect was increased by GEM addition (Figure 37).

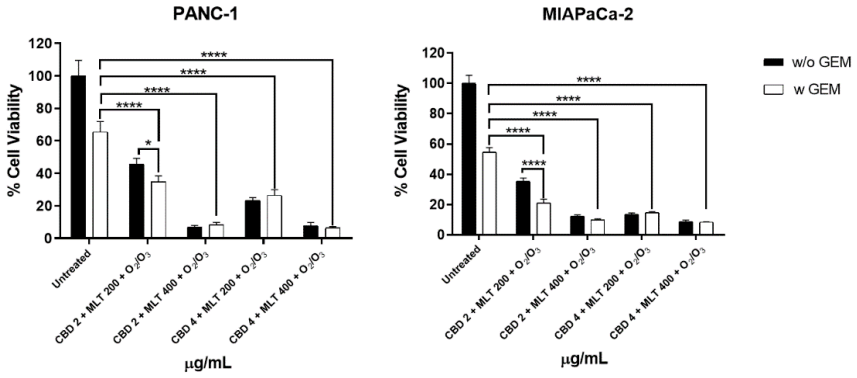


*Figure 37. MLT + CBD + O_2/O_3 + GEM effect on PANC-1 and MIAPaCa-2 cell lines after 72 h post treatment. Cell viability was determined by MTT assay. Data shown are expressed as the mean ± SD of three separate experiments. *p < 0.05, ****p < 0.0001.*

4.11 Ras pathway modulation in PANC-1 cells

To better investigate the mechanism of action of MLT + CBD + O_2/O_3 + GEM treatment, at molecular levels, the modulation in phosphorylated BRAF (pBRAF), CRAF (pCRAF), and MEK1 (pMEK1), and the total Ras protein by MILLIPLEX® RAS-RAF Oncoprotein Magnetic Bead Panel 6-plex was investigated in PDAC cell lines. PDAC cells were treated with CBD 2 µg/mL, MLT 200 µg/mL and O_2/O_3. Results showed that in PANC-1 all the treatments compared to the untreated cells, induced a negative modulation of total Ras protein, excepted GEM alone, (Figure 38A) and reduced pBRAF, excepted in O_2/O_3 treatment (Figure 38B). pCRAF was reduced by all treatments respect to untreated cells, while pMEK1 was not statistically modulated (Figure 38C, D). Similar results were obtained in MIAPaCa-2 line (result not showed). In addition, the results evidenced that high statistically significant modulation, compared to GEM, was observed in total Ras reduction and pBRAF in all the treatments (Figure 38A, B).

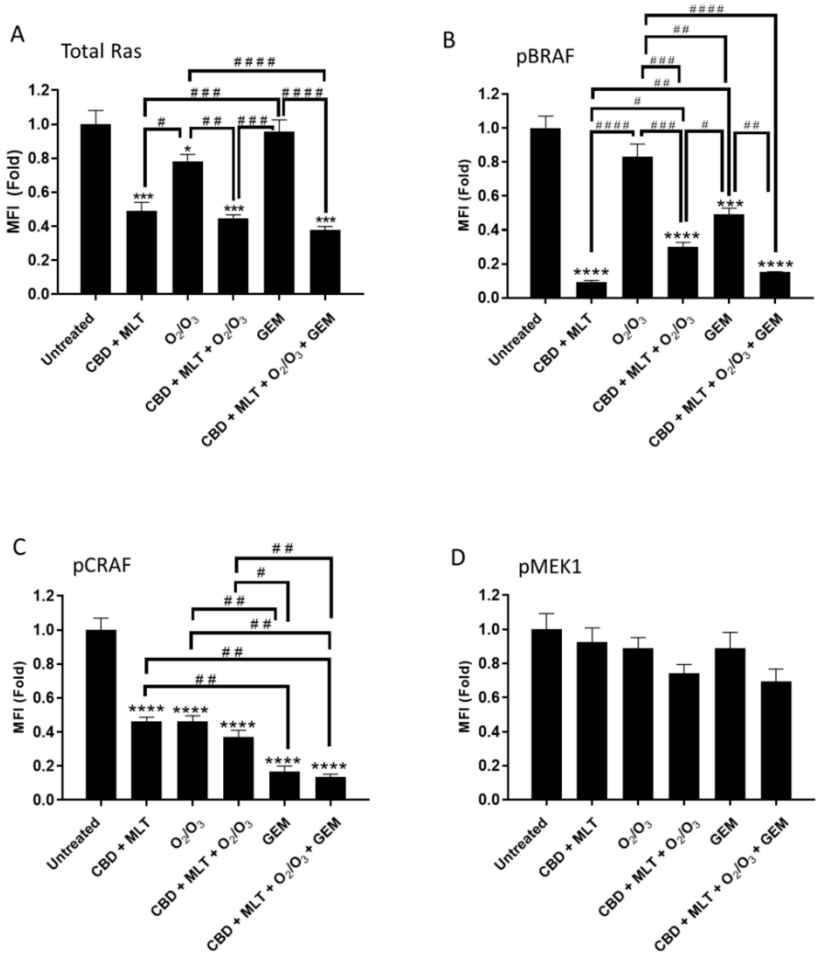
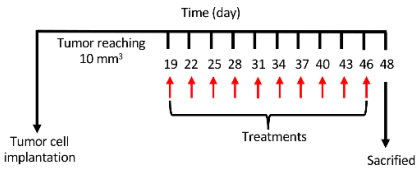


*Figure 38. Treatments modulated total Ras protein (A) and pBRAF (B), pCRAF (C) and pMEK1 (D) in PANC-1 cells. PANC-1 cells were treated with the different treatments for 48 h and the expression of total Ras, pBRAF, pCRAF, pMEK1was evaluated by Milliplex multiplex assay and MFI was measured with the Luminex ® 200 system. Data shown are expressed as the mean ± SD of three separate experiments. *p <0.05, ***p<0.001, ****p<0.0001 treated cells vs untreated; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#}p< 0.0001$.*

4.12 Effect of CBD + MLT and O_2/O_3 in mouse model of PDAC

To further study the effectiveness of the combination on PDAC, in vivo experiments were performed. Mice were subdivided in six groups: vehicle (Vhc) (Group 1), MLT + CBD (Group 2), O_2/O_3 (Group 3), MLT + CBD + O_2/O_3 (Group 4), GEM (Group 5), MLT + CBD + O_2/O_3 + GEM (Group 6) (Figure 39A, B).

A



B

Groups	Number of animals	Treatment	Dose
1	5	Vhc (saline)	/
2	5	MLT + CBD	400 mg/kg MLT 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 10 mg/kg CBD 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 10 mg/kg CBD 2.5 ml/kg O_2/O_3 50 mg/kg GEM

Figure 39. (A) Experimental design used for the treatment of mice inoculated with PANC-1 cells. When tumor size reached a volume of approximately 10 mm3, animals were treated i.p. every 3 days, for a total of 10 treatments. (B) Treatments of each animals group and dose received.

During the treatment, ultrasound (US) and photoacoustic (PA) imaging was performed to evaluate the engraftment and the development of tumor mass, once a week. At the end-point mice were sacrificed and macroscopic necroscopy was performed. All the tumors were explanted, weighted and photographed (Figure 40A).

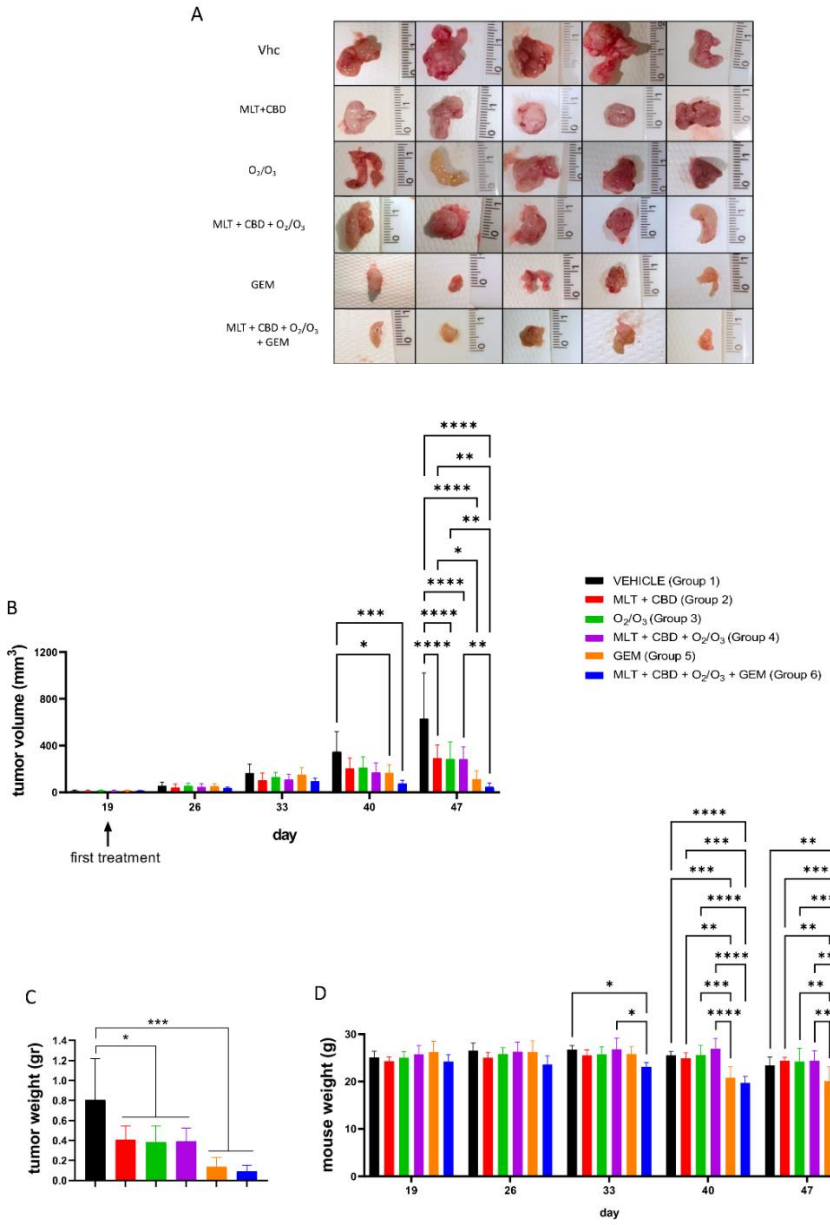


*Figure 40. Effect of treatments on PDAC mouse model. (A) Representative photographs of tumors at the end of treatments after the explantation. (B) Tumor volumes of PANC-1 inoculated mice, treated as described in Figure 6. Volumes were measured by US and PA imaging once a week. (C) Tumor weight at the end of treatments. (D) Body weight of the mice during the treatments. The values are expressed as the mean ± SD of 5 animals in each group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001*

Data showed no significant tumor volume variation until day 33 of treatment (Figure 40B). At day 40, it was observed a statistically significant reduction of tumor volume in the group 5 compared with the group 1, which was more noticeable in the group 6 (Figure 40B). At the end point (day 47), a significant reduction of tumor volumes in all the treated groups compared to the Vhc group was observed (Figure 40B), both in the groups with and without GEM. Moreover, also if the reduction was non statistically significant, comparing the groups 5 and 6, it can be noted that the addition of CBD + MLT + O_2/O_3 slightly improved GEM reduction of tumor volume. Similar results were obtained analysing the explanted tumor mass, in which tumor weight reduction was statistically significant in all treated groups compared to the Vhc group, in particular in groups 5 and 6 (Figure 40A, C). A strong reduction of mouse weight was observed in the groups 5 and 6 already after the day 33, while no decrease was observed in groups 2, 3 and 4, neither at the end point, suggesting that this effect was associated with GEM presence in the treatments, while the other treatments did not induce animal toxicity (Figure 40D). Summarizing, data evidenced as all the treatments reduced tumor growth, as the groups 2, 3 and 4 had a tumor mass and volume reduction of about 50% compared to the Vhc without showed toxicity to the groups 5 and 6, and as GEM effect on tumor volume, was improved by CBD + MLT + O_2/O_3 addition, after three weeks of treatment (Figure 40). Moreover, when at the end-point mice were sacrificed and macroscopic necroscopy was performed, also liver, brain and heart of the animals were explanted, weighted. Changes in liver, heart and brain weight resulted not significative (Figure 41).

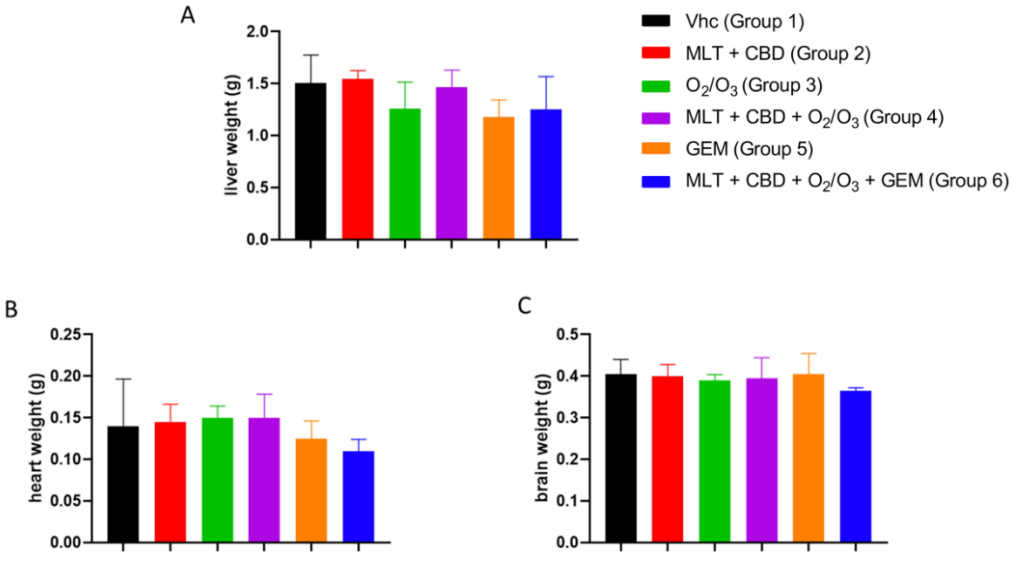


Figure 41. Effect of treatments on organ weight in PDAC mice model. (A, B, C) Liver (5 animals of each group), heart and brain (2 animals of each group) weight. Not statistical differences were observed.

5. Discussion

Despite significant treatment efforts, most of PDAC cases, are still incurable (98, 107). PDAC poor prognosis is mainly due to an aggressive behavior and, tumor heterogeneity and metabolic changes. This has prompted the investigation of the mechanisms underlying chemoresistance and new effective therapeutic approaches by selectively targeting genetic pathways (98, 107). The standard first-line treatment for PDAC is GEM administration (107). The search of integrative therapies that can ameliorate chemotherapy side effects or improve their effect are constantly under study (108). Recent research evidenced that the efficacy of GEM or others chemotherapeutic drugs can be improved by combining them with evidence-based integrative compounds (52, 85, 109, 110) *Cannabis sativa L*. contains more than 100 PyCBs and, for some of them, several biological properties are well known. Beside a direct anticancer effect, mainly demonstrated for CBD, in *in vitro* and *in vivo* experiments, PyCBs were suggested to ameliorate numerous important side effects induced by chemotherapeutics (111). In this study, CBG showed a dose-dependent cytotoxicity in PDAC cells, as also evidenced in other human preclinical cancer models as in glioblastoma multiforme (GBM) (59). Further, THC, CBD and synthetic cannabinoids reduced PANC-1 and MIAPaCa-2 cell growth and viability, as reported in several studies and, in line with our evidence, MIAPaCa-2 was more sensitive than PANC-1 to treatments with PyCBs (49, 52). In several pathologies, including cancer such as in glioma cells, cannabinoids have been demonstrated to activate autophagy and apoptotic cell death, through the interaction between apoptosis and autophagy signaling mechanisms (40, 112). Several pathways are mediated through the multiprotein complex involved in EGF/EGFR, including RAS and mTOR. These pathways suppress autophagy and promote proliferation and resistance to chemotherapy (113). The simultaneous inhibition of EGFR and RAS/mTOR was demonstrated to provide a synergistic antitumor effect in various human cancers, indeed the PI3K/AKT/mTOR axis, a frequently dysregulated pathway in PDAC, is responsible for the control of cell proliferation and resistance (114), and these pathways can be inhibited by cannabinoids (29, 40). The present data evidenced as CBG induced autophagy by reducing the AKT-mTOR pathways with consequent LC-3 conversion and autophagic vesicle formation, as previously observed with others PyCBs. In particular, increased LC3-II conversion was mainly in the more sensitive MIAPaCa-2. For example, THC inhibits AKT/mTOR, reducing the proliferation of glioma cells (112). A similar finding was observed in hepatocarcinoma cells, where THC inhibits AKT/mTORC1 through ER stress-dependent activation of AMPK (115). Herein, CBG reduced mTOR protein expression and, in line with this, some studies demonstrated that CBD inhibits mTOR signaling pathway in breast cancer and in human glioma (29, 116). Autophagy has a double function: to induce cancer resistance to chemotherapy and protect cancer cells from death,

or to be correlated with cancer cell death. Herein, it was evidenced that CBG induced autophagy and apoptosis in both cell lines. Cannabinoids are known to be involved in reducing cancer cell growth, by the EGFR-RAS-RAF-MAPK pathway. In pancreatic cancer, mutated KRAS upregulates endogenous EGFR expression, and hyperactivation results in a transformation from acinar to ductal metaplasia (117). Herein, CBG suppresses EGFR expression in PANC-1 and MIAPaCa-2. Up to now, there are no data about the ability of CBG to reduce the EGFR expression; however, some studies demonstrated that CBD and THC can reduce EGFR expression in A549, H460 and H1792 cells and suppress EGF/EGFR signaling pathways in breast cancer (25). Moreover, data evidenced as CBG was able to reduce down-stream RAS signaling. Herein, Annexin V positive cells and Caspase-3 cleavage confirmed CBG induction of apoptosis. In line with our results, CBG induced Caspase-3/-7 dependent apoptosis in glioblastoma and Caco-2 cells (59, 118). Moreover, in PANC-1 and MIAPaCa-2 cells also CBD induced apoptosis and Caspase-3 activation (52). Lastly, many studies demonstrated that cannabinoids could increase the chemotherapeutic drugs efficacy, reducing tumor growth and overcoming drugs resistance (119). Herein, the combination of CBG with GEM or PTX, increased the cytotoxicity compared to the administration of the drug alone, also showing a synergistic effect for some combinations. In GBM cells, CBD or CBG plus TMZ did not show additive effect, but in cholangiocarcinoma cells, CBG synergized with GEM and cisplatin (55, 59). Moreover, in PDAC CBD showed the ability to increase GEM and PTX efficacy in *in vitro* tests and KPC mice treated with CBD and GEM showed a survival three times longer than mice treated with GEM (49, 52). Overall, data evidenced the ability of CBG to induce autophagy, reduce EGFR/AKT/RAS pathways, promote apoptotic cell death, and increase the sensitivity of PDAC cell lines to chemotherapeutic drugs. Further study on CBG will be necessary to better understand the role of this compounds in PDAC progression, and *in vivo* studies will be useful to deeply investigate its anticancer effects.

MLT reduced cancer cell viability and induced cell death on PANC-1 and MIAPaCa-2 cells as obtained in SW-1990 and in other pancreatic cancer cell lines (AsPc-1 and Panc-28) with the induction of pro apoptotic and pro necrotic effects (84, 85). In PDAC, MLT synergized with sorafenib to suppress PDAC growth both *in vitro* and in mice, increasing apoptosis of PDAC cells by blockade of PDGFR-β/STAT3 signaling pathway (109). MLT inhibited NF-κB by suppressing IκBα phosphorylation and consequently the expression of NF-κB target genes in MiaPaCa-2, AsPc-1, Panc-28 cells and enhances GEM cytotoxicity both *in vitro* than *in vivo* (85). Herein, the aim of this research was to evaluate, for the first time, a potential antitumoral role of a combination therapy composed by MLT, CBD and O_2/O_3 in preclinical models of human PDAC. Previous data evidenced as CBD and O_2/O_3, alone or in combination, were able to induce cell death and to enhance the efficacy of GEM (52). *In vitro* results evidenced that CBD plus MLT improved the

cytotoxicity compared to CBD and MLT alone, and this effect was significantly potentiated by O_2/O_3 addition. Moving on *in vivo* models, the efficacy was confirmed. Results showed a significant tumoral mass decreased both in the animal groups administrated with MLT + CBD, with O_2/O_3 alone, and with the triple combination even though without significant differences, contrary to was observed *in vitro*. Anyway, already after three weeks of treatment, the triple combination with GEM was found to increase GEM efficacy as showed from the higher statistical significance observed in the group 6 compared to the Vhc group. More than 90% of PDAC cases harbour activating RAS (mainly KRAS) and Ras pathways (120). So, seemed promising to inhibit RAS and/or downstream target in the MAPK pathway such as BRAF, CRAF or MEK1/2, for a better response to chemotherapy or to overcome resistance mechanisms. The effect of MLT on RAS pathways was never proved, while for CBD and O_2/O_3 was previously evidenced as mainly CBD was able to reduce KRAS at transcriptional levels as well as some downstream signals (52). In this experimental work, it was demonstrated that CBD, MLT and O_2/O_3, with different efficacy, were able to reduce total RAS, pBRAF and pCRAF. The effect of the quadruple combination was observed both on pBRAF and pCRAF reduction, and it could be related with MLT + CBD effect on pBRAF and with GEM effect on pCRAF. This pathway could be one of the molecular mechanisms that induces *in vitro* and *in vivo* reduction of PDAC growth. In conclusion, data presented in this work, evidenced as MLT + CBD, O_2/O_3 or their combination exerted anticancer activity, halving tumor volume without inducing toxicity in the animals as differently observed in groups treated with GEM. Then, the quadruple combination showed a more significant tumor mass reduction after three weeks of treatment and this effect was observed at the end point too. Finally, data suggested that the combination therapy could be useful in improving GEM efficacy given that its effect was not hampered by the MLT, CBD and O_2/O_3 addition. Definitely, more studies are needed mainly to confirm the response obtained in *in vivo* model. It would also be useful to assess the possibility to use these integrative compounds with lower GEM doses aiming to obtain the same anticancer effect reducing chemotherapeutics toxicity.

6. Conclusions

Data revealed the potentiality of these different molecules as integrative therapy. They exerted a direct anticancer effect in human PDAC preclinical model. Moreover, they could potentiate and support chemotherapeutic drugs. In particular, CBG was able to induce autophagy followed by apoptosis in PDAC cell lines and to modulate EGFR/Akt/mTOR pathway. MLT, CBD and O_2/O_3 modulated Ras pathway. Interesting results obtained in *in vivo* model suggest that the use of the combination could inhibit tumoral mass growth and could be a support to chemotherapy.

7. Bibliography

1. Zimmermann-Klemd AM, Reinhardt JK, Winker M, Gründemann C. Phytotherapy in Integrative Oncology-An Update of Promising Treatment Options. Molecules. 2022 May 17;27(10):3209. doi: 10.3390/molecules27103209.
2. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023 Jan;73(1):17-48. doi: 10.3322/caac.21763.
3. Witt CM, Balneaves LG, Cardoso MJ, Cohen L, Greenlee H, Johnstone P, Kücük Ö, Mailman J, Mao JJ. A Comprehensive Definition for Integrative Oncology. J Natl Cancer Inst Monogr. 2017 Nov 1;2017(52). doi: 10.1093/jncimonographs/lgx012.
4. Jentzsch V, Davis JAA, Djamgoz MBA. Pancreatic Cancer (PDAC): Introduction of Evidence-Based Complementary Measures into Integrative Clinical Management. Cancers (Basel). 2020 Oct 23;12(11):3096. doi: 10.3390/cancers12113096.
5. Halbrook CJ, Lyssiotis CA, Pasca di Magliano M, Maitra A. Pancreatic cancer: Advances and challenges. Cell. 2023 Apr 13;186(8):1729-1754. doi: 10.1016/j.cell.2023.02.014.
6. Kim A, Ha J, Kim J, Cho Y, Ahn J, Cheon C, Kim SH, Ko SG, Kim B. Natural Products for Pancreatic Cancer Treatment: From Traditional Medicine to Modern Drug Discovery. Nutrients. 2021 Oct 26;13(11):3801. doi: 10.3390/nu13113801.
7. Radwan MM, Chandra S, Gul S, ElSohly MA. Cannabinoids, Phenolics, Terpenes and Alkaloids of Cannabis. Molecules. 2021 May 8;26(9):2774. doi: 10.3390/molecules26092774.
8. Walsh KB, McKinney AE, Holmes AE. Minor Cannabinoids: Biosynthesis, Molecular Pharmacology and Potential Therapeutic Uses. Front Pharmacol. 2021 Nov 29;12:777804. doi: 10.3389/fphar.2021.777804.
9. Solymosi K, Köfalvi A. Cannabis: A Treasure Trove or Pandora's Box? Mini Rev Med Chem. 2017;17(13):1223-1291. doi: 10.2174/1389557516666161004162133.
10. Moreno E, Cavic M, Krivokuca A, Casadó V, Canela E. The Endocannabinoid System as a Target in Cancer Diseases: Are We There Yet? Front Pharmacol. 2019 Apr 5;10:339. doi: 10.3389/fphar.2019.00339.
11. Mangal N, Erridge S, Habib N, Sadanandam A, Reebye V, Sodergren MH. Cannabinoids in the landscape of cancer. J Cancer Res Clin Oncol. 2021 Sep;147(9):2507-2534. doi: 10.1007/s00432-021-03710-7.
12. Di Marzo V, Piscitelli F. The Endocannabinoid System and its Modulation by PyCBs. Neurotherapeutics. 2015 Oct;12(4):692-8. doi: 10.1007/s13311-015-0374-6.
13. Di Marzo V. New approaches and challenges to targeting the endocannabinoid system. Nat Rev Drug Discov. 2018 Sep;17(9):623-639. doi: 10.1038/nrd.2018.115.

14. An D, Peigneur S, Hendrickx LA, Tytgat J. Targeting Cannabinoid Receptors: Current Status and Prospects of Natural Products. Int J Mol Sci. 2020 Jul 17;21(14):5064. doi: 10.3390/ijms21145064.
15. Muller C, Morales P, Reggio PH. Cannabinoid Ligands Targeting TRP Channels. Front Mol Neurosci. 2019 Jan 15;11:487. doi: 10.3389/fnmol.2018.00487.
16. De Petrocellis L, Nabissi M, Santoni G, Ligresti A. Actions and Regulation of Ionotropic Cannabinoid Receptors. Adv Pharmacol. 2017;80:249-289. doi: 10.1016/bs.apha.2017.04.001.
17. Castillo-Arellano J, Canseco-Alba A, Cutler SJ, León F. The Polypharmacological Effects of Cannabidiol. Molecules. 2023 Apr 6;28(7):3271. doi: 10.3390/molecules28073271.
18. Kis B, Ifrim FC, Buda V, Avram S, Pavel IZ, Antal D, Paunescu V, Dehelean CA, Ardelean F, Diaconeasa Z, Soica C, Danciu C. Cannabidiol-from Plant to Human Body: A Promising Bioactive Molecule with Multi-Target Effects in Cancer. Int J Mol Sci. 2019 Nov 25;20(23):5905. doi: 10.3390/ijms20235905.
19. Fraguas-Sánchez AI, Martín-Sabroso C, Torres-Suárez AI. Insights into the effects of the endocannabinoid system in cancer: a review. Br J Pharmacol. 2018 Jul;175(13):2566-2580. doi: 10.1111/bph.14331.
20. Britch SC, Babalonis S, Walsh SL. Cannabidiol: pharmacology and therapeutic targets. Psychopharmacology (Berl). 2021 Jan;238(1):9-28. doi: 10.1007/s00213-020-05712-8.
21. Heider CG, Itenberg SA, Rao J, Ma H, Wu X. Mechanisms of Cannabidiol (CBD) in Cancer Treatment: A Review. Biology (Basel). 2022 May 26;11(6):817. doi: 10.3390/biology11060817.
22. Hamad H, Olsen BB. Cannabidiol Induces Cell Death in Human Lung Cancer Cells and Cancer Stem Cells. Pharmaceuticals (Basel). 2021 Nov 17;14(11):1169. doi: 10.3390/ph14111169.
23. Misri S, Kaul K, Mishra S, Charan M, Verma AK, Barr MP, Ahirwar DK, Ganju RK. Cannabidiol Inhibits Tumorigenesis in Cisplatin-Resistant Non-Small Cell Lung Cancer via TRPV2. Cancers (Basel). 2022 Feb 24;14(5):1181. doi: 10.3390/cancers14051181.
24. Milián L, Monleón-Guinot I, Sancho-Tello M, Galbis JM, Cremades A, Almenar-Ordaz M, Peñaroja-Martinez J, Farras R, Martín de Llano JJ, Carda C, Mata M. In Vitro Effect of Δ9-Tetrahydrocannabinol and Cannabidiol on Cancer-Associated Fibroblasts Isolated from Lung Cancer. Int J Mol Sci. 2022 Jun 17;23(12):6766. doi: 10.3390/ijms23126766.
25. Elbaz M, Nasser MW, Ravi J, Wani NA, Ahirwar DK, Zhao H, Oghumu S, Satoskar AR, Shilo K, Carson WE 3rd, Ganju RK. Modulation of the tumor microenvironment and

inhibition of EGF/EGFR pathway: novel anti-tumor mechanisms of Cannabidiol in breast cancer. Mol Oncol. 2015 Apr;9(4):906-19. doi: 10.1016/j.molonc.2014.12.010.

26. Patel N, Kommineni N, Surapaneni SK, Kalvala A, Yaun X, Gebeyehu A, Arthur P, Duke LC, York SB, Bagde A, Meckes DG Jr, Singh M. Cannabidiol loaded extracellular vesicles sensitize triple-negative breast cancer to doxorubicin in both in-vitro and in vivo models. Int J Pharm. 2021 Sep 25;607:120943. doi: 10.1016/j.ijpharm.2021.120943.
27. Nabissi M, Morelli MB, Santoni M, Santoni G. Triggering of the TRPV2 channel by cannabidiol sensitizes glioblastoma cells to cytotoxic chemotherapeutic agents. Carcinogenesis. 2013 Jan;34(1):48-57. doi: 10.1093/carcin/bgs328.
28. Nabissi M, Morelli MB, Amantini C, Liberati S, Santoni M, Ricci-Vitiani L, Pallini R, Santoni G. Cannabidiol stimulates Aml-1a-dependent glial differentiation and inhibits glioma stem-like cells proliferation by inducing autophagy in a TRPV2-dependent manner. Int J Cancer. 2015 Oct 15;137(8):1855-69. doi: 10.1002/ijc.29573.
29. Huang T, Xu T, Wang Y, Zhou Y, Yu D, Wang Z, He L, Chen Z, Zhang Y, Davidson D, Dai Y, Hang C, Liu X, Yan C. Cannabidiol inhibits human glioma by induction of lethal mitophagy through activating TRPV4. Autophagy. 2021 Nov;17(11):3592-3606. doi: 10.1080/15548627.2021.1885203.
30. Abrams DI, Velasco G, Twelves C, Ganju RK, Bar-Sela G. Cancer Treatment: Preclinical & Clinical. J Natl Cancer Inst Monogr. 2021 Nov 28;2021(58):107-113. doi: 10.1093/jncimonographs/lgab010.
31. Kosgodage US, Mould R, Henley AB, Nunn AV, Guy GW, Thomas EL, Inal JM, Bell JD, Lange S. Cannabidiol (CBD) Is a Novel Inhibitor for Exosome and Microvesicle (EMV) Release in Cancer. Front Pharmacol. 2018 Aug 13;9:889. doi: 10.3389/fphar.2018.00889.
32. Mahmoud AM, Kostrzewa M, Marolda V, Cerasuolo M, Maccarinelli F, Coltrini D, Rezzola S, Giacomini A, Mollica MP, Motta A, Paris D, Zorzano A, Di Marzo V, Ronca R, Ligresti A. Cannabidiol alters mitochondrial bioenergetics via VDAC1 and triggers cell death in hormone-refractory prostate cancer. Pharmacol Res. 2023 Mar;189:106683. doi: 10.1016/j.phrs.2023.106683.
33. Kargl J, Andersen L, Hasenöhrl C, Feuersinger D, Stančić A, Fauland A, Magnes C, El-Heliebi A, Lax S, Uranitsch S, Haybaeck J, Heinemann A, Schicho R. GPR55 promotes migration and adhesion of colon cancer cells indicating a role in metastasis. Br J Pharmacol. 2016 Jan;173(1):142-54. doi: 10.1111/bph.13345.
34. Jeong S, Kim BG, Kim DY, Kim BR, Kim JL, Park SH, Na YJ, Jo MJ, Yun HK, Jeong YA, Kim HJ, Lee SI, Kim HD, Kim DH, Oh SC, Lee DH. Cannabidiol Overcomes Oxaliplatin Resistance by Enhancing NOS3- and SOD2-Induced Autophagy in Human

Colorectal Cancer Cells. Cancers (Basel). 2019 Jun 5;11(6):781. doi: 10.3390/cancers11060781.

35. Nabissi M, Morelli MB, Offidani M, Amantini C, Gentili S, Soriani A, Cardinali C, Leoni P, Santoni G. Cannabinoids synergize with carfilzomib, reducing multiple myeloma cells viability and migration. Oncotarget. 2016 Nov 22;7(47):77543-77557. doi: 10.18632/oncotarget.12721.
36. Morelli MB, Offidani M, Alesiani F, Discepoli G, Liberati S, Olivieri A, Santoni M, Santoni G, Leoni P, Nabissi M. The effects of cannabidiol and its synergism with bortezomib in multiple myeloma cell lines. A role for transient receptor potential vanilloid type-2. Int J Cancer. 2014 Jun 1;134(11):2534-46. doi: 10.1002/ijc.28591.
37. Olivas-Aguirre M, Torres-López L, Valle-Reyes JS, Hernández-Cruz A, Pottosin I, Dobrovinskaya O. Cannabidiol directly targets mitochondria and disturbs calcium homeostasis in acute lymphoblastic leukemia. Cell Death Dis. 2019 Oct 14;10(10):779. doi: 10.1038/s41419-019-2024-0.
38. Maggi F, Morelli MB, Tomassoni D, Marinelli O, Aguzzi C, Zeppa L, Nabissi M, Santoni G, Amantini C. The effects of cannabidiol via TRPV2 channel in chronic myeloid leukemia cells and its combination with imatinib. Cancer Sci. 2022 Apr;113(4):1235-1249. doi: 10.1111/cas.15257.
39. Fonseca BM, Correia-da-Silva G, Teixeira NA. Cannabinoid-induced cell death in endometrial cancer cells: involvement of TRPV1 receptors in apoptosis. J Physiol Biochem. 2018 May;74(2):261-272. doi: 10.1007/s13105-018-0611-7.
40. Marinelli O, Morelli MB, Annibali D, Aguzzi C, Zeppa L, Tuyaerts S, Amantini C, Amant F, Ferretti B, Maggi F, Santoni G, Nabissi M. The Effects of Cannabidiol and Prognostic Role of TRPV2 in Human Endometrial Cancer. Int J Mol Sci. 2020 Jul 29;21(15):5409. doi: 10.3390/ijms21155409.
41. Fraguas-Sánchez AI, Torres-Suárez AI, Cohen M, Delie F, Bastida-Ruiz D, Yart L, Martin-Sabroso C, Fernández-Carballido A. PLGA Nanoparticles for the Intraperitoneal Administration of CBD in the Treatment of Ovarian Cancer: In Vitro and In Ovo Assessment. Pharmaceutics. 2020 May 9;12(5):439. doi: 10.3390/pharmaceutics12050439.
42. Michalski CW, Oti FE, Erkan M, Sauliunaite D, Bergmann F, Pacher P, Batkai S, Müller MW, Giese NA, Friess H, Kleeff J. Cannabinoids in pancreatic cancer: correlation with survival and pain. Int J Cancer. 2008 Feb 15;122(4):742-50. doi: 10.1002/ijc.23114.
43. Fogli S, Nieri P, Chicca A, Adinolfi B, Mariotti V, Iacopetti P, Breschi MC, Pellegrini S. Cannabinoid derivatives induce cell death in pancreatic MIA PaCa-2 cells via a receptor-

independent mechanism. FEBS Lett. 2006 Mar 20;580(7):1733-9. doi: 10.1016/j.febslet.2006.02.024.

44. Brandi J, Dando I, Palmieri M, Donadelli M, Cecconi D. Comparative proteomic and phosphoproteomic profiling of pancreatic adenocarcinoma cells treated with CB1 or CB2 agonists. Electrophoresis. 2013 May;34(9-10):1359-68. doi: 10.1002/elps.201200402.
45. Dando I, Donadelli M, Costanzo C, Dalla Pozza E, D'Alessandro A, Zolla L, Palmieri M. Cannabinoids inhibit energetic metabolism and induce AMPK-dependent autophagy in pancreatic cancer cells. Cell Death Dis. 2013 Jun 13;4(6):e664. doi: 10.1038/cddis.2013.151.
46. Carracedo A, Gironella M, Lorente M, Garcia S, Guzmán M, Velasco G, Iovanna JL. Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes. Cancer Res. 2006 Jul 1;66(13):6748-55. doi: 10.1158/0008-5472.CAN-06-0169.
47. Donadelli M, Dando I, Zaniboni T, Costanzo C, Dalla Pozza E, Scupoli MT, Scarpa A, Zappavigna S, Marra M, Abbruzzese A, Bifulco M, Caraglia M, Palmieri M. Gemcitabine/cannabinoid combination triggers autophagy in pancreatic cancer cells through a ROS-mediated mechanism. Cell Death Dis. 2011 Apr 28;2(4):e152. doi: 10.1038/cddis.2011.36.
48. Yang Y, Huynh N, Dumesny C, Wang K, He H, Nikfarjam M. Cannabinoids Inhibited Pancreatic Cancer via P-21 Activated Kinase 1 Mediated Pathway. Int J Mol Sci. 2020 Oct 28;21(21):8035. doi: 10.3390/ijms21218035.
49. Ferro R, Adamska A, Lattanzio R, Mavrommati I, Edling CE, Arifin SA, Fyffe CA, Sala G, Sacchetto L, Chiorino G, De Laurenzi V, Piantelli M, Sansom OJ, Maffucci T, Falasca M. GPR55 signaling promotes proliferation of pancreatic cancer cells and tumor growth in mice, and its inhibition increases effects of gemcitabine. Oncogene. 2018 Dec;37(49):6368-6382. doi: 10.1038/s41388-018-0390-1.
50. Garofano F, Sharma A, Abken H, Gonzalez-Carmona MA, Schmidt-Wolf IGH. A Low Dose of Pure Cannabidiol Is Sufficient to Stimulate the Cytotoxic Function of CIK Cells without Exerting the Downstream Mediators in Pancreatic Cancer Cells. Int J Mol Sci. 2022 Mar 29;23(7):3783. doi: 10.3390/ijms23073783.
51. Sakarin S, Meesiripan N, Sangrajrang S, Suwanpidokkul N, Prayakprom P, Bodhibukkana C, Khaowroongrueng V, Suriyachan K, Thanasittichai S, Srisubat A, Surawongsin P, Rattanapinyopituk K. Antitumor Effects of Cannabinoids in Human Pancreatic Ductal Adenocarcinoma Cell Line (Capan-2)-Derived Xenograft Mouse Model. Front Vet Sci. 2022 Jul 22;9:867575. doi: 10.3389/fvets.2022.867575.

52. Luongo M, Marinelli O, Zeppa L, Aguzzi C, Morelli MB, Amantini C, Frassineti A, di Costanzo M, Fanelli A, Santoni G, Nabissi M. Cannabidiol and Oxygen-Ozone Combination Induce Cytotoxicity in Human Pancreatic Ductal Adenocarcinoma Cell Lines. Cancers (Basel). 2020 Sep 27;12(10):2774. doi: 10.3390/cancers12102774.
53. Nachnani R, Raup-Konsavage WM, Vrana KE. The Pharmacological Case for Cannabigerol. J Pharmacol Exp Ther. 2021 Feb;376(2):204-212. doi: 10.1124/jpet.120.000340.
54. Borrelli F, Pagano E, Romano B, Panzera S, Maiello F, Coppola D, De Petrocellis L, Buono L, Orlando P, Izzo AA. Colon carcinogenesis is inhibited by the TRPM8 antagonist cannabigerol, a Cannabis-derived non-psychotropic cannabinoid. Carcinogenesis. 2014 Dec;35(12):2787-97. doi: 10.1093/carcin/bgu205.
55. Viereckl MJ, Krutsinger K, Apawu A, Gu J, Cardona B, Barratt D, Han Y. Cannabidiol and Cannabigerol Inhibit Cholangiocarcinoma Growth In Vitro via Divergent Cell Death Pathways. Biomolecules. 2022 Jun 20;12(6):854. doi: 10.3390/biom12060854.
56. Wyrobnik I, Steinberg M, Gelfand A, Rosenblum R, Eid Mutlak Y, Sulimani L, Procaccia S, Ofran Y, Novak-Kotzer H, Meiri D. Decreased melanoma CSF-1 secretion by Cannabigerol treatment reprograms regulatory myeloid cells and reduces tumor progression. Oncoimmunology. 2023 Jun 11;12(1):2219164. doi: 10.1080/2162402X.2023.2219164.
57. Colvin EK, Hudson AL, Anderson LL, Kumar RP, McGregor IS, Howell VM, Arnold JC. An Examination of the Anti-Cancer Properties of Plant Cannabinoids in Preclinical Models of Mesothelioma. Cancers (Basel). 2022 Aug 5;14(15):3813. doi: 10.3390/cancers14153813.
58. Schoeman R, Beukes N, Frost C. Cannabinoid Combination Induces Cytoplasmic Vacuolation in MCF-7 Breast Cancer Cells. Molecules. 2020 Oct 14;25(20):4682. doi: 10.3390/molecules25204682.
59. Lah TT, Novak M, Pena Almidon MA, Marinelli O, Žvar Baškovič B, Majc B, Mlinar M, Bošnjak R, Breznik B, Zomer R, Nabissi M. Cannabigerol Is a Potential Therapeutic Agent in a Novel Combined Therapy for Glioblastoma. Cells. 2021 Feb 5;10(2):340. doi: 10.3390/cells10020340.
60. Vasey C, McBride J, Penta K. Circadian Rhythm Dysregulation and Restoration: The Role of Melatonin. Nutrients. 2021 Sep 30;13(10):3480. doi: 10.3390/nu13103480.
61. Tonon AC, Pilz LK, Markus RP, Hidalgo MP, Elisabetsky E. Melatonin and Depression: A Translational Perspective From Animal Models to Clinical Studies. Front Psychiatry. 2021 Apr 8;12:638981. doi: 10.3389/fpsyt.2021.638981.

62. Talib WH, Alsayed AR, Abuawad A, Daoud S, Mahmod AI. Melatonin in Cancer Treatment: Current Knowledge and Future Opportunities. Molecules. 2021 Apr 25;26(9):2506. doi: 10.3390/molecules26092506.
63. Tarocco A, Caroccia N, Morciano G, Wieckowski MR, Ancora G, Garani G, Pinton P. Melatonin as a master regulator of cell death and inflammation: molecular mechanisms and clinical implications for newborn care. Cell Death Dis. 2019 Apr 8;10(4):317. doi: 10.1038/s41419-019-1556-7.
64. Cecon E, Oishi A, Jockers R. Melatonin receptors: molecular pharmacology and signaling in the context of system bias. Br J Pharmacol. 2018 Aug;175(16):3263-3280. doi: 10.1111/bph.13950.
65. Liu J, Clough SJ, Hutchinson AJ, Adamah-Biassi EB, Popovska-Gorevski M, Dubocovich ML. MT1 and MT2 Melatonin Receptors: A Therapeutic Perspective. Annu Rev Pharmacol Toxicol. 2016;56:361-83. doi: 10.1146/annurev-pharmtox-010814-124742.
66. Tordjman S, Chokron S, Delorme R, Charrier A, Bellissant E, Jaafari N, Fougerou C. Melatonin: Pharmacology, Functions and Therapeutic Benefits. Curr Neuropharmacol. 2017 Apr;15(3):434-443. doi: 10.2174/1570159X14666161228122115.
67. Chitimus DM, Popescu MR, Voiculescu SE, Panaitescu AM, Pavel B, Zagrean L, Zagrean AM. Melatonin's Impact on Antioxidative and Anti-Inflammatory Reprogramming in Homeostasis and Disease. Biomolecules. 2020 Aug 20;10(9):1211. doi: 10.3390/biom10091211.
68. Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M, Qin L. Melatonin as an antioxidant: under promises but over delivers. J Pineal Res. 2016 Oct;61(3):253-78. doi: 10.1111/jpi.12360.
69. Hardeland R. Melatonin and inflammation-Story of a double-edged blade. J Pineal Res. 2018 Nov;65(4):e12525. doi: 10.1111/jpi.12525.
70. Chen X, Hao B, Li D, Reiter RJ, Bai Y, Abay B, Chen G, Lin S, Zheng T, Ren Y, Xu X, Li M, Fan L. Melatonin inhibits lung cancer development by reversing the Warburg effect via stimulating the SIRT3/PDH axis. J Pineal Res. 2021 Sep;71(2):e12755. doi: 10.1111/jpi.12755.
71. Chao CC, Chen PC, Chiou PC, Hsu CJ, Liu PI, Yang YC, Reiter RJ, Yang SF, Tang CH. Melatonin suppresses lung cancer metastasis by inhibition of epithelial-mesenchymal transition through targeting to Twist. Clin Sci (Lond). 2019 Mar 15;133(5):709-722. doi: 10.1042/CS20180945.

72. Liu Z, Sang X, Wang M, Liu Y, Liu J, Wang X, Liu P, Cheng H. Melatonin potentiates the cytotoxic effect of Neratinib in HER2+ breast cancer through promoting endocytosis and lysosomal degradation of HER2. Oncogene. 2021 Nov;40(44):6273-6283. doi: 10.1038/s41388-021-02015-w.
73. Alonso-González C, Menéndez-Menéndez J, González-González A, González A, Cos S, Martínez-Campa C. Melatonin enhances the apoptotic effects and modulates the changes in gene expression induced by docetaxel in MCF-7 human breast cancer cells. Int J Oncol. 2018 Feb;52(2):560-570. doi: 10.3892/ijo.2017.4213.
74. Zhang Y, Liu Q, Wang F, Ling EA, Liu S, Wang L, Yang Y, Yao L, Chen X, Wang F, Shi W, Gao M, Hao A. Melatonin antagonizes hypoxia-mediated glioblastoma cell migration and invasion via inhibition of HIF-1α. J Pineal Res. 2013 Sep;55(2):121-30. doi: 10.1111/jpi.12052.
75. Martín V, Sanchez-Sanchez AM, Puente-Moncada N, Gomez-Lobo M, Alvarez-Vega MA, Antolín I, Rodriguez C. Involvement of autophagy in melatonin-induced cytotoxicity in glioma-initiating cells. J Pineal Res. 2014 Oct;57(3):308-16. doi: 10.1111/jpi.12170.
76. Zhou L, Zhang C, Yang X, Liu L, Hu J, Hou Y, Tao H, Sugimura H, Chen Z, Wang L, Chen K. Melatonin inhibits lipid accumulation to repress prostate cancer progression by mediating the epigenetic modification of CES1. Clin Transl Med. 2021 Jun;11(6):e449. doi: 10.1002/ctm2.449.
77. Gao Y, Xiao X, Zhang C, Yu W, Guo W, Zhang Z, Li Z, Feng X, Hao J, Zhang K, Xiao B, Chen M, Huang W, Xiong S, Wu X, Deng W. Melatonin synergizes the chemotherapeutic effect of 5-fluorouracil in colon cancer by suppressing PI3K/AKT and NF-κB/iNOS signaling pathways. J Pineal Res. 2017 Mar;62(2). doi: 10.1111/jpi.12380.
78. Lee JH, Yun CW, Han YS, Kim S, Jeong D, Kwon HY, Kim H, Baek MJ, Lee SH. Melatonin and 5-fluorouracil co-suppress colon cancer stem cells by regulating cellular prion protein-Oct4 axis. J Pineal Res. 2018 Nov;65(4):e12519. doi: 10.1111/jpi.12519.
79. Tian T, Li J, Li Y, Lu YX, Tang YL, Wang H, Zheng F, Shi D, Long Q, Chen M, Garcia-Manero G, Hu Y, Qin L, Deng W. Melatonin enhances sorafenib-induced cytotoxicity in FLT3-ITD acute myeloid leukemia cells by redox modification. Theranostics. 2019 May 31;9(13):3768-3779. doi: 10.7150/thno.34327.
80. Tang YL, Sun X, Huang LB, Liu XJ, Qin G, Wang LN, Zhang XL, Ke ZY, Luo JS, Liang C, Peng CJ, Tang WY, Li Y, Huang W, Luo XQ, Deng W. Melatonin inhibits MLL-rearranged leukemia via RBFOX3/hTERT and NF-κB/COX-2 signaling pathways. Cancer Lett. 2019 Feb 28;443:167-178. doi: 10.1016/j.canlet.2018.11.037.

81. Kanishi Y, Kobayashi Y, Noda S, Ishizuka B, Saito K. Differential growth inhibitory effect of melatonin on two endometrial cancer cell lines. J Pineal Res. 2000 May;28(4):227-33. doi: 10.1034/j.1600-079x.2000.280405.x.
82. Gu C, Yang H, Chang K, Zhang B, Xie F, Ye J, Chang R, Qiu X, Wang Y, Qu Y, Wang J, Li M. Melatonin alleviates progression of uterine endometrial cancer by suppressing estrogen/ubiquitin C/SDHB-mediated succinate accumulation. Cancer Lett. 2020 Apr 28;476:34-47. doi: 10.1016/j.canlet.2020.02.009.
83. Leja-Szpak A, Jaworek J, Pierzchalski P, Reiter RJ. Melatonin induces pro-apoptotic signaling pathway in human pancreatic carcinoma cells (PANC-1). J Pineal Res. 2010 Oct;49(3):248-55. doi: 10.1111/j.1600-079X.2010.00789.x.
84. Xu C, Wu A, Zhu H, Fang H, Xu L, Ye J, Shen J. Melatonin is involved in the apoptosis and necrosis of pancreatic cancer cell line SW-1990 via modulating of Bcl-2/Bax balance. Biomed Pharmacother. 2013 Mar;67(2):133-9. doi: 10.1016/j.biopha.2012.10.005.
85. Ju HQ, Li H, Tian T, Lu YX, Bai L, Chen LZ, Sheng H, Mo HY, Zeng JB, Deng W, Chiao PJ, Xu RH. Melatonin overcomes gemcitabine resistance in pancreatic ductal adenocarcinoma by abrogating nuclear factor-κB activation. J Pineal Res. 2016 Jan;60(1):27-38. doi: 10.1111/jpi.12285.
86. Cui P, Yu M, Peng X, Dong L, Yang Z. Melatonin prevents human pancreatic carcinoma cell PANC-1-induced human umbilical vein endothelial cell proliferation and migration by inhibiting vascular endothelial growth factor expression. J Pineal Res. 2012 Mar;52(2):236-43. doi: 10.1111/j.1600-079X.2011.00933.x.
87. Ruiz-Rabelo JF, Vázquez R, Perea MD, Cruz A, González R, Romero A, Muñoz-Villanueva MC, Túnez I, Montilla P, Muntané J, Padillo FJ. Beneficial properties of melatonin in an experimental model of pancreatic cancer. J Pineal Res. 2007 Oct;43(3):270-5. doi: 10.1111/j.1600-079X.2007.00472.x.
88. Padillo FJ, Ruiz-Rabelo JF, Cruz A, Perea MD, Tasset I, Montilla P, Túnez I, Muntané J. Melatonin and celecoxib improve the outcomes in hamsters with experimental pancreatic cancer. J Pineal Res. 2010 Oct;49(3):264-70. doi: 10.1111/j.1600-079X.2010.00791.x.
89. Uguz AC, Cig B, Espino J, Bejarano I, Naziroglu M, Rodríguez AB, Pariente JA. Melatonin potentiates chemotherapy-induced cytotoxicity and apoptosis in rat pancreatic tumor cells. J Pineal Res. 2012 Aug;53(1):91-8. doi: 10.1111/j.1600-079X.2012.00974.x.
90. de Sire A, Agostini F, Lippi L, Mangone M, Marchese S, Cisari C, Bernetti A, Invernizzi M. Oxygen-Ozone Therapy in the Rehabilitation Field: State of the Art on Mechanisms of Action, Safety and Effectiveness in Patients with Musculoskeletal Disorders. Biomolecules. 2021 Feb 26;11(3):356. doi: 10.3390/biom11030356.

91. Baeza-Noci J, Pinto-Bonilla R. Systemic Review: Ozone: A Potential New Chemotherapy. Int J Mol Sci. 2021 Oct 30;22(21):11796. doi: 10.3390/ijms222111796.
92. de Sire A, Marotta N, Ferrillo M, Agostini F, Sconza C, Lippi L, Respizzi S, Giudice A, Invernizzi M, Ammendolia A. Oxygen-Ozone Therapy for Reducing Pro-Inflammatory Cytokines Serum Levels in Musculoskeletal and Temporomandibular Disorders: A Comprehensive Review. Int J Mol Sci. 2022 Feb 25;23(5):2528. doi: 10.3390/ijms23052528.
93. Yanchu L, Rong P, Rong C, Li Z, Xiaoyan Y, Feng W. Ozone therapy for high-grade glioma: an overview. Front Oncol. 2023 May 24;13:1161206. doi: 10.3389/fonc.2023.1161206.
94. Clavo B, Ruiz A, Lloret M, López L, Suárez G, Macías D, Rodríguez V, Hernández MA, Martín-Oliva R, Quintero S, Cuyás JM, Robaina F. Adjuvant Ozonetherapy in Advanced Head and Neck Tumors: A Comparative Study. Evid Based Complement Alternat Med. 2004 Dec;1(3):321-325. doi: 10.1093/ecam/neh038.
95. Li J, Zeng T, Tang S, Zhong M, Huang Q, Li X, He X. Medical ozone induces proliferation and migration inhibition through ROS accumulation and PI3K/AKT/NF-κB suppression in human liver cancer cells in vitro. Clin Transl Oncol. 2021 Sep;23(9):1847-1856. doi: 10.1007/s12094-021-02594-w.
96. Schulz S, Häussler U, Mandic R, Heverhagen JT, Neubauer A, Dünne AA, Werner JA, Weihe E, Bette M. Treatment with ozone/oxygen-pneumoperitoneum results in complete remission of rabbit squamous cell carcinomas. Int J Cancer. 2008 May 15;122(10):2360-7. doi: 10.1002/ijc.23382.
97. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. Genes Dev. 2006 May 15;20(10):1218-49. doi: 10.1101/gad.1415606.
98. Grossberg AJ, Chu LC, Deig CR, Fishman EK, Hwang WL, Maitra A, Marks DL, Mehta A, Nabavizadeh N, Simeone DM, Weekes CD, Thomas CR Jr. Multidisciplinary standards of care and recent progress in pancreatic ductal adenocarcinoma. CA Cancer J Clin. 2020 Sep;70(5):375-403. doi: 10.3322/caac.21626.
99. Klein AP. Pancreatic cancer epidemiology: understanding the role of lifestyle and inherited risk factors. Nat Rev Gastroenterol Hepatol. 2021 Jul;18(7):493-502. doi: 10.1038/s41575-021-00457-x.
100. Grant TJ, Hua K, Singh A. Molecular Pathogenesis of Pancreatic Cancer. Prog Mol Biol Transl Sci. 2016;144:241-275. doi: 10.1016/bs.pmbts.2016.09.008.

101. Collisson EA, Bailey P, Chang DK, Biankin AV. Molecular subtypes of pancreatic cancer. Nat Rev Gastroenterol Hepatol. 2019 Apr;16(4):207-220. doi: 10.1038/s41575-019-0109-y.

102. Qian Y, Gong Y, Fan Z, Luo G, Huang Q, Deng S, Cheng H, Jin K, Ni Q, Yu X, Liu C. Molecular alterations and targeted therapy in pancreatic ductal adenocarcinoma. J Hematol Oncol. 2020 Oct 2;13(1):130. doi: 10.1186/s13045-020-00958-3.

103. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet. 2011 Aug 13;378(9791):607-20. doi: 10.1016/S0140-6736(10)62307-0.

104. Mann KM, Ying H, Juan J, Jenkins NA, Copeland NG. KRAS-related proteins in pancreatic cancer. Pharmacol Ther. 2016 Dec;168:29-42. doi: 10.1016/j.pharmthera.2016.09.003.

105. Qin C, Yang G, Yang J, Ren B, Wang H, Chen G, Zhao F, You L, Wang W, Zhao Y. Metabolism of pancreatic cancer: paving the way to better anticancer strategies. Mol Cancer. 2020 Mar 2;19(1):50. doi: 10.1186/s12943-020-01169-7.

106. Ianevski A, Giri AK, Aittokallio T. SynergyFinder 2.0: visual analytics of multi-drug combination synergies. Nucleic Acids Res. 2020 Jul 2;48(W1):W488-W493. doi: 10.1093/nar/gkaa216.

107. Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in Survival and Clinical Benefit With Gemcitabine as First-Line Therapy for Patients With Advanced Pancreas Cancer: A Randomized Trial. J Clin Oncol. 2023 Dec 20;41(36):5482-5492. doi: 10.1200/JCO.22.02777.

108. Frenkel M, David A, Sapire K, Hausner D. Complementary and Integrative Medicine in Pancreatic Cancer. Curr Oncol Rep. 2023 Mar;25(3):231-242. doi: 10.1007/s11912-023-01370-z.

109. Fang Z, Jung KH, Yan HH, et al. Melatonin Synergizes with Sorafenib to Suppress Pancreatic Cancer via Melatonin Receptor and PDGFR-β/STAT3 Pathway. Cell Physiol Biochem. 2018;47(5):1751-1768. doi:10.1159/000491058

110. Serra MEG, Baeza-Noci J, Mendes Abdala CV, Luvisotto MM, Bertol CD, Anzolin AP. The role of ozone treatment as integrative medicine. An evidence and gap map. Front Public Health. 2023 Jan 16;10:1112296. doi: 10.3389/fpubh.2022.1112296.

111. Afrin F, Chi M, Eamens AL, Duchatel RJ, Douglas AM, Schneider J, Gedye C, Woldu AS, Dun MD. Can Hemp Help? Low-THC Cannabis and Non-THC Cannabinoids for the Treatment of Cancer. Cancers (Basel). 2020 Apr 23;12(4):1033. doi: 10.3390/cancers12041033.

112. Salazar M, Carracedo A, Salanueva IJ, Hernández-Tiedra S, Lorente M, Egia A, Vázquez P, Blázquez C, Torres S, García S, Nowak J, Fimia GM, Piacentini M, Cecconi F, Pandolfi PP, González-Feria L, Iovanna JL, Guzmán M, Boya P, Velasco G. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. J Clin Invest. 2009 May;119(5):1359-72. doi: 10.1172/jci37948.

113. Wu M, Zhang P. EGFR-mediated autophagy in tumourigenesis and therapeutic resistance. Cancer Lett. 2020 Jan 28;469:207-216. doi: 10.1016/j.canlet.2019.10.030.

114. Stanciu S, Ionita-Radu F, Stefani C, Miricescu D, Stanescu-Spinu II, Greabu M, Ripszky Totan A, Jinga M. Targeting PI3K/AKT/mTOR Signaling Pathway in Pancreatic Cancer: From Molecular to Clinical Aspects. Int J Mol Sci. 2022 Sep 4;23(17):10132. doi: 10.3390/ijms231710132.

115. Vara D, Salazar M, Olea-Herrero N, Guzmán M, Velasco G, Díaz-Laviada I. Anti-tumoral action of cannabinoids on hepatocellular carcinoma: role of AMPK-dependent activation of autophagy. Cell Death Differ. 2011 Jul;18(7):1099-111. doi: 10.1038/cdd.2011.32.

116. Shrivastava A, Kuzontkoski PM, Groopman JE, Prasad A. Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy. Mol Cancer Ther. 2011 Jul;10(7):1161-72. doi: 10.1158/1535-7163.MCT-10-1100.

117. Blasco MT, Navas C, Martín-Serrano G, Graña-Castro O, Lechuga CG, Martín-Díaz L, Djurec M, Li J, Morales-Cacho L, Esteban-Burgos L, Perales-Patón J, Bousquet-Mur E, Castellano E, Jacob HKC, Cabras L, Musteanu M, Drosten M, Ortega S, Mulero F, Sainz B Jr, Dusetti N, Iovanna J, Sánchez-Bueno F, Hidalgo M, Khiabanian H, Rabadán R, Al-Shahrour F, Guerra C, Barbacid M. Complete Regression of Advanced Pancreatic Ductal Adenocarcinomas upon Combined Inhibition of EGFR and C-RAF. Cancer Cell. 2019 Apr 15;35(4):573-587.e6. doi: 10.1016/j.ccell.2019.03.002.

118. Lamtha T, Tabtimmai L, Songtawee N, Tansakul N, Choowongkomon K. Structural analysis of cannabinoids against EGFR-TK leads a novel target against EGFR-driven cell lines. Curr Res Pharmacol Drug Discov. 2022 Sep 30;3:100132. doi: 10.1016/j.crphar.2022.100132.

119. Yan C, Li Y, Liu H, Chen D, Wu J. Antitumor mechanism of cannabidiol hidden behind cancer hallmarks. Biochim Biophys Acta Rev Cancer. 2023 Jul;1878(4):188905. doi: 10.1016/j.bbcan.2023.188905.

120. Godfrey LK, Forster J, Liffers ST, Schröder C, Köster J, Henschel L, Ludwig KU, Lähnemann D, Trajkovic-Arsic M, Behrens D, Scarpa A, Lawlor RT, Witzke KE, Sitek B, Johnsen SA, Rahmann S, Horsthemke B, Zeschnigk M, Siveke JT. Pancreatic cancer acquires resistance to MAPK pathway inhibition by clonal expansion and adaptive DNA hypermethylation. Clin Epigenetics. 2024 Jan 16;16(1):13. doi: 10.1186/s13148-024-01623-z.

APPENDIX

1. Participation in other projects

“Cannabinoids and endometrial cancer”

“Medical cannabis in multiple myeloma” financed by Entourage Biosciences

“Modulating synaptic neurotransmission to reactivate the immune reaction against brain tumors” PRIN 2020 financed by MUR

“Valutazioni di formulazioni di Cannabis terapeutica nella malattia di Crohn” financed by ASUR e Regione Marche

“An innovative platform against SARS-CoV-2 and other emerging viruses grounded on copper-based materials” FAR 2022 PNRR

Projects in collaboration with Prof. Maggi Filippo laboratory and with prof. Bonacucina Giulia laboratory

2. Conferences and seminars

Speaker at the conference “Alimenti e nutraceutici: salute e prevenzione attraverso il cibo” carried out in UNICAM. Title: “Evening Primrose oil effects in human Pancreatic Ductal Adenocarcinoma cell lines” (July, 2021)

Speaker at the 2nd Global virtual summit on Cancer & Cancer Research. Title: “Improving chemotherapeutic effect in human Pancreatic Ductal Adenocarcinoma cell lines with Evening Primrose Oil” (May, 2022)

Poster presenter at European Association for Cancer Research (EACR) 2022 Congress - Innovative Cancer Science: Translating Biology to Medicine carried out in Seville. Title: “TRPV channels activation induces cell death in human Pancreatic Ductal Adenocarcinoma cell lines” (June, 2022)

Poster presenter at Cannabinoid Conference 2022 carried out in Basel. Title:”Anti-cancer effects of Cannabidiol and Oxygen-ozone combination in Human Pancreatic Ductal Adenocarcinoma cell lines” (October, 2022)

Speaker at the Congress ETS AMOR "Cancro e dolore: ossigeno ozono, cannabis e terapie integrate" carried out in Naples. Title: “Ossigeno ozono terapia e terapie integrate in modelli preclinici di tumore al pancreas” (November, 2022)

Poster presenter at European Association for Cancer Research (EACR) 2022 Congress - EACR 2023 Congress - Innovative Cancer Science carried out in Turin. Title: “Effect of Cannabidiol, Melatonin and Oxygen/Ozone combination in preclinical models of human Pancreatic Ductal Adenocarcinoma” (June, 2023)

3. Publications

Pavela R, Pavoni L, Bonacucina G, Cespi M, Cappellacci L, Petrelli R, Spinozzi E, Aguzzi C, **Zeppa L**, Ubaldi M, et al. (2021). Encapsulation of Carlina acaulis essential oil and carlina oxide to develop long-lasting mosquito larvicides: microemulsions versus nanoemulsions. Journal of Pest Science 94, 899–915.

Maggi F, Morelli MB, Nabissi M, Marinelli O, **Zeppa L**, Aguzzi C, Santoni G, Amantini C. Transient Receptor Potential (TRP) Channels in Haematological Malignancies: An Update.Biomolecules. 2021 May 20;11(5):765. doi: 10.3390/ biom11050765. PMID: 34065398; PMCID:PMC8160608.

Susana Sánchez-Gómez, Rafael Pagán, Roman Pavela, Eugenia Mazzara, Eleonora Spinozzi, Oliviero Marinelli, **Laura Zeppa**, Mohammad Reza Morshedloo, Filippo Maggi, Angelo Canale, Giovanni Benelli. Lethal and sublethal effects of essential oil-loaded zein nanocapsules on a zoonotic disease vector mosquito, and their non-target impact, Industrial Crops and Products, Volume 176, 2022, 114413, ISSN 0926-6690,https://doi.org/10.1016/j.indcrop.2021.114413.

Maggi F, Morelli MB, Tomassoni D, Marinelli O, Aguzzi C, **Zeppa L**, Nabissi M, Santoni G, Amantini C. The effects of cannabidiol via TRPV2 channel in chronic myeloid leukemia cells and its combination with imatinib. Cancer Sci. 2022 Apr; 113(4):1235-1249. doi:10.1111/cas.15257. Epub 2022 Mar 4. PMID: 34971020; PMCID: PMC8990867.

Zeppa L, Aguzzi C, Versari G, Luongo M, Morelli MB, Maggi F, Amantini C, Santoni G, Marinelli O, Nabissi M. Evening Primrose Oil Improves Chemotherapeutic Effects in Human Pancreatic Ductal Adenocarcinoma Cell Lines-A Preclinical Study. Pharmaceuticals (Basel).2022 Apr 12;15(4):466. doi: 10.3390/ph15040466. PMID: 35455464; PMCID: PMC9024477.

Morelli MB, Marinelli O, Aguzzi C, **Zeppa L**, Nabissi M, Amantini C, Tomassoni D, Maggi F, Santoni M, Santoni G. Unveiling the Molecular Mechanisms Driving the Capsaicin-Induced Immunomodulatory Effects on PD-L1 Expression in Bladder and Renal Cancer Cell Lines. Cancers (Basel). 2022 May 26;14(11):2644. doi: 10.3390/cancers14112644. PMID: 35681623; PMCID: PMC9179445.

Badalamenti N, Bruno M, Pavela R, Maggi F, Marinelli O, **Zeppa L**, Benelli G, Canale A. Acaricidal Activity of Bufadienolides Isolated from Drimia pancration against Tetranychusurticae, and Structural Elucidation of Arenobufagin-3-O-α-L- rhamnopyranoside. Plants (Basel). 2022 Jun 21;11(13): 1629. doi: 10.3390/ plants11131629. PMID: 35807580; PMCID: PMC9268777.

Maggi F, Morelli MB, Aguzzi C, **Zeppa L**, Nabissi M, Polidori C, Santoni G, Amantini C. Calcium influx, oxidative stress, and apoptosis induced by TRPV1 in chronic myeloid leukemia cells: Synergistic effects with imatinib. Front Mol Biosci. 2023 Feb 15;10:1129202. doi:10.3389/fmolb.2023.1129202. PMID: 36876044; PMCID: PMC9975599.

Spinozzi E, Ferrati M, Baldassarri C, Maggi F, Pavela R, Benelli G, Aguzzi C, **Zeppa L**, Cappellacci L, Palmieri A, Petrelli R. Synthesis of Carlina Oxide Analogues and Evaluation of Their Insecticidal Efficacy and Cytotoxicity. J Nat Prod. 2023 May 12. doi:10.1021/acs.jnatprod.3c00137. Epub ahead of print. PMID: 37172063.

Antonini M, Aguzzi C, Fanelli A, Frassineti A, **Zeppa L**, Morelli MB, Pastore G, Nabissi M, Luongo M. The Effects of a Combination of Medical Cannabis, Melatonin, and Oxygen–Ozone Therapy on Glioblastoma Multiforme: A Case Report. Reports. 2023; 6(2):22.https://doi.org/10.3390/reports6020022.

Aguzzi C, Perinelli DR, Cespi M, **Zeppa L**, Mazzara E, Maggi F, Petrelli R, Bonacucina G, Nabissi M. Encapsulation of Hemp (Cannabis sativa L.) Essential Oils into Nanoemulsions for Potential Therapeutic Applications: Assessment of Cytotoxicological Profiles. Molecules. 2023 Sep 7;28(18):6479. doi: 10.3390/molecules28186479.

Zeppa L, Aguzzi C, Morelli MB, Marinelli O, Giangrossi M, Luongo M, Amantini C, Santoni G, Nabissi M. Cannabigerol Induces Autophagic Cell Death by Inhibiting EGFR-RAS Pathways in Human Pancreatic Ductal Adenocarcinoma Cell Lines. International Journal of Molecular Sciences. 2024; 25(4):2001. https://doi.org/10.3390/ijms25042001

4. Patents

Co-author of three provisional patents registered in USA:

“Use of phytocannabinoids for treating Multiple Myeloma” PCT/CA2021/050801

“Use of phytocannabinoids for treating Endometrial cancer and Endometriosis” PCT/US22/36758

“Methods and compounds of cannabidiol, melatonin and AKBA for treating Pancreatic cancer” PCT/US22/23244