UNIVERSITÀ DEGLI STUDI DI CAMERINO

School of Advanced Studies

DOCTORAL COURSE IN

Molecular Biology and Cellular Biotechnology

Life and Health Sciences

XXXVI cycle

ANTICANCER EFFECT OF MINOR PHYTOCANNABINOIDS IN PRECLINICAL MODELS OF MULTIPLE MYELOMA

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Abstract

Multiple myeloma (MM) is a blood cancer caused by uncontrolled growth of clonal plasmacells. Bone disease is responsible for the severe complications of MM and is caused by myeloma cells infiltrating the bone marrow and inducing osteoclast activation. To date, no treatment for MM is truly curative, since patients relapse and become refractory to all drug classes. Cannabinoids are already used as palliative in cancer patients. Furthermore, their proper anticancer effect was demonstrated in many cancer models *in vitro*, *in vivo* and in clinical trials. Anyway, only little is known about the effect of cannabinoids on MM and no data has been provided on minor phytocannabinoids such as cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN) and cannabidivarin (CBDV). Scientific literature reported also cannabinoids beneficial effect against bone disease. Here, we investigated the cytotoxic effect of CBG, CBC, CBN and CBDV *in vitro* in MM cell lines, obtaining that the phytocannabinoids inhibited MM cell growth and induced necrotic cell death. Moreover, the phytocannabinoids reduced the invasion of MM cells toward osteoblast cells and the bone resorption *in vitro*. Subsequently, according to the *in vitro* results, we selected CBN for *in vivo* studies in a MM xenograft mice model. Results showed that CBN reduced tumor mass *in vivo*. Together, our results suggest that CBG, CBC, CBN and CBDV can be promising anticancer agents for MM.

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1. Introduction

1.1. Multiple myeloma

Multiple myeloma (MM) is a blood cancer caused by abnormal and uncontrolled growth of clonal plasmacells (Figure 1). They accumulate in the bone marrow and produce monoclonal immunoglobulin (also known as M-protein or paraprotein), detectable in serum and/or urine, and causing hypercalcemia and organ dysfunction such as acute kidney injury with renal insufficiency, anaemia and destructive bone lesions.[1-3]

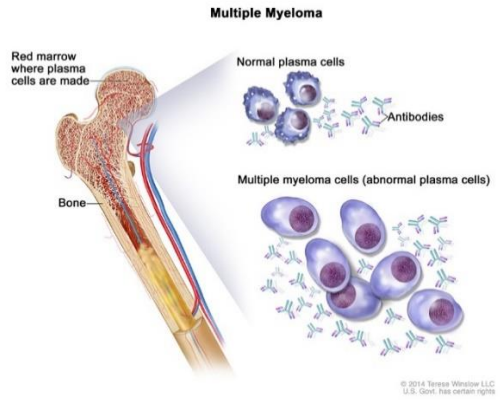


Figure 1. MM cells are abnormal plasma cells produced in the bone marrow.
(https://www.cdc.gov/cancer/myeloma/index.htm)

MM evolves almost always from an asymptomatic pre-malignant stage termed monoclonal gammopathy of undetermined significance (MGUS). MGUS is present in approximately 3-5% of the population above the age of 50 and progresses to MM or related malignancy at a rate of 1% per year. In some patients, an intermediate asymptomatic, but more advanced pre-malignant stage called smoldering multiple myeloma (SMM), can be recognized clinically. SMM progresses to MM at a rate of approximately 10% per year over the first 5 years following diagnosis. Patients with t(4;14) translocation, del(17p), and gain(1q) are at a higher risk of progression from MGUS or SMM to MM.[4,5]

1.1.1. Epidemiology

MM constitutes 1% of all neoplastic diseases and approximately 10% of all haematological malignancies, being the third most common haematological malignancy after non-Hodgkin lymphoma and leukaemia in 2020.[1,4,6] The median age at onset of MM is 69 years, and approximately 63% of patients diagnosed with this disease are older than 65 years. [2] The median overall survival in patients with MM is 6 years. Between patients eligible for autologous stem cell transplantation (ASCT), the median overall survival (OS) is higher, approximately 10 years, but only 5 years among elderly patients (>75 years). Instead, the OS is 4-5 years in patients not eligible for transplantation. [1,4] The majority of patients have numerous relapses of the disease and, ultimately, they die for the disease itself or for treatment-related complications. [1] In 2020, it was estimated that

176 404 new cases of MM were reported worldwide with a global incidence of 1,78 per 100 000 people (Figure 2A). The highest incidence was reported in Australia and New Zealand, followed by northern America and northern Europe. Anyway, the lower MM burden in low-income and middle-income countries could be attributable to the poor diagnostic capacity compared with high-income countries. There were also disparities between men and women: men had a 47% higher incidence rate than women. What about mortality, in 2020 it was estimated 117 077 MM-associated deaths worldwide, with a global mortality of 1,14 per 100 000 people (Figure 2B). The highest mortality was observed in Polynesia, followed by western Europe, northern Europe, Australia and New Zealand, and southern Africa. The poor outcomes of MM in low-income and middle-income countries could be attributable to a poor access to care and development of health services, while in high-income countries, to a higher prevalence of environmental risk factors and metabolic diseases. Men were found to have higher mortality than women and it might be related to a higher prevalence of environmental risk factors and occupational factors. Over time, there was an overall increase in MM incidence, that might be related to a better diagnosis of the disease and increasing prevalence of environmental and metabolic risk factors. By contrast, an overall decreasing trend of MM mortality was observed over time, thanks to the availability of better therapies.[6]

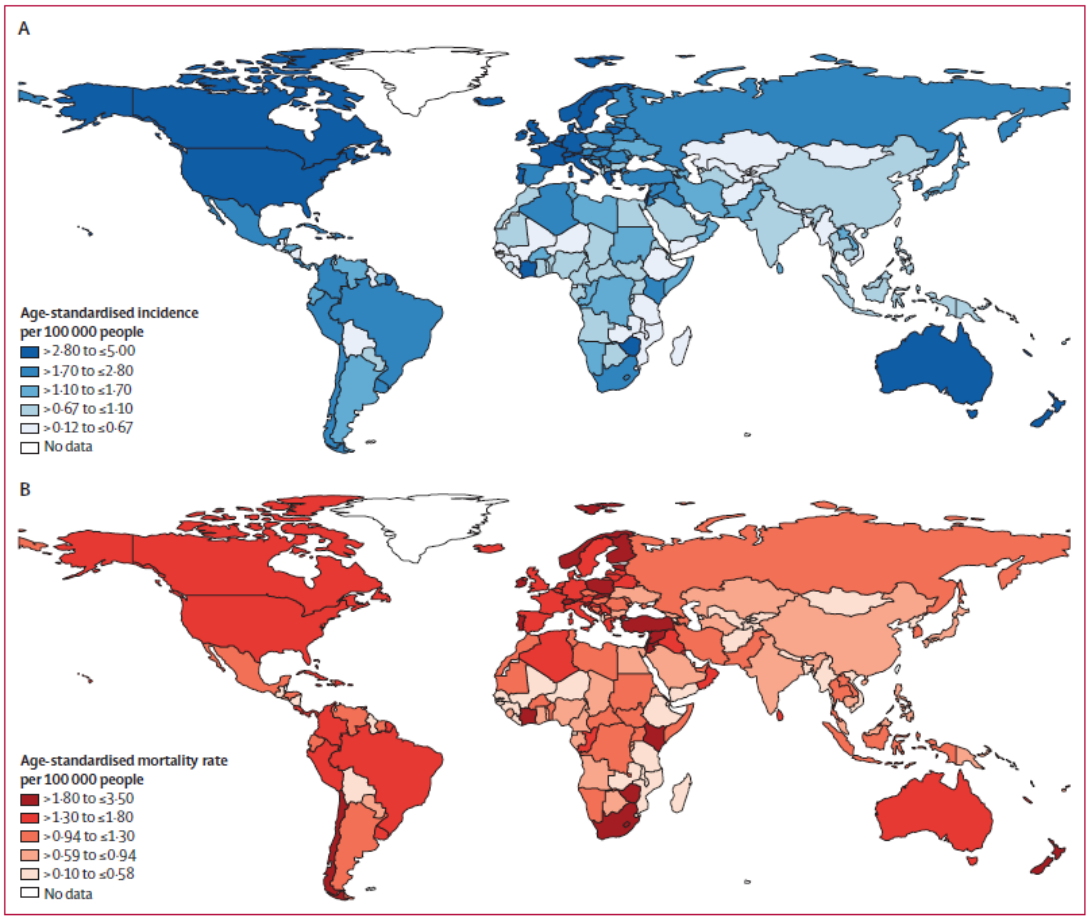


Figure 2. Global MM incidence (A) and mortality (B) in 2020.[6]

1.1.2. Risk factors

The cause of MM remains unclear, but the risk factors that increase the incidence of this malignancy include physical inactivity and obesity, diabetes, chronic inflammation and exposure to pesticides, organic solvents, or radiation, while studies did not find an association between smoking and alcohol drinking and the risk of MM. Inherited genetic variants also contribute to the development of MM.[1,6]

1.1.3. Pathogenesis

In normal conditions, immature B lymphocyte differentiate in the bone marrow and express functional IgM after rearrangement of heavy and light-chain immunoglobulin genes. The naïve B lymphocytes migrate in secondary lymphatic tissues, where the exposure to antigen, such as viral or bacterial infections, induces naïve B cells to proliferate and 1) differentiate in short-lived plasma cells which produce IgM (low affinity antigen response), if the antigen is not a protein; 2) undergo somatic hypermutation of heavy-chain and light-chain gene sequences, resulting in selection of activated B lymphocytes expressing a high-affinity binding site in the immunoglobulin, if the antigen is a protein and thus the response is T-dependent. Somatically mutated IgM-positive B cells may escape into the blood as memory cells, or differentiate to post-germinal-centre plasmablasts, which switch immunoglobulin production from IgM to IgG and IgA (occasionally to IgD or IgE). Isotype-switched B cells then migrate to the bone marrow and terminally differentiate to form long-lived plasma cells that reside in the bone marrow and that secrete large amounts of antibodies. [2,7] The development of an abnormal clonal plasma cell population mimics these normal biological processes, but results in excessive amounts of intact immunoglobulin.[2]

In patients with MM, plasma cells show characteristic features of long-lived bone-marrow cells. They generally express CD138, CD38, and other heterogeneous immunophenotypic markers. Myelomatous plasma cells have extensive somatic hypermutations of rearranged immunoglobulin genes, and, in the vast majority of cases, they express an immunoglobulin isotype other than IgM, which indicates post-follicular B-cell origin. In the rare case of an IgM MM, B cells are arrested shortly before isotype-switching has occurred (pre-switched B lymphocyte).[7]

Primary cytogenetic abnormalities that initiate the malignant development are the acquisition of hyperdiploidy, in particular trisomies, present in 40-55% of patients and affecting mainly chromosomes 3, 5, 7, 9, 11, 15, 19, 21, or a translocation involving the immunoglobulin heavy chain (IgH) gene locus on chromosome 14q32, which is altered in 45-50% of patients. These clonal events are present in the precursor MGUS conditions and SMM. The IgH translocations are mediated primarily by errors in IgH switch recombination and result in activation of oncogenes, because they are controlled by the enhancer regions in the IgH gene locus. The more frequent translocations are the t(4;14) translocation that dysregulates *MMSET* (also known as *NSD2*) and *FGFR3* gene, resulting in their overexpression; t(6;14) dysregulates *CCND3* and t(11;14) *CCND1* gene,

leading to hyperexpression of respectively Cyclin D3 and Cyclin D1; t(14;16) deregulate *C-MAF* oncogene and t(14;20) *MAFB*, resulting in their overexpression.[1,2,4,7,8]

Secondary cytogenetic abnormalities are additional genetic events that arise along the disease course and are found in sub-clonal populations, with increased frequency as the disease evolves from a precursor condition into MM. Acquired genetic events include:

- Copy number abnormalities. They result in chromosomal regions of loss or gain. Loss of tumour suppressor genes results from a deletion (del) of the chromosome's short arm (p) or long arm (q). For example, del(1p) results in the loss of *CDKN2C*, *FAF1*, and *FAM46C* (also known as *TENT5C*), del(11q) *BIRC2* and *BIRC3*, del(13q) *RB1* and *DIS3*, and del(17p) *TP53*. Gain in the long arm of chromosome 1, gain(1q), is found in around 40% of patients, often in association with t(4;14).
- Secondary translocations. They usually involve *MYC*, either via t(8;14) or without involving the IgH gene.
- Somatic mutations. Rates of somatic mutation are highly variable between MM patients and studies showed that each patient show more than 400 canonical somatic mutations. The most mutated genes include immunoglobulin heavy and light chain genes, and genes of the RAS/MAPK pathway, that are mutated in almost 50% of patients (*KRAS* 22%, *NRAS* 17%, *BRAF* 8%).[1,4]

Most of these genetic events converge to dysregulate the cell cycle, leading to cell proliferation and clonal growth (Figure 3).[1]

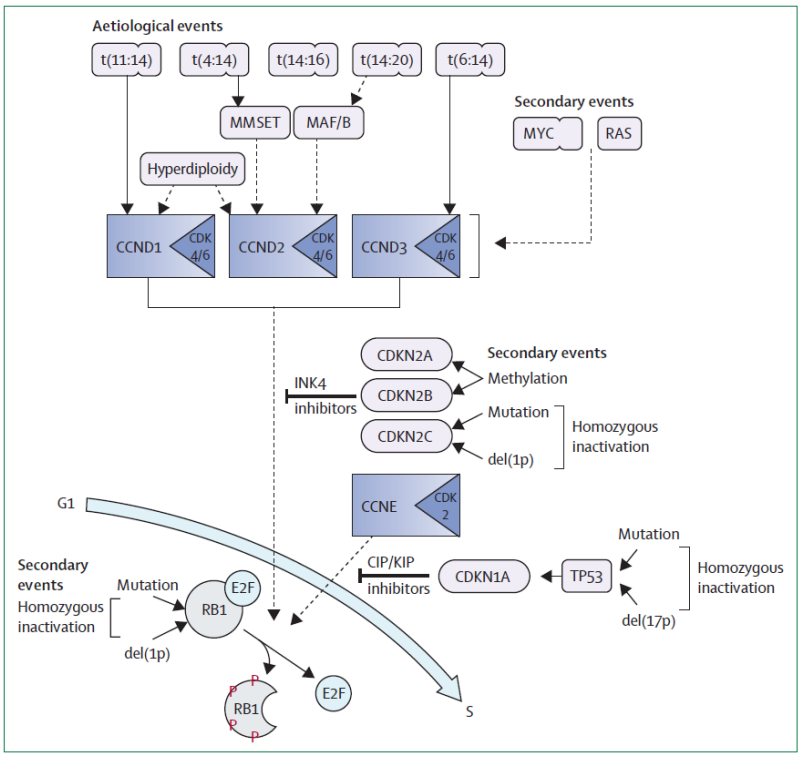


Figure 3. Cell cycle dysregulation in MM. [1]

Other signalling pathways affected include the nuclear factor κB (NF-κB) pathway (affected by copy number loss, mutation, and translocations) and the Phosphatidylinositol 3-Kinase (PI3K) pathway (dysregulated in the absence of genetic change). Apoptotic pathway dysregulation occurs too, with BCL2 dependency in patients with t(11;14) and MCL1 dependency in other patients. Normal plasma-cell differentiation signalling is altered, with upregulation of IRF4, which occurs via a positive autoregulatory loop and the loss of the negative feedback to *MYC* expression via PRDM1. Epigenetic dysregulation is also important in MM. MMSET is a histone 3 lysine 36 methyltransferase and is upregulated in 15% of patients with t(4;14), which have a distinct histone methylation and DNA methylation pattern, which might be responsible for downstream gene expression differences.[1]

Ultimately, there is coevolution of the MM clone and the bone marrow microenvironment, with an increase in tumour-promoting immune cells and a loss of anti-tumour immunity as the disease evolves. In this regard, damage to the bone induced by MM cells induces a feedback loop that drives further MM cell proliferation and immune suppression, resulting from the release of IGF1 and TGF-β during bone resorption[1]

1.1.4.Diagnosis

The diagnostic criteria for MM require the presence of 10% or more clonal plasma cells in the bone marrow (and/or a biopsy proven plasmacytoma) plus one or more myeloma defining events (MDE). The MDE are:

- Biomarkers of malignancy: bone marrow clonal plasma cells ≥60%, serum involved to uninvolved free light chain ratio ≥100, and more than 1 focal lesion (5mm or more in size) on magnetic resonance imaging.
- End-organ damage attributable to the plasma-cell disorder, known as the CRAB criteria (hypercalcemia, renal insufficiency, anaemia, or bone lesions). Hypercalcaemia (serum calcium >11 mg/dL); renal insufficiency (creatinine clearance <40 mL/min or serum creatinine >2 mg/dL); anaemia (haemoglobin value <100 g/L); lytic bone lesions (one or more osteolytic lesions on skeletal radiography, CT, or PET-CT, ≥5 mm in size).[1,4]

The asymptomatic precursor conditions MGUS and SMM are negative for the myeloma defining events, and have M-protein <30 g/l, and urinary M-protein <500 mg/24 h, and clonal bone marrow plasma cells <10%.

Moreover, bone marrow studies at the time of initial diagnosis should include fluorescent cytogenetic analysis by fluorescence in situ hybridization (FISH) to detect the high-risk lesions t(11;14), t(4;14), t(14;16), t(6;14), t(14;20), trisomies, and del(17p).[1,4]

1.1.5.Systemic effects of multiple myeloma

Infections, cardiovascular disease, and renal failure are major causes of early death in MM. Before the introduction of novel drugs, around 10% of patients died within 60 days of diagnosis because of complications caused by either the disease or therapy.[1,2]

Complications of the disease results in a complex symptom burden in myeloma survivors affecting several organ systems.

- Infections. Increased risk of both bacterial and viral infections occurs because of combined effects of MM-related immunodeficiency (deficits in humoral and cellular immunity, B-cell and T-cell disfunction), patient comorbidities and anti-MM therapy (immunomodulatory drugs, steroids, multidrug combinations, drugs that suppress bone marrow function and cellular immunity). It is often observed the reactivation of varicella zoster virus and hepatitis B.
- Bone disease. Up to 80% of patients with MM suffer from osteolytic bone disease at diagnosis or at relapse. Patient show pain and often fractures occur (compression fractures of the spine may cause cord compression). It is the result of increased bone resorption and reduced bone formation.
- Renal disease. 20% of patients with MM have serum creatinine ≥2 mg/dL, and a minority will need dialysis. It is caused by light-chain tubular precipitation causing nephropathy, amyloidosis, dehydration (often associated with hypercalcaemia), infections, use of nephrotoxic drugs (anti-MM drugs can cause or precipitate renal injury).
- Thromboembolic and cardiovascular disease. Patient with MM have increased risk of venous and arterial thromboembolism. Risk is increased by immunomodulatory drugs therapy, especially in combination with high-dose dexamethasone or other chemotherapy. Patient-related risk factors are obesity, immobility, history of venous thromboembolism, recent surgery, comorbidities such as diabetes and infections.
- Peripheral neuropathy. It is common in patients with MM and symptoms are tingling, pain or numbness in hands and feet. It is caused by direct effects of disease (amyloid deposition, reactivity of the M-protein to nerve components, cytokine-mediated nerve damage) or anti-MM therapy (some immunomodulatory and proteasome inhibitors agents, in particular thalidomide and bortezomib).[1,2]

1.1.5.1. Focus on bone disease

In patients with MM, bone disease (Figure 4) is responsible for the severe complications of MM, such as fractures that is seen in 50-60% of patients, and that induce debilitating pain and increase the mortality risk up to 20%. Moreover, bone involvement can cause hypercalcemia, due to the release of calcium after bone destruction, and spinal cord compression syndromes, impacting quality of life and survival.[3,9] Bone involvement is caused by myeloma cells infiltrating the bone marrow and inducing unbalanced bone remodelling, thus resulting in excessive bone destruction and lytic lesions.[10] In normal conditions, in fact, bone undergoes remodelling thanks to the action of osteoclasts and osteoblasts, that keep bone resorption and bone formation in balance.[9] In MM, instead, it is observed an increased activity of osteoclasts and a reduced activity of osteoblasts, leading to both increased bone resorption and reduced bone formation. [3,11] MM cells directly stimulate osteoclast formation in the bone by the release of osteoclast-stimulating factors, such as receptor activator of NF-κB (RANK) ligand (RANKL), macrophage inflammatory protein 1α (MIP-1α), tumour necrosis factor α (TNF-α), interleukin (IL)-3, IL-6, parathyroid hormone-related protein (PTHrP), and hepatocyte growth factor (HGF). MM cells can also indirectly activate osteoclasts, by interaction with bone marrow stromal cells, osteocytes, resident T cells and adipocytes. The RANK/RANK-L signalling pathway is

a regulator of normal and pathological bone remodelling. RANKL is a transmembrane protein that can exists also in the secreted soluble form and in the bone is expressed by stromal cells, osteoblasts, osteocyte and also by activated T lymphocytes. It is not clear if also MM cells can express RANKL.[3,9] RANKL binds its receptor RANK, a transmembrane protein member of the TNF receptor superfamily that is expressed on the surface of osteoclasts and osteoclasts precursors, inducing differentiation, survival, and activity of osteoclasts.[3] An aberrant expression of RANKL increases the osteolytic bone resorption and the development of lytic bone lesions.[9] On the other side, osteoblasts are not able to repair the bone lesions because MM cells can inhibit osteoblasts differentiation, mainly by the suppression of the activity and function of the transcription factor Runx2 in mesenchymal and osteoprogenitor cells, but also through the effect of cytokines like IL-7, or TNF-α, or by the secretion of Wnt antagonists, since Wnt signalling has been reported to regulate osteoblast proliferation and survival, or by other osteoblast inhibitory factors, such as Dickkopf-Related Protein 1 (DKK1) and sclerostin.[1,3,9] With bone resorption it is established a cycle that auto-aliments and supports bone disease. In fact, the bone resorption induced by MM tumour cells, mobilizes growth factors stored in the bone matrix (such as IGF1 and TGF-β) which further stimulates MM cells growth and the production of osteolytic and osteoblast-suppressive factor by MM cells and/or cells that reside in the bone microenvironment.[1,3]

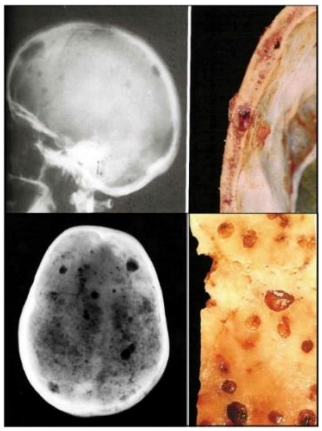


Figure 4. MM bone disease [3]

1.1.6. Treatments

Since no treatment for myeloma is truly curative, the goal of the primary treatment is to induce a deep response to have longer time to relapse and to increase overall survival and quality of life by mitigating disease-related complications. The therapy aims at reducing the abundance of malignant plasma cells in the bone marrow.[1,2] The class of drug used to treat MM are mainly four:

- Proteasome inhibitors (bortezomib, ixazomib, carfilzomib) that inhibit the 20S proteasome
- Immunomodulatory agents (thalidomide, lenalidomide, pomalidomide) that bind to cereblon and activate the cereblon E3 ubiquitin ligase complex, resulting in rapid ubiquitination and degradation of disease-related proteins, in particular two specific B cell transcription factors;

- Glucocorticoids (dexamethasone and prednisolone) that indirectly repress target genes via interaction with NF-κB and Activator protein-1 (AP-1) transcription factors [1,2,4] (AP-1 family include the JUN, FOS, ATF, and MAF multigene subfamilies)[12], important factors in MM pathogenesis;
- Monoclonal antibodies directed against myeloma cell surface antigens (daratumumab and isaxituzimab against CD-38; elotuzumab against SLAMF7).

Moreover, other types of drugs used in MM are alkylator agents (melphalan, cyclophosphamide) that are nitrogen mustard compounds that alkylate DNA, leading to DNA damage and cellular death; antibody drug-conjugated (such as Belantamab mafodotin, that target B-cell maturation antigen (BCMA) on plasma cells and is conjugated to monometyl auristatin-F, a microtubule disrupting agent); CAR-T products targeting BCMA (idecabtagene vicleucel, clitacabtagene autoleucel); selective inhibitors of nuclear export (Selinexor) that, through the blockade of exportin 1, inhibits nuclear export of tumour suppressor proteins and growth factor, leading to apoptosis of myeloma cells.[1,2,4]

In eligible patients, ASCT preceded by an induction therapy is the initial strategy. [1,2] Patients are selected for ASCT on the basis of organ function (they have to be in a good performance status) and age (generally younger than 70 years). Before ASCT patients receive 3 to 6 cycles of induction therapy with a multidrug regimen, usually triplet regimen bortezomib-thalidomide-dexametasone or bortezomib-lenalidomide-dexametasone or bortezomib-cyclophosphamide-dexametasone or daratumumab- lenalidomide-dexametasone or the quadruple regimen with anti-CD38 antibody. After the induction therapy, hematopoietic stem cells are mobilised into the pheripheral blood by colony stimulating factor or chemotherapy (typically cyclophosphamide) or both, harvested, frozen down and then reinfused 1-2 day after high dose chemotherapy (usually melphalan). After ASCT, the standard of care is "consolidation" therapy, a treatment of limited duration using the same induction regimen, followed by "maintenance" therapy that is a long-term continuous treatment with low-dose administration of active antimyeloma agent, usually lenalidomide or bortezomib, to prolong remission.[1,2,4]

In patients not eligible for ASCT, the therapy is usually a triplet regimen similar to patients candidate for ASCT, bortezomib-lenalidomide-dexametasone, or bortezomib-cyclophosphamyde-prednisone, or bortezomib-melphalan-prednisone, or triple with CD38 antibody daratumumab-lenalidomide-dexamethasone, or the quadruple daratumumab-bortezomib-melphalan-prednisolone. Maintenance regimens is with lenalidomide or daratumumab or ixazomib.[1,2,4]

After treatment, almost all patients with MM relapse. The choice of agents after relapsing, depends on patient features, disease characteristics and response to previous treatments. Lenalidomide-dexametasone or bortezomib-dexamethasone or carfilzomib-dexamethasone are usually used. Sometimes it is added a third drug at lenalidomide-dexamethasone, with a different mechanism of action, like carfilzomib-lenalidomide-dexamethasone or ixazomib-lenalidomide-dexamethasone or elatuzumab-lenalidomide-dexamethasone or daratumumab-lenalidomide-dexametasone. To the doublet bortezomib-dexamethasone is usually added daratumumab or pomalidomide. Daratumumab or isatuximab can be added to the carfilzomib-dexamethasone doublet. Moreover, CAR-T cell therapy targeting BCMA shown clinical activity. In patients refractory to

immunomodulatory drugs, proteasome inhibitors and CD-38 targeting antibodies, the options are Selinexor and Belantamab mafodotin.

Despite the multiple options available, patients usually become refractory to all drug classes.[1,4] Thus, new therapies that have different mechanism of action are still needed for these patients.[1]

1.1.6.1. Supportive care

For the complications of the disease, often patients need to take further drugs. For the increased risk of infections, patients receive an antibacterial prophylaxis and also antiviral prophylaxis like acyclovir to prevent varicella zoster virus reactivation. Moreover, anti influenza vaccination is recommended and, in some patients, it is suggested immunoglobulin supplementation. For bone lesions, it is used bisphosphonates and RANK-L neutralising antibody, such as denosumab. For the renal impairment, it is sometimes needed to reduce the doses of anti-myeloma drugs. To prevent thromboembolic events, prophylaxis with aspirin, or with heparin or direct oral anticoagulants in high-risk patients, is recommended in patients receiving immunomodulatory drugs treatment. For the peripheral neuropathy, a prompt identification is essential to interrupt or modulate the treatment and pharmacological intervention may include neuroleptic agents, antidepressants, antiepileptic drugs.[1,2]

1.2. The endocannabinoid system

The endocannabinoid system (ECS) is a complex molecular/biological system distributed throughout the body and discovered in 1988 by scientists Allyn Howlett and W.A. Devane.[13,14] The ECS comprises the cannabinoid receptors, the endocannabinoids ligands and the enzymes that drives their biosynthesis, degradation and transport[15,16,17] The ECS plays a critical role in the organism's physiological processes to maintain the homeostasis in the body[13,15,16] and is not an isolated system, but it influences and is influenced by many other signalling pathways[17], as showed in Figure 5. In fact, endocannabinoids can activate different receptors and the pathways involved in their synthesis and metabolism are often shared with other mediators, so, the ECS is considered to be part of an expanded signalling system, called "the endocannabinoidome".[18] The ECS maintains homeostasis in brain, skin, digestive tract, liver, and in respiratory, cardiovascular, and reproductive systems, by regulating brain development, neurotransmitters, and cytokine release from microglia. ECS regulate anxiety, feeding behaviour/appetite, emotional behaviour, depression, reward, cognition, learning, memory and pain sensation.[13,14] ECS is involved in developing nervous system, and in the mature nervous system it modulates neuronal activity and network function[17], regulating nervous functions, neurogenesis and neuroprotection[13]**.** An alteration in the endocannabinoid signalling has been associated with many diseases[15,19], such as neurodegenerative disorders, multiple sclerosis, inflammation, epilepsy, schizophrenia, glaucoma, cardiovascular diseases, obesity and cancer.[14,15]

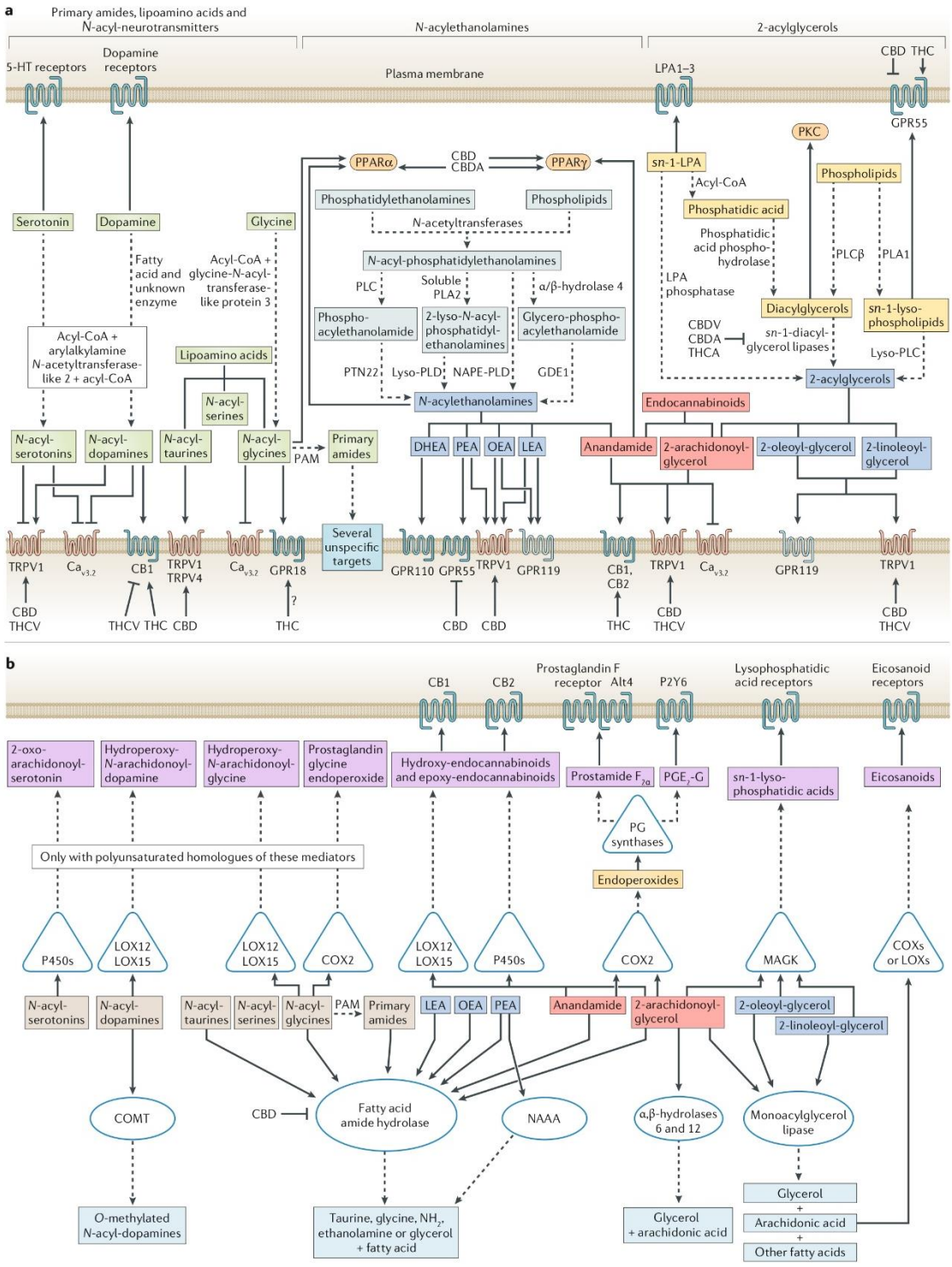


Figure 5. The expanded endocannabinoid system [18]

1.2.1.Cannabinoid receptors

CB1 and CB2 are the canonical cannabinoid receptors (CBRs).[15] They are G protein-coupled receptors (GPCRs), a class of cell surface proteins important for transducing signals from the extracellular space to intracellular signalling molecules. [16] CB1 and CB2 receptors primarily couple to inhibitory G proteins (Gi/o),

and once activated, they modulate many cellular functions through receptor internalization, interaction with other GPCRs, inhibition of adenylyl cyclase activity, changing the activity of calcium and potassium channels, increasing phosphorylation of various mitogen-activated protein kinases (MAPK), and many other functions.[20] CB1 and CB2 receptors have functional selectivity, that means that different ligands can favour a different conformation of the receptor, leading to the activation of a different signalling pathway.[17] So, agonists with a different structure may stimulate different pathways, leading to divergent biological effects[17]. Moreover, ligands have different intrinsic efficacy, so they can activate in a high or low way a particular signalling pathway. For example, THC is a CB1 agonist with low efficacy, while 2-AG with high efficacy. [17] Also, some ligands can bind allosteric sites of receptors, rather than the canonical site (orthosteric site), leading to alteration of the affinity and/or efficacy of the ligand. GPCRs can act not only as monomeric proteins, but can dimerize or multimerize to form homomers, or heteromers with other GPCRs, and an allosteric modulator can modify the affinity/efficacy of a ligand in the heteromers. In this regard, cannabinoid receptors have been demonstrated to form GPCR homomers and heteromers. In particular, CB1 receptor showed to form homomers but also heteromers with adenosine A2A receptors, dopamine D2 receptors, opioid μ and δ receptors, orexin OX1 receptors, angiotensin AT1 receptors, CB2 receptor, adrenergic β2 receptor, 5HT2A serotonin receptor, GPR55 receptor. Less is known about heteromers of CB2 receptor, but there is evidence of interaction with GPR55 and CXCR4.[16,17]

CB1 receptors are mainly present in the central nervous system (CNS) where they modulate the release of neurotransmitters, but also in peripheral tissues including liver, heart, skeletal muscle, adipose tissue, gastro-intestinal tract and skin. [17,18,21] This receptor is highly present in GABAergic neurons, but is found also in glutamatergic, cholinergic, glycinergic, serotonergic neurons.[17] In the CNS CB1 is mostly located presynaptically in excitatory and inhibitory neurons[18], reflecting its major role in modulating synaptic transmission[17]. For example, CB1 can inhibit voltage-gate Ca^{2+} channels and inhibit vesicular release of GABA and glutamate, thus giving to endocannabinoids, mainly 2-AG, through CB1, the role of inhibitory retrograde neuromodulators that can modulate the excitatory or inhibitory neurotransmission. In accord, in neurological disorders it was observed alterations of CB1 function. CB1 is expressed also postsynaptically where it can change the expression of precursors of appetite-controlling peptides in the hypothalamus. Moreover, postsynaptic CB1 was found in the external membrane of mitochondria where it inhibits electron transport and the respiratory chain, thus affecting brain metabolism and memory formation.[18] CB1 is expressed also by astrocytes of some brain area, while the expression on oligodendrocytes and microglia is less frequent.[17] Activation of CB1 was also shown to stimulate proliferation of adult stem cells and their differentiation into neurons or astrocytes, thus confirming that alterations in CB1 can be involved in neurodegenerative disorders.[18] CB2 receptors are instead mainly expressed in peripheral immune cells[17,18], but also in microglia,[17] although their expression has also been observed in the CNS [22] and other tissues. Therefore, the role of CB2 is primarily the immune modulation and modulation of inflammation.[18,21] The activation of microglial CB2 receptor is usually anti-inflammatory[17] and, in accordance, studies showed that CB2 reduced pro-inflammatory cytokine

release from activated microglia.[18] CB2 activation showed to stimulate adult neurogenesis, as CB1 activation does, and it probably regulates the blood-brain barrier permeability.[18]

Other receptors are included in the ECS because have been reported to mediate cannabinoid activity, and are called non-canonical CBRs. They are ion channels such as the transient receptor potential (TRP) channels, in particular the TRP vanilloid (TRPV), TRP ankyrin (TRPA) and TRP melastatin (TRPM) subfamilies (TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8), or GPCRs, such as GPR55, GPR18, GPR119, and nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs, PPARα and PPARγ).[13,15,16]

TRP channels are a family of trans-membrane ion channels activated by heterogeneous stimuli, including endogenous and exogenous chemical mediators and physical stimuli.[23,24] TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, and TRPM8, in particular showed to be activated also by cannabinoids and are called "ionotropic cannabinoid receptors" [24] TRP channels were showed to mediate various physiological and pathological functions, such as pain, temperature sensation, vision, inflammation, pressure and cancers.[23,24] In fact, they mainly mediate calcium influx into the cells and cytoplasmic calcium level is critical for regulating many cellular processes, such as motility, secretion, action potential generation and propagation, the release of neurotransmitters, and gene expression.[23] TRPV1, TRPV2, TRPA1 and TRPM8 are expresses in neurons, TRPV3 is expressed in the dorsal root ganglia, trigeminal ganglia, and in the brain, but also peripheral tissues such as testis, skin and tongue. TRPV4 is widely expressed throughout the body and can be found in the CNS, epithelial cells, osteoblasts, blood vessels, and other tissues including those of the heart, liver, and kidney.[24]

PPARα is distributed in metabolically active tissues, such as liver, heart and muscle and it controls fatty acid catabolism and inflammatory processes. PPARγ isoforms instead are expressed ubiquitous, in adipose tissue, in macrophages and involved in adipocyte formation, insulin sensitivity and inflammation.[21] PPARγ showed to be expressed in neurons, astrocytes and microglia in the brain, and have anti-inflammatory and neuroprotective effect during acute and chronic neuroinflammatory insults, and is involved in neuronal differentiation.[18]

GPR55 showed to be expressed in different tissues comprised brain, spleen, bones, adipose tissue, gastro-intestinal tract and was seen to contribute to vascular functions, bone turnover, motor coordination, and to have some implications in neuropathic/inflammatory pain, neurological disorders and metabolic/immune dysregulation.[21] It showed to stimulate excitatory hippocampal neurons.[18]

However, it has been documented also receptor-independent effects of endocannabinoids.[16]

1.2.2. Endocannabinoids

Endocannabinoids (eCBs) are the endogenous ligands of CBRs and the first discovered, most known and most abundant are anandamide (N-arachidonoyl ethanolamine, AEA) and 2-arachidonoyl glycerol (2-AG).[16,17] AEA belongs to N-acylethanolamines (NAEs) family of lipids, while 2-AG belongs to the 2-acylglycerols (2-AcGs) family of lipids.[19] They are synthetised from membrane phospholipids precursors[17,21] and are derivatives of ω-6 arachidonic acid (ARA), an essential polyunsaturated fatty acid (PUFA).[16] In particular, 2-AG is synthetized

from arachidonoyl-containing phosphatidyl inositol bis phosphate through the action of phospholipase C (PLC) that removes inositol triphosphate and then through the action of diacylglycerol lipase (DAG lipase, DGLα or DGLβ) that removes the acyl group in the 1 position. AEA is produced by the hydrolysis of N-arachidonoyl phosphatidyl ethanolamine by a *N*-acylphosphatidylethanolamine (NAPE)-specific phospholipase d-like hydrolase (NAPE-PLD).[16,17,19] 2-AG and AEA can activate many GPCRs, nuclear receptors, and ion channels.[17] In particular, 2-AG and AEA showed to activate CB1 and CB2 receptors with high affinity and efficacy. Moreover, AEA can activate TRPV1, PPARα, PPARγ and inhibit TRPM8 receptor, while 2-AG can activate TRPV1, PPARα, PPARγ and $GABA_A$ receptors.[18,21] Both AEA and 2-AG showed to modulate GRP55.[21]

Other unsaturated lipid-based molecules discovered and classified as eCBs are 2-arachidonoyl glyceryl ether (noladin ether, 2-AGE), O-arachidonoylethanolamine (virodhamine), N-arachidonoyl dopamine (NADA), oleic acid amide (oleamide, OA), N-oleoyl dopamine (ODA), homo linoleoyl ethanolamide (HEA), linoleoyl ethanolamide (LEA), N-stearoyl ethanolamide (SEA), docosa tetraenyl ethanolamide (DEA), N-palmitoyl ethanolamide (PEA), N-oleoyl ethanolamide (OEA), eicosapentaenoyl ethanolamide (EPEA), sphingosine, hemopressin.[15,16,25] Some eCBs are showed in Figure 6.

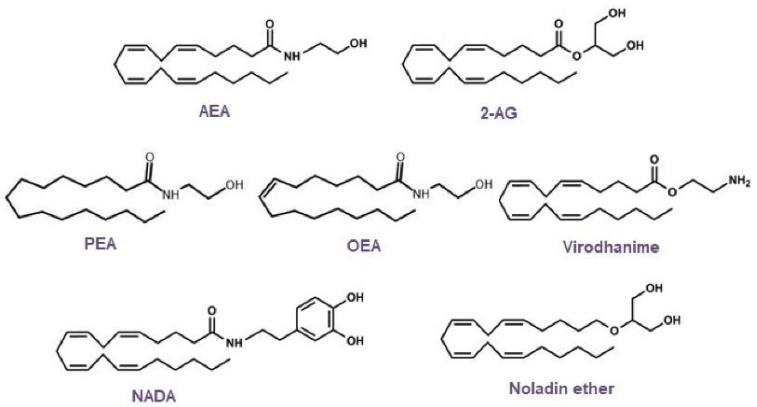


Figure 6. Chemical structures of the main eCBs.[26]

The synthesis of eCBs is "on demand", in fact they are released after a specific endogenous or environmental stimulus that compromise internal homeostasis, to exert a regulatory role of adaptive cellular response[16,17] eCBs effect is mostly restricted locally, in the site of their biosynthesis and release, and after exerting their effect on the receptors of the postsynaptic neurons, they are transported back to the presynaptic neuron and subjected to enzymatic hydrolysis. [16] This on-demand effect of eCBs means that they are released in a precise temporal and spatial manner, on the contrary of what happens when exogenous cannabinoid ligands are administered, activating receptors indiscriminately in space and time (minutes or longer for exogenous cannabinoids, seconds or less for eCBs). Therefore, the effect of administered cannabinoids may differ from the effect of physiologically released eCBs.[17]

After their synthesis from phospholipids in the inner side of the membrane, eCBs can't pass across cell membrane by simple diffusion, but they need transporters.[16,17] There is evidence that AEA and 2-AG are

transported by the same endocannabinoid membrane transporter (EMT), and that these latter are ATP- or Na^{2+}-requiring transporters.[17] In particular, heat shock proteins, serum albumin and other fatty acid binding proteins such as FAAH-like anandamide transporter (FLAT), cholesterol and ceramides, have been described as EMT.[16] Moreover, since eCBs degrading enzymes are primarily intracellular, transporters are necessary to bring eCBs back in cells too.[16,17] The degradation of AEA occurs in the endoplasmic reticulum through the hydrolysis of the arachidonic group from the ethanolamine by fatty acid amide hydrolase (FAAH), while the degradation of 2-AG is the hydrolysis of the arachidonic group from the glycerol by, primarily, monoacylglycerol lipase (MAGL), and, secondary, by FAAH and some serine hydrolases (ABHD6, ABHD12).[16,17,19] The arachidonic acid liberated can be a substrate for cyclooxygenases (COXs) to produce prostaglandin.[17] Also, the COX-2, 12- and 15-lipoxygenases and some cytochrome p450 oxygenases can directly degrade AEA and 2-AG, directing eCBs to alternative catabolic routes and generating the corresponding prostaglandin ethanolamines and prostaglandin glycerol esters, respectively, thus contributing to inflammation.[17,21,25] Thus, the degradation of the eCBs is not only the end of a signal, but can be the transition to a different type of signalling,[17] and can cause pro-inflammatory effects.[19]

1.3. Phytocannabinoids

Phytocannabinoids are molecules isolated from *Cannabis sativa L.,* a genus within the family Cannabaceae.[27,28] Cannabis contains more than 500 compounds, included phytocannabinoids, terpenoids and flavonoids, and the phytocannabinoids isolated are more than 100.[14,28] Phytocannabinoids are stored in glandular trichomes, located all over the aerial part of the plant and in higher density in the female flower. The root does not produce and accumulate phytocannabinoids.[14,27,28] Phytocannabinoids are classified into cannabigerols (CBGs), cannabichromenes (CBCs), cannabidiols (CBDs), (-)Δ^9-trans-tetrahydrocannabinols (Δ^9THCs), (-)Δ^8-trans-tetrahydrocannabinols (Δ^8-THCs), cannabicyclols (CBLs), cannabielsoins (CBEs), cannabinols (CBNs), cannabinodiols (CBNDs), cannabitriols (CBTs), and the miscellaneous cannabinoids.[27] The most abundant and also pharmacologically relevant phytocannabinoids are Δ9-tetrahydrocannabinol (Δ^9THC or THC) and cannabidiol (CBD), followed by cannabichromene (CBC) and cannabigerol (CBG), that are all neutral molecules, together with their respective acid forms (THCA, CBDA, CBCA, and CBGA).[14,27] The main phytocannabinoids are showed in Figure 7.

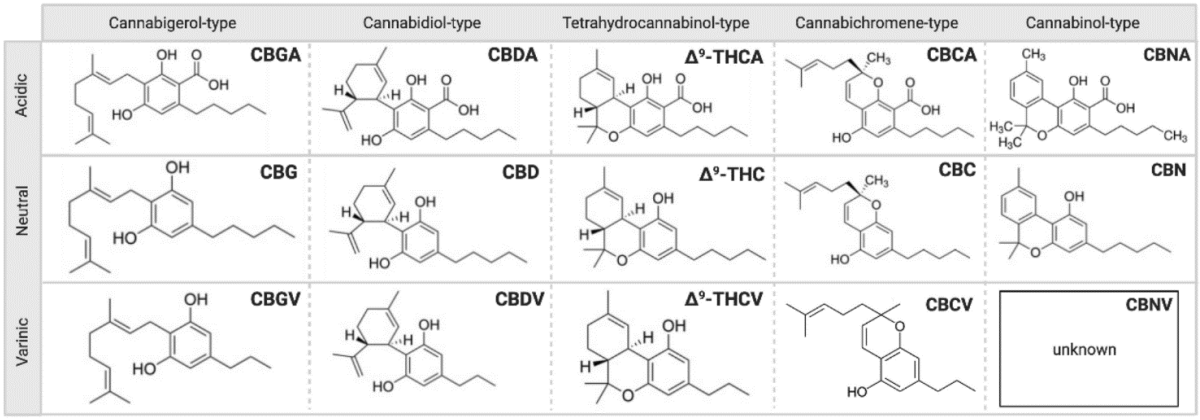


Figure 7. The chemical structures of CBG, CBD, THC, CBC and CBN-type neutral, varinic and acidic phytocannabinoids.[29]

The biosynthesis of phytocannabinoids is split between different cell types and organelles: the cytosol of the gland cells, the plastids, and the extracellular storage cavity (Figure 8).[27,28] In the cytosol, the precursor molecule hexanoic acid is generated from fatty acids, such as palmitic acid, through oxidative cleavage, in fact they are sequentially desaturated, peroxygenated and cleaved into a C6 (the hexanoic acid) and another product by the action of desaturases, lipoxygenases, and hydroperoxide lyases, respectively. Then the hexanoic acid is converted into the activated thioester hexanoyl-CoA, reaction catalyzed by acyl-activating enzyme 1 (AAE1). Then there is the elongation with malonyl-CoA as a C2 donor in a reaction catalyzed by olivetol synthase (OLS). Finally, cyclization by olivetolic acid cyclase (OAC) produces olivetolic acid (OA). OA is transported in the plastids and here OA is prenilated with geranyl diphosphate (GPP) by cannabigerolic acid synthase (CBGAS) catalyzes, forming the cannabigerolic acid (CBGA) that is the first cannabinoid compound and the direct precursor for the other cannabinoids. CBGA is transported in the extracellular space and is converted by the flavoproteins Δ9-tetrahydrocannabinolic acid synthase (THCAS), cannabidiolic acid synthase (CBDAS), cannabichromenic acid synthase (CBCAS) to Δ^9-THCA, CBDA and CBCA respectively, through an oxidative cyclization reaction, via reduction of molecular oxygen (O_2), that generate hydrogen peroxide (H_2O_2) as a side-product. Δ^9-THCA, CBDA, and CBCA are the end-products of the enzymatic biosynthesis of cannabinoids with a pentyl side chain. When exposed to heat, radiation, or spontaneously during storage, the compounds undergo decarboxylation and spontaneous rearrangement reactions, generating the neutral compounds CBG, THC, CBD and CBC.[27,28] CBN is obtained by the degradation of Δ^9-THC.[14]

Cannabinoids with unusual alkyl side chains (C1–C4) are produced by the same enzymes, but from the respective short-chain fatty acyl-CoAs and with lower affinity (acetyl-CoA, propanoyl-CoA, butanoyl-CoA, or pentaoyl-CoA, respectively, instead of hexanoil-CoA). It is the case of CBDV.[27]

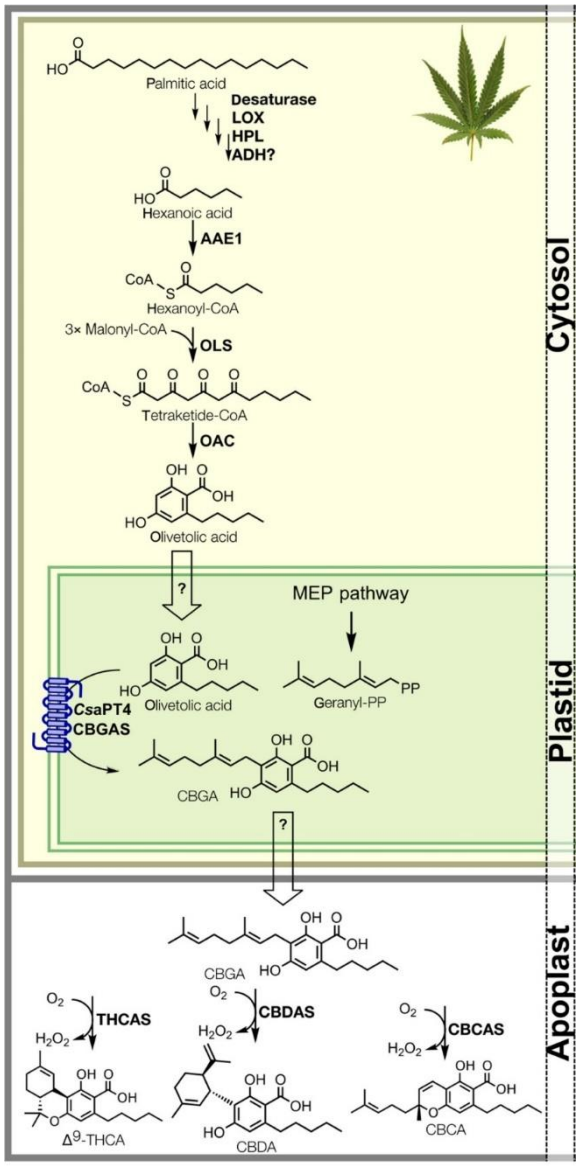


Figure 8. Phytocannabinoid biosynthesis in *Cannabis sativa* [27]

Phytocannabinoids play a role in human physiological processes, mainly through their interaction with GPCRs CB1 and CB2, TRP ion channels, and PPARs receptors, like eCBs.[27,28]

THC is the major psychotropic component of *Cannabis sativa L.* [14,27,30] and this effect is due to its agonist effect on CB1 receptor, which activation in the CNS leads to the classical psychoactive effect of cannabis, hypolocomotion, hypothermia, catalepsy and analgesia.[14,27] On the other hand, THC has neuroprotective, antispasmodic and anti-inflammatory effect, by the activation of other receptors, mainly CB2 and PPARs.[14,27] THC showed also anti-cancer effects *in vitro*, *in vivo* in mouse models and also in clinical trials.[14] THC is a partial agonist of CB1 and CB2 receptors, so it can have an agonist or antagonist effect, depending on the cell type, expression of receptors, and presence of endocannabinoids or other full agonists.[14] THC acts as a potent agonist for GPR18 and GPR55 receptors, and agonist for TRPV2, TRPV3 and TRPV4 channels, while it does not affect TRPV1, and is agonist of PPARs, specifically PPARγ.[14] THC is mainly administered orally and,

thanks to his lipophilicity, it is highly bound by plasma proteins and readily distributed to vascularized tissues such as liver, heart, spleen and lungs, and it accumulates in adipose tissues and spleen.[14,30] Taken orally, THC usually peaks in the circulation within 1–2 h, and it readily crosses the blood–brain barrier and can be found in high quantities in the brain. THC is metabolized in the liver.[14]

CBD is a non-psychotropic cannabinoid [14,27] that does not affect motor and cognitive functions or body temperature.[14] CBD acts on the CNS and in periphery by the action mainly on CB2 receptors and works as an inverse agonist or antagonist.[14,27] CBD showed inti-inflammatory, anticonvulsive, antianxiety, antipsychotic, antinausea, anti-oxidant, analgesic and immunomodulatory effects and showed curative effects in diabetes, neurodegenerative disorders and inflammation.[14,27] Many studied suggested an anti-cancer effect of CBD *in vitro*, *in vivo* in animal models and also in clinical trials.[14,30] CBD showed to reduce the psychotic effects of THC, such as tachycardia, anxiety, and hunger [14,19,27] and to inhibit the cellular uptake of the endogenous CB1 receptor ligand, AEA, directly affecting endocannabinoid tone.[14] CBD is also an agonist of PPARγ, TRPV1, TRPV2, [14,20] TRPV3, TRPA1 [20] and a GRP55 antagonist.[19,20] CBD also worked as an allosteric modulator of the μ-opioid receptor,[27] and to act as 5-hydroxytryptamin (5HT1A) agonist, inhibiting the uptake of serotonin, noradrenaline, dopamine and GABA, contributing to its anxiolytic properties.[14] In addition, CBD showed receptor-independent effects, such as the inhibition of adenosine uptake that contribute to anti-inflammatory and immunosuppressive effects.[14] CBD is most commonly administered orally and is usually prepared as an oil.[30] CBD is highly lipophilic, has a poor oral bioavailability, accumulates in body fat, is metabolized in the liver and the intestine by cytochrome P450 and others, and is excreted through feces (84%) and urine (8%).[14]

Little is known about the other phytocannabinoids.
CBG is another non-psycothropic phytocannabinoid [14,27,30] with low affinity for CB1 and CB2 receptors,[14,27] but significant activity on TRP channels, being agonist of TRPV1 and TRPA1, and potent inhibitor of TRPM8.[27] Moreover, CBD inhibits AEA uptake, affecting the ECS.[14] CBG can also activate the α2 adrenergic receptor, slightly block the serotonin 5HT1A receptor and potentially target COX-1 and COX-2 enzymes. CBG was shown to exert antibacterial, antioxidant and antiproliferative effects, showing potential anticancer effects *in vitro* and *in vivo* animal models.[14,30]

CBC is a non-psychotropic phytocannabinoid [14,27,30] with low/absent interaction with CB1 and CB2 receptors, but it inhibits eCBs inactivation and AEA uptake, and can act on TRP channels.[14,27] It is a potent agonist of TRPA1, while with lower potency activate TRPV3 and TRPV4 and block TPRM8.[14] CBC showed antinociceptive and anti-inflammatory effects *in vitro* and *in vivo* [14] and antiproliferative effects *in vitro* on cancer cells.[14,30]

CBN is the degradation product of THC [14] and has weak psychoactive effect, estimated to be 10 times lower than THC.[14,30] CBN has higher affinity for CB2 than CB1 receptor, is potent agonist of TRPA1 and antagonist of TRPM8.[14] Evidences showed CBN anticancer effects *in vitro*.[14,30]

CBDV's structure is similar to cannabidiol except with a shortened side chain and is another non-psychotropic cannabinoid.[14,30] It has weak affinity to CB1 receptor, while more activity on CB2 receptors. It showed to be an antagonist of GPR55 receptor and an agonist of TRPA1, TRPV1, and TRPV2 channels. Moreover, CBDV showed to be partial agonist for dopamine D2-like receptors. CBDV showed anticonvulsant effects and inti-inflammatory effects on intestinal inflammation [14] and antiproliferative effects *in vitro* on cancer cells.[14,30]

1.4. Endocannabinoid system as pharmacological target

The ECS showed to be a good pharmacological target for many diseases, including general pain, headache, migraine, glaucoma, mood and anxiety disorders, obesity/metabolic syndrome, osteoporosis, neuromotor, neuropsychological and neurodegenerative diseases, respiratory diseases such as asthma, cardiovascular diseases such as stroke, atherosclerosis, myocardial infarction, metabolic disorders, arrhythmias, and hypertension.[13]

For example, ECS is thought to ameliorate neuropathic disorders including anxiety, depression and post-traumatic disorder, through the activation of CB1 receptor in peripheral and central nervous system.[13,19] In fact, CB1 receptor is densely populated in the brain, in areas responsible for the mediation of reward, such as the amygdala, hippocampus, and orbitofrontal cortex. Also, the ECS potentially modulates synaptic transmission of neurotransmitters, such as mesocorticolimbic dopamine, acetylcholine, glutamate, opiate peptides, and GABA, which play significant roles in the control of emotions and behaviours.[13]

ECS can be targeted to manage pain, included cronic, neuropatic and inflammatory pain, through CB1,[13,19] CB2, GPR55, GPR12, opioid/serotonin, TRPV1 and PPARγ receptor.[13]

Cannabinoids are well-known anti-emetic drugs, mainly by the action on CB1 receptor, which attenuate the emetic reflex by inhibiting the release of excitatory transmitters. Indeed, CB1 receptor was found on dopaminergic, noradrenergic, and other neurons situated in the brain regions regulating nausea and vomiting.[14] Moreover, antinausea effect of cannabinoids could also be due to a direct effect on gastrointestinal function.[13]

ECS can be targeted to modulate inflammation and treat inflammatory disease, mainly through the activation of CB2 receptor, expressed in particular in immune tissues and cells.[19] Cannabinoids, in fact, showed to be potent anti-inflammatory agents, and endocannabinoids, such as AEA and 2-AG, and phytocannabinoids, such as THC and CBD, have demonstrated anti-inflammatory and immune-suppressive properties via CB1 and CB2 receptors. Cannabinoids showed to downregulate cytokine and chemokine production thus suppressing inflammatory responses.[13]

ECS can be targeted to manage also neurological diseases, such as epilepsy and neurodegenerative diseases, included Parkinson's and Alzheimer's disease, that are characterized by inflammation and dysregulation of the neurons function. In fact, cannabinoids are known to play a role in the modulation of inflammation (neuroinflammation), and to enhance neuroprotection. What about epilepsy, many scientific evidences confirm the success of CBD in reducing the frequency of seizure episodes.[13,14] Also, the ECS implication in the

modulation of the blood–brain barrier, is another reason for ECS to be a potential target for the management of neurological or psychiatric diseases such as schizophrenia and epilepsy.[13]

In addition, ECS can be targeted to treat some autoimmune disorders, since it has been implicated in immunoregulation. Phytocannabinoids (such as THC and CBD) have demonstrated immunosuppressive properties, mainly through CB1 receptor, and could have a beneficial effect in these pathologies also thanks to analgesic effect.[13,19] Some examples are rheumatoid arthritis and multiple sclerosis, in which THC and CBD ameliorate neuropathic pain and spasticity. Moreover, in inflammatory bowel diseases CBD showed therapeutic potential, since components of the ECS are distributed in colonic tissue and CB1 receptors are distributed throughout the enteric nervous system and the gut-brain axis, a communication network between the brain and the gut.[13]

Components of the ECS are found also in the skin and evidences showed that cannabinoids have therapeutic effects against skin lesions, skin burns and pruritus in several dermatologic diseases such as allergic contact dermatitis and atopic dermatitis. The ECS was showed to maintain skin homeostasis by regulating many aspects of cell proliferation, differentiation, and inflammatory signalling.[13,14]

1.4.1. Approved cannabinoid drugs

Dronabinol (Marinol®) is a drug containing synthetic THC, used as antiemetic for patients receiving cancer chemotherapy and as an appetite stimulant for people with acquired immunodeficiency syndrome (AIDS). Nabilone (Cesamet®) is a drug containing a synthetic derivative of THC, that showed efficacy as antiemetic in cancer patients and recently for neuropathic and chronic pain, and for spasticity related to multiple sclerosis.[14,27,31] Both compounds are approved from FDA are available in many European countries, but not in all the member states of the European Union because they didn't pass a centralized authorization procedure.[14,31]

Epidiolex® is a CBD containing drug approved from FDA and EMA for the treatment of two forms of severe and resistant childhood epilepsy.[14,19,27,32]

Nabiximols (Sativex®) is a 1:1 ratio of standardized extracts from two different cultivars of *C. sativa* enriched in CBD and THC, approved from FDA and EMA for multiple sclerosis related spasticity and in Canada for neuropathic pain associated with multiple sclerosis and cancer.[14,19,32]

Moreover, in Italy other commercial products with standardized concentrations of THC and CBD are available, under the trade names of: "Bedrocan®" (22% THC and CBD < 1%), "Bedrobinol®" (13.5% THC and CBD < 1%), "Bediol®" (6% THC and 8% CBD), "Bedrolite®" (THC < 0.4% and 9% CBD) and "Bedica®" (14% THC and CBD < 1%).[33] Also, two different types of cannabis produced in the Stabilimento Chimico Farmaceutico Militare in Florence are available, "cannabis FM2" (5–8% THC and 7.5–12% CBD), introduced in December 2016, and "cannabis FM1" (13–20% THC and CBD < 1%), available from July 2018.[33]

1.4.2. Endocannabinoid system as target for cancer therapy

Cannabinoids are already used in cancer patients who receive chemotherapy and radiotherapy, for their palliative properties, such as analgesic, antinauseant, antidepressant and antiemetic effects.[13] However, after the first studies showing tumour-regressive effects of cannabinoids on 1975,[25] always more studies have analysed the proper anticancer effect of cannabinoids in last years.[13,25] In accord, if we consider the complexity of the ECS and the distribution of its component in all the organism, it is sensible that cannabinoids potentially interfere with many cancer-related signalling pathways.[15] In fact, cannabinoids showed to have anticancer effect through many mechanisms of action, such as inhibition of tumour cell growth, by inhibition of proliferation, blockade of cell cycle, induction of cell death, inhibition of cancer cell invasion and metastasis, inhibition of tumour angiogenesis and interaction with the immune system.[13,15,16,20,25,34,35] The anticancer effect of cannabinoids has been demonstrated in many cancer models such as breast, lung, prostate, testicular, endometrial, gastric, pancreatic, skin, colon, bone cancer, multiple myeloma, glioblastoma, lymphoma, leukaemia and neuroblastoma, *in vitro* and in some *in vivo* animal model [13,15,36,37] and also in some clinical trials.[14] Cannabinoids showed to mediate these antitumour effect via receptors, but also independently of receptors.[25] Together with the use of exogenous cannabinoids (both phyto- and endo-cannabinoids), new strategies to target the ECS comprise the use of agents that inhibit endocannabinoid-degrading enzymes.[19,25] Indeed, inhibiting FAAH and MAGL not only activate cannabinoid receptor through the increased presence of 2-AG and AEA, but also reduces the formation of tumour-promoting fatty acids.[25]

Many studies observed an altered expression of the ECS components in tumour tissues. Anyway, the relation between the expression of ECS components and the tumour progression, is not clear. Results are contradictory, in fact the expression of endocannabinoids, cannabinoid receptors, and endocannabinoid-degrading enzymes has been found to be upregulated in cancer tissues compared to healthy tissues in some studies, while downregulated in other studies, and sometimes high levels correlated with better prognosis, and other times correlated with worst prognosis. Thus, the expression of ECS components can't be a marker for predicting tumour progression and for prognosis. Anyway, lot of works suggest an overall increased expression of ECS components in cancer tissues.[16,25,34]

Mechanisms and pathways by which cannabinoids were found to induce anticancer effect in many publications in years, are the following:

- Inhibition of tumour cell proliferation

Publications showing cancer growth inhibition effect of cannabinoids are a lot.[25,35]

Regulation of the cell cycle is important for the survival of a cell, including the detection and repair of genetic damage and the control of correct cell division. Several studies have demonstrated that cannabinoids mediated cell cycle dysregulation and inhibition of proliferation in cancer cells.[34] For example, proliferation was inhibited after cell cycle blockage by cannabinoids in the G1/S transition, through downregulation of cyclin-dependent kinase 1 (CDK1), induction of p21, or induction of p27kip1, decrease in cyclin A and E, degradation

of cell division cycle 25 A and inactivation of CDK2.[35] Also, WIN55 blocked cell cycle in the G0/G1 phase via CB_2 receptor, bringing upregulation of p27 and reduction of CDK4 expression and phosphorylated retinoblastoma protein (pRb).[34,35] CBD had antiproliferative effect on human gastric cancer cells, accompanied by upregulation of ataxia telangiectasia mutated (ATM) gene and p21 protein expression and downregulation of p53 protein expression, which resulted in decreased levels of CDK2 and Cyclin E and cell cycle arrest in the G0/G1 phase.[34,35] THC, through activation of CB2 receptor, blocked cell cycle at the G2-M phase, by reduction of Cdc2 and induction of ROS synthesis, provoking cell death in breast cancer cell lines, while in MM cell lines, THC stopped cells in the G1 phase, and combined with CBD the effect was more evident.[34] In human breast carcinoma cell lines, AEA, through CB1 receptor activation, caused inhibition of cAMP synthesis and cell cycle arrest in the G1/S phase, while in human endometrial cell line, AEA, by a receptor-independent mechanism, induced an arrest in the G2-M phase through the inhibition of the Akt pathway.[34]

Inhibition of the pro-survival protein kinase B (PKB or Akt) appears to be an important mechanism driving growth inhibition. For example, in melanoma cells, cannabinoids induced cell cycle arrest at the G1/S transition via inhibition of the protein Akt and hypo-phosphorylation of pRb tumour suppressor protein. CB_2 receptor-mediated inhibition of Akt has been reported in breast cancer mouse model. Inactivation of the Akt pathway was also involved in the antitumour activity of cannabinoids on human gastric cancer, non-small cell lung cancer and hepatocellular carcinoma cells.[35]

CBD appears to inhibit cancer cell proliferation also via apoptosis signalling. In fact, CBD suppressed the proliferation and growth of head and neck squamous cell carcinomas by inducing the apoptotic and autophagy activity of Dual Specificity Phosphatase 1 (DUSP1), that interfere as a negative regulator with EGFR-initiated mitogen activated protein kinase (MAPK) signalling and associated proliferation.[35] (Figure 9)

- Induction of apoptosis

Cannabinoids activate apoptosis either through CB1 or CB2 receptors, other receptors, or receptor-independent mechanism.[34,35]

An important role in cannabinoid-induced apoptosis is played by the pro-apoptotic sphingolipid ceramide. In fact, THC and other cannabinoids induced glioma cells death via cannabinoid receptor-dependent de novo synthesis of ceramide.[35] For example, in rat glioma cells, THC via activation of CB1 receptor induced ceramide accumulation and Raf1/ERK activation.[34]

Another mechanism by which apoptosis is induced is via CB2 receptor in glioma cells, with following increase in the stress-associated transcriptional coactivator p8 as an upstream regulator of the endoplasmic reticulum (ER) stress-related proteins activating transcription factor (ATF)-4 and tribbles pseudokinase 3 (TRB3).[35]

The eicosanoid system also revealed to play an important role in the proapoptotic effect of cannabinoids. For example, in human cervical carcinoma cells after Met-AEA administration, the PPARγ-activating eicosanoids PGD2 and 15-deoxy-Δ12,14-PGJ2 were identified as apoptosis mediators.[34,35] Moreover, in tumour tissue of xenografted tumour mice model, CBD upregulated COX-2 and PPARγ and induced tumour regression that

was reversed by a PPARγ antagonist.[35] AEA induced apoptosis of nonmelanoma cancer cells via ER stress, because of its metabolization by COX-2 to the prostaglandin 15d-PGJ2-EA, which diminishes intracellular glutathione levels leading to oxidative stress.[34,35] AEA induced apoptotic death of human neuroblastoma and lymphoma cells also via TRPV1, by an increase in intracellular calcium, oxidative stress, cytochrome c release, activation of caspase. AEA reduced viability of cholangiocarcinoma cells via activation of GPR55, and subsequent activation of the death complex Fas/FasL.[34,35] In prostate cancer cells, AEA and 2-AG via CB1 receptor induced apoptosis, by increasing the levels of activated caspase-3, reducing the levels of Bcl-2, activating Erk pathway and inhibiting Akt pathway.[34]

CBD showed pro-apoptotic effect on human bladder cancer cells via activation of TRPV2. However, the pro-apoptotic effects of CBD have been associated mainly with receptor independent mechanisms. For example, CBD induced apoptosis of mammary carcinoma and human gastric cancer cells via the formation of reactive oxygen species (ROS). Moreover, CBD induced a CB2 receptor dependent mitochondrial apoptosis in human leukaemia cells, which was accompanied by ROS production, increased expression of the NAD(P)H oxidases Nox4 and p22phox and release of Cytochrome c. Also, CBD modulated the mitochondrial voltage-dependent anion channel (VDAC), inducing oxidative stress, mitochondrial Ca^{2+} overload, Cytochrome c release into the cytosol and induction of LC3-phosphatidylethanolamine conjugate (LC3-II) and caspase activation in leukaemia cells.[35] In gastric cancer, CBD promoted cell death by suppressing X-linked inhibitor apoptosis (XIAP) (by acting on Smac and resulting in increased XIAP ubiquitination, and by stimulation of ER stress-related genes) or by cleavage of PARP, caspase-3, caspase-8, caspase-9, increasing Noxa, Bax and decreasing Bcl-2 expression levels, determining an increase of mitochondrial membrane permeability and a decrease of mitochondrial transmembrane potential, ROS production, the release of cytochrome C into the cytosol.[34] In breast cancer CBD inhibited the survival and induced apoptotic cell death via the activation of caspase-8, the translocation of BID to the mitochondria, enhanced ROS generation, the release of cytochrome c and SMAC into the cytosol, and increased levels of Fas-L.[34]

THC, via CB1 receptor activation, inhibited both PI3K/Akt and RASMAPK/ ERK survival pathways in colorectal carcinoma cell lines and inhibited ERK and Akt signalling in human leukemia T cells. CBD with THC, CBG, CBN induced cell cycle block in G2 phase and consequent apoptosis in the breast cancer cell line, also by increasing levels of Endoplasmic Reticulum Chaperone Protein Glucose-regulated Protein 78 (GRP78), and consequent ER stress.[34] (Figure 9)

- Induction of autophagy

Autophagy can have a dual role of bringing cells to survival or death.[35] Many publications showed that cannabinoids can induce toxicity via autophagy signalling pathways in several cell lines and mouse models of cancer.[34,35]

As for apoptosis, also cannabinoid induction of autophagy can be due to ceramide accumulation. For example, THC induced ceramide accumulation, subsequent ER stress response and, via this, induction of autophagy via

the TRB3-dependent inhibition of the Akt/mTORC1 axis, that brought human glioma cells death.[34,35] Moreover, in glioma cells, the same pathway was modulated by THC with decreased phosphorylation of p70S6 kinase, a mTORC1 downstream target, and its substrate phospho-S6 ribosomal protein, causing upregulation of ER-stress-related TRIB3, mTORC1 inhibition, induction of autophagy, which led to apoptotic glioma cell death.[34]

THC and the CB2 agonist JWH-015 induced autophagy in human hepatocellular carcinoma cells due to upregulation of TRB3 and subsequent inhibition of the Akt/mTORC1 axis and adenosine monophosphate-activated kinase (AMPK) stimulation. In accord, tumour regressive effects of THC and JWH-015 in *in vivo* model of hepatocellular carcinoma were annulled when autophagy was genetically or pharmacologically inhibited.[35] In melanoma cells, THC-induced autophagy and cell death was annulled if autophagy was blocked.[35]

In medulloblastoma cell lines, CBD and THC induced increase in LC3-II levels as an autophagy marker, together with an increase in poly (ADP-ribose) polymerase (PARP) cleavage.[35]

In MM cell lines, THC and CBD increased the sub-G1 cell accumulation due to an autophagic-cell death process, showed by the conversion of LC3-I to form LC3-II.[34] (Figure 9)

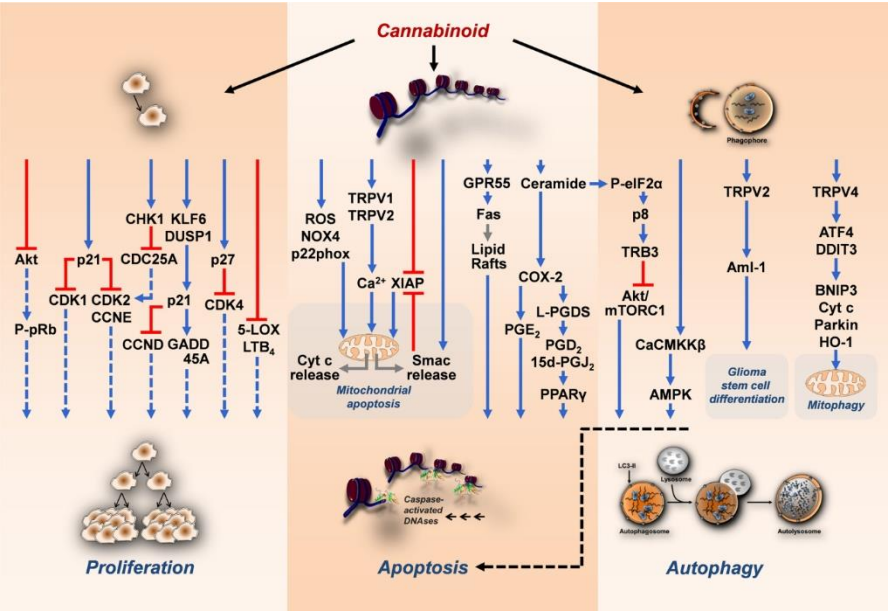


Figure 9. Mechanisms of anti-proliferative, pro-apoptotic and pro-autophagic effects of cannabinoids on cancer cells.[35]

- Inhibition of tumour cell invasion

A large number of publications show an anticancer effect of cannabinoids by inhibiting tumour cell migration, invasion and *in vivo* metastasis.[34,35]

The first studies to reveal these effects were about 2-AG that inhibited prostate cancer cell invasion through CB1 receptor, AEA that showed anti-invasive effect in glioma and lung cancer cells, and the AEA derivative Met-AEA that showed anti-invasive properties on human cervical and lung cancer cells via CB1, CB2 and TRPV1 receptors.[35]

Then studies explained that the anti-invasive effect of THC, Met-AEA and CBD in cervical and lung cancer cells occurred through CB1 and CB2 (for THC, Met-AEA, CBD) and TRPV1 (for Met-AEA, CBD) by induction of metallopeptidase inhibitor-1 (TIMP-1) expression, a protein that inhibits collagen-degrading enzymes such as matrix metalloproteinase (MMP)-2 and MMP-9, which contribute to promote cancer metastasis. AEA, OEA, 2-AG, MAGL inhibitor JZL184, FAAH inhibitors N-arachidonoyl-serotonin and URB597 showed anti-invasive effect through TIMP-1 induction too, in lung cancer cells.[35] THC administration downregulated TIMP-1 expression in mice bearing gliomas and also in two patients with recurrent glioblastoma multiforme.[34] The modulation of extracellular matrix proteolysis as anti-invasive cannabinoid action was demonstrated also by other publications. THC downregulated MMP-2 in glioma cells. CB1 (ACEA) or CB2 agonists (CB65) downregulated MMP-2 and -9 in hepatocellular carcinoma cells.[35] Synthetic cannabinoid agonist WIN-55,212–2 mesylate (WIN-55) downregulated MMP-2 and -9 thus blocking migration of osteosarcoma cells.[34,35]

What about other mechanisms, the CBD-THC combination was able to reduce cell migration of the MM cell line by downregulating expression of the chemokine receptor CXCR4 and the CD147 plasma membrane glycoprotein.[34] In human breast and prostate cancer cells, CB2 receptor formed heterodimer with CXCR4 receptor, and cannabinoids CB2-activator reduced CXCR4-mediated formation of phosphorylated p42/44 MAPK (P-p42/44) and thus reduced cancer cell chemotaxis.[35] In human non-small cell lung cancer cell lines, THC reduced the EGF-induced phosphorylation of ERK1/2, JNK1/2, and AKT, thus inhibiting growth, chemotaxis, and chemo-invasion via CB receptors. Moreover, THC inhibited lung cancer growth and metastasis in *in vivo* murine model.[34] THC anti-invasive effect on cholangiocarcinoma cells was associated with reduced activation of Akt and p42/44 MAPK.[35] CBD had inti-invasive effect in breast cancer, glioblastoma and salivary gland cancer cells, by downregulation of Id-1, an inhibitor of basic helix-loop-helix transcription factors. Moreover, CBD antimetastatic effect on breast cancer cells in a mouse model was linked to a downregulation of Id-1 too.[35] In human breast cancer cells Met-F-AEA inhibited adhesion and migration, via decreased tyrosine phosphorylation of focal adhesion kinase (FAK).[34]

JWH-133 (CB2 receptor synthetic agonist) and WIN55 (CB1 and CB2 receptors agonist) inhibited cell proliferation and migration *in vitro* and reduced tumour growth and metastasis in various *in vivo* mouse model, and the effect was reversed by CB1 and CB2 receptor antagonists.[34] CBD or a selective GPR55 antagonist decreased adhesion to endothelial cells and migration of metastatic colon cancer cell line, suggesting an involvement of GPR55 receptor.[34] Inhibition of cancer cell migration showed to be also receptor independent, as seen in bladder urothelial carcinoma after treatment with CBD alone or combined with THC.[35] (Figure 10)

- Inhibition of endothelial mesenchymal transition (EMT)

Many publications showed that cannabinoids can modulate EMT, a process by which cancer cells acquire migratory and invasive properties.[34,35]

Met-F-AEA reduced levels of β-catenin, one of the key factors involved in EMT, and inhibited mesenchymal markers such as vimentin, N-cadherin, fibronectin and EMT transcription factors as Snail1, Slug and Twist, and upregulated epithelial markers such as E-cadherin and cytokeratin 18.[35] CBD reversed IL-1β-induced EMT in human breast cancer cells, by relocalising E-cadherin and β-catenin at adherens junctions, thereby preventing nuclear translocation of β-catenin and inhibited the expression of the EMT marker ΔNp63, an isoform of tumour protein 63 (TP63), baculoviral IAP repeat-containing protein 3 (BIRC3) and Id-1.[35] THC and CBD on lung cancer cells reversed the TGF-β-induced inhibition of E-cadherin expression and upregulation of N-cadherin and vimentin [35] and reduced proliferation, EMT, and migration *in vitro* in lung cancer model.[34] AEA upregulated epithelial markers, such as E-cadherin, and reduced mesenchymal markers, including vimentin and SNAIL1 in human breast cancer cell line.[34] AM251 (CB1 antagonist and GPR55 agonist) suppressed EMT independently from receptors, by reducing the expression of several EMT transcription factors such as SNAIL1 and the AP-1 transcription factors FOSB and JUNB and downregulated collagen 1A1 and E-cadherin.[34] JWH-015 inhibited EMT by regulating EGFR signalling targets such as ERK and STAT3 in lung cancer cells. THC inhibited EMT and downregulated MMP 9 gene expression in aggressive human endometrial cancer cells.[34] WIN55 treatment downregulated COX-2 expression and decreased the phosphorylation of AKT, and inhibited EMT in human gastric adenocarcinoma cell line.[34] (Figure 10)

- Inhibition of tumour cell metastasis

Many studies also analysed the effect of cannabinoids in experimental metastasis models.

THC, CBD, AEA, 2-AG, Met-F-AEA, OEA, PEA and ECs degradation inhibitors (AA-5HT, URB597, JZL184) showed an inhibitory effect on metastatic infiltration of the lung after injection of lung carcinoma cells. The mechanism of CBD antimetastatic effect showed to be increased ICAM-1 expression, while binding to CB1 receptor was considered as an initial mechanism of the antimetastatic effect of Met-F-AEA and JZL184.[35] Also, cannabinoids inhibited cell metastasis of breast cancer, salivary gland cancer and melanoma cells.[35] JZL184 impaired bone metastasis of osteotropic prostate and breast cancer cells in mice and inhibited metastasis of osteosarcoma cells.[35] Knockdown of MAGL reduced lymph node metastasis in MAGL-overexpressing nasopharygeal carcinoma cells.[35] JWH-133 and WIN55 reduced tumour growth and metastasis in many *in vivo* mouse models.[34] (Figure 10)

- Inhibition of tumour angiogenesis

Several studies showed that cannabinoids inhibit tumour neovascularisation in mouse xenograft models.[35] Cannabinoids seem to downregulate proangiogenic parameters, mainly vascular endothelial growth factor (VEGF), but also placental growth factor (PlGF), angiopoietin-2 (Ang-2) and MMP-2.[34,35]

Cannabinoids down-regulated VEGF and the active forms of its main receptors VEGFR-1 and VEGFR-2 in skin carcinomas, gliomas, and thyroid carcinomas.[34] CB2 receptor agonist JWH-133 modulated hypoxia-related angiogenesis markers, such as downregulation of VEGF-A and -B, hypoxia inducible factor-1α (HIF-

1α), connective tissue growth factor (CTGF), Id-3, Ang-2. Moreover, JWH-133 down-regulated VEGFR-2 *in vivo* in glioma xenografts mouse model.[35]

Cannabinoids can have indirect effects on endothelial cells through the secretome of tumour cells. For example, conditioned media from AEA-treated breast cancer cells inhibited endothelial cell proliferation and VEGF concentration and other proangiogenic factors such as leptin and thrombopoietin (THPO). Also, conditioned media from CBD-, THC-, Met-AEA- and JWH-133-treated lung cancer cells had indirect effect on endothelial cell formation and migration, by increasing anti-angiogenic TIMP-1.[35]

CB1 inactivation with an antagonist, or CB1 knockdown, inhibited fibroblast growth factor (β-FGF)-induced proliferation, migration and capillary-like tube formation of human umbilical vein endothelial cells, through pathways involving decreased activation of focal adhesion kinase, c-Jun N-terminal kinase, RhoA and MMP-2. Moreover, CB1 antagonism *in vivo* inhibited β-FGF induced neovascularisation in the rabbit cornea.[35]

Moreover, ceramides can be involved also in anti-angiogenetic effect of cannabinoids. In fact, antitumour and anti-angiogenic effect of cannabinoids that target CB1 and CB2 receptors in glioma xenografts, and the reduced VEGF production by glioma cells *in vitro* and *in vivo*, was dependent of ceramide.[34] (Figure 10)

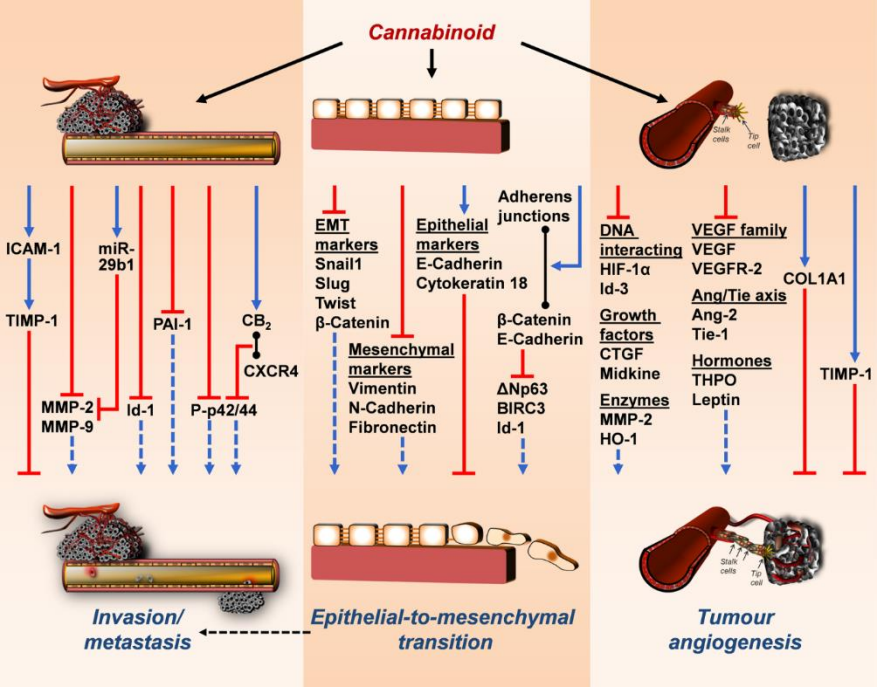


Figure 10. Mechanisms of anti-invasive, antimetastatic, anti-epithelial-to-mesenchymal-transition and anti-angiogenic effects of cannabinoids on cancer cells.[35]

- Interaction with immune system

The role of immune cells present in the tumour stroma was taken into consideration in relation to cannabinoid effects on cancer progression.[35]

Cannabinoids reduced expression of programmed death ligand 1 (PD-L1) via decreasing PAK1 activity, thereby enhancing immune checkpoint blockade of pancreatic cancer cells.[35] CB1 (ACEA) and CB2 agonists

(JWH-133) decreased tumour growth via inhibition of angiogenesis, through action on peripheral cells such as neutrophil and granulocytes, while CBD, THC and Met-AEA increased killer cell mediated lysis of lung cancer cells.[35] 2-AG modulated subpopulations of immune cells involved in the progression of pancreatic ductal adenocarcinoma in an orthotopic mouse model, in particular increased the proportion of $CD83^+$, $CD86^+$ and $MHCII^+$ cells in $CD11C^+$ cell populations in the spleen of mice.[35] WIN 55 inhibited cancer growth more efficiently in immunocompetent compared to immunodeficient murine melanoma models.[35] FAAH inhibitor URB597 suppressed the shedding of the proteins major histocompatibility complex class I polypeptide-related sequence A (MICA) and B (MICB) on the surface of human hepatocellular cells, due to increased expression of TIMP-3. This effect improved antitumour immunity, since MICA/B proteins are recognised by cytotoxic lymphocytes expressing the natural killer group 2D (NKG2D) receptor and tumour cells are subsequently eliminated. Similarly, AEA, 2-AG and the CB2 receptor agonist AM1241 enhanced TIMP-3 expression together with a reduction of MICA/B shedding.[35] (Figure 11)

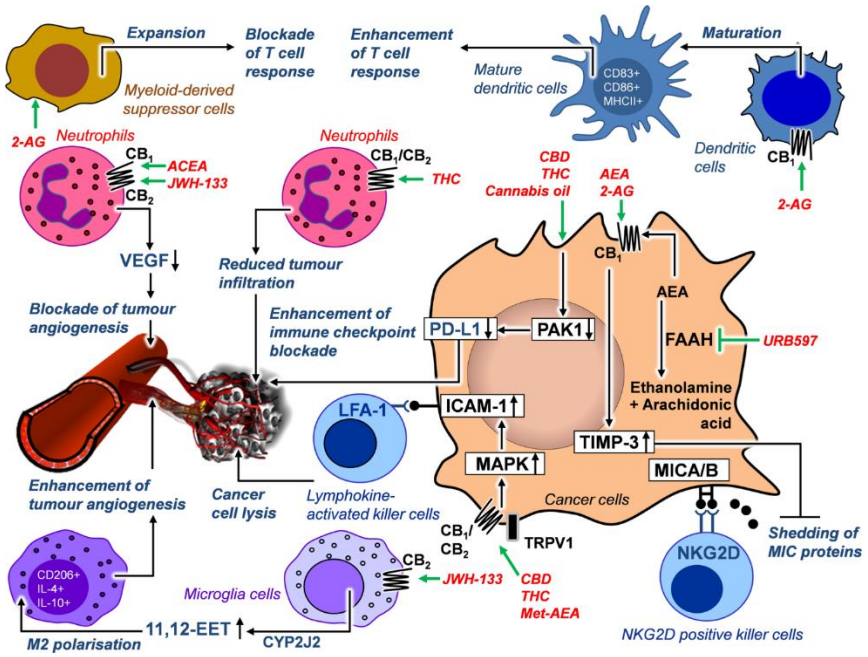


Figure 11. Effects of cannabinoid compounds on tumour-immune interaction [35]

- Increasing effect of anticancer agents

Cannabinoid compounds could be used combined with currently used anticancer agents, since are gaining successful clinical trials.[35]

THC and CBD showed to enhance the effect of various cytostatic drugs, such as vinca alkaloids, cytarabine, doxorubicin, mitoxantrone, carmustine, temozolomide (TMZ), bortezomib, carfilzomib and cisplatin.[35] For example, CBD and TMZ showed synergistic effect in reducing tumour size and prolonging survival in patient-derived neurosphere cultures and orthotopic mouse models.[35] Also, THC increased the sensitivity of TMZ-resistant glioma cells in mice models, and combined with CBD enhanced the action of TMZ in mouse models.[20]

Moreover, CBD enhanced the effect of cisplatin in glioblastoma cells and in a murine model of squamous cell carcinoma of the head and neck. CBD showed synergistic effects of cytotoxicity also in combination with cisplatin, 5-fluorouracil or paclitaxel on human squamous cell carcinoma cells of the head and neck.[35]

Regarding the mechanisms of these synergies, THC- and CBD-induced enhancement of vinblastine effect in resistant leukaemia cells was accompanied by downregulation of P-glycoprotein, while THC-induced enhancement of the cytostatic effect of cytarabine, doxorubicin and vincristine in resistant leukaemia cells was attributed to reduced p42/44 MAPK activity.[35] Also, CBD increased the uptake into and toxicity on glioma cells of doxorubicin, TMZ and carmustine via an increase in TRPV2 activity and associated increased calcium influx, and these results were obtained also with doxorubicin in triple negative breast cancer cells.[35] CBD and THC combined with bortezomib inhibited the expression of the immunoproteasome subunit β5i in MM cells. .[35] CBD showed to overcome oxaliplatin resistance of cancer cells via inhibition of superoxide dismutase 2 and activation of autophagic response.[20]

Moreover, THC and CBD increased sensitivity of glioma cells to ionising radiation, also in a murine model, and CBD effect was explained by inhibition of ATM kinase, a serine/threonine protein kinase that is recruited and activated by DNA double-strand breaks.[20,35]

An important aspect to consider in the combined administration of cannabinoids with chemotherapeutic drugs is their pharmacokinetics. In fact, *in vitro* studies showed that CBD can inhibit cytochrome P450, which is responsible for the metabolism of many drugs, including conventional chemotherapeutics. As a result, a high concentration of CBD may increase the toxicity and decrease the potency of standard anti-cancer therapy.[20] In this regard, a clinical trial found for example that nabiximols showed not to interact with TMZ pharmacokinetic in glioblastoma patients.[38]

1.4.3. Evidences of anticancer effect of minor cannabinoids cannabigerol, cannabichromen, cannabinol and cannabidivarin

THC and CBD have been deeply studied in years for their anticancer effect *in vitro*, *in vivo*, as already described, and also in clinical trials, as will be showed later. Instead, the minor cannabinoids are less studied.

What about CBG, studies showed that it reduced viability of human pancreatic ductal adenocarcinoma,[39] human breast cancer cells,[40,41,42] human oral epithelioid carcinoma cells,[43] human colorectal cancer cells,[42,44] human gastric adenocarcinoma cells,[42] human cholangiocarcinoma cells,[45] human glioblastoma cells and glioma stem cells,[46] human mesothelioma cells,[47] and human prostate carcinoma.[42,48] Moreover, CBG showed to induce apoptosis, ROS production, and *in vivo* reduced tumour growth in a xenograft mouse model of colon adenocarcinoma.[44] CBG induced autophagy by inhibition of EGFR and Akt/mTOR pathway, and subsequent apoptosis, reduced RAS downstream pathway and increased the cytotoxicity of chemotherapeutic drugs in pancreatic ductal adenocarcinoma.[39] CBG combined with CBD CBN and THC induced cell cycle arrest in G2 phase, apoptosis and ER stress.[40] CBG induced cell-cycle arrest in the G1 phase, apoptosis and reduced invasion in 3D cell spheroid of glioblastoma.[46] CBG reduced cells in the S phase, inhibited migration and

induced cell death by apoptotic pathway in cholangiocarcinoma cell lines.[45] CBG blocked cell cycle in G0/G1 phase, induced apoptosis, inhibited migration and invasion, influenced cannabinoid-related gene targets, for example increased expression of CB1 receptor, GPR55 receptor, CXCR4, but tested *in vivo* failed to prolong survival in a syngeneic orthotopic rat mesothelioma model.[47] CBG reduced CSF-1 secretion by murine melanoma cells and restored $CD8^+$ T-cell activation, decreased tumour volume and weight in a mouse melanoma model.[49]

Only few studies involving CBC exist. It showed to inhibit cell viability in human prostate carcinoma cells,[48] human colorectal cancer cells,[44] human breast cancer cells [42] and human mesothelioma cells.[47]

Only few evidences are showed also for CBN, that induced antiproliferative effect in human prostate cancer cells,[48] human breast cancer cell lines [40,41] and human mesothelioma cells.[47] Moreover, CBN combined with CBD CBG and THC induced cell cycle arrest in G2 phase, apoptosis and ER stress.[40]

CBDV was found to reduce the growth of human prostate cancer cells,[48] human colon carcinoma cells [44] and human mesothelioma cells.[47]

1.4.4. Effect of cannabinoids on multiple myeloma

While cannabinoids effect on many cancer types has been studies a lot as, already seen, only little is still known about the effect of cannabinoids and ECS component on MM.[50] Only six papers were published,[36,51-55] among which only one showed *in vivo* analysis.[53]

Our laboratory in 2013 studied the anticancer effect of CBD, alone and combined with the drug Bortezomib, on MM cell lines, and the involvement of TRPV2 receptor.[51] It was found that CBD induces cytotoxicity in MM cells and that this effect was amplified in TRPV2 transfected cells. Moreover, the effect of CBD on non-transfected MM cell lines was independent of the CB1 and CB2 receptors, TRPs and PPARγ. The coadministration of CBD and BORT synergistically reduced the viability of MM cell lines, with higher effect in TRPV2-transfected MM cell lines. CBD alone, but in higher way with BORT, inhibited proliferation, induced cell cycle arrest at the G1 phase and accumulation of cells in the subG1 phase, induced mitochondrial and ROS-dependent necrosis, reduced ERK and AKT signals and switched off ERK, AKT phosphorylation and cyclinD1 levels, inhibited the classical and alternative NF-kB pathways. Importantly, all these effects were obtained in higher levels in TRPV2-transfected MM cells. CBD and BORT do not affect $CD34^+$ cells growth, suggesting not to have cytotoxic effects on haematopoietic progenitor cells. To reassume, it was suggested that TRPV2 activation may represent a promising target to deregulate MM signalling pathways and that CBD could be promising as combination therapy with Bortezomib in MM.

Feng et al. in 2015 studied the effect of CB2 anticancer effect by the use of the inverse agonist of CB2, phenylacetylamide (PMA).[52] They found that CB2 receptor was highly expressed in MM cell lines and primary $CD138^+$ cells, while CB1 was not expressed. PMA inhibited proliferation of MM cells, through induction of

apoptosis, while was less cytotoxic on human normal mononuclear cells. Regarding the mechanism involved, it was demonstrated that ERK and JNK are activated, while p38 MAPK was less affected, however, blocking JNK and ERK did not reverse the cytotoxic and apoptotic effect of PAM, suggesting that these pathways are not involved in PAM's anti-MM activities. Moreover, caspae-8 and Bid were activated, Bcl-2 was slightly decreased, together with survivin, a member of the IAP anti-apoptotic proteins family. The cytotoxic effect of PMA was reversed by CB2 gene silencing and by antagonizing CB2 receptor, thus supporting CB2 involvement. The cytotoxic effect was not obtained instead with CB1 inverse agonist SR141716. PAM negatively regulated MM cell cycle, in fact it reduced the number of cells in S and G2-M phase and highly increased cells in the subG0/G1, decreased the expression of cell cycle related protein cdc25C, and inactivated mitotic regulator serine/threonine-protein kinase 6, resulting in reduced phosphorylation of CDK substrates and block of MM cell mitotic division. Moreover, PMA was effective in MM cells resistant to dexamethasone or melphalan.

In 2016 our laboratory also studied the anticancer effect of THC alone and in combination with CBD in MM cell lines.[36] It was found that THC reduced cell viability in a dose-dependent manner, blocked cell cycle, increasing the G1 and subG1 cell population, and the effect of the combination with CBD was higher. The cytotoxic effect of CBD and THC was not CB2 receptor dependent, since the CB2 antagonist AM630 did not interfere with the effect. CBD alone induced a slight increase of LC3-II/LC3-I ratio, THC has no effect, while the THC-CBD combination strongly increased the cleaved LC3-II form and the LC3-II/ LC3-I ratio, suggesting an autophagy induction. Blockade of autophagy reverted the cytotoxic effect of CBD-THC treatment, suggesting a cytotoxicity autophagy-dependent. Moreover, a necrotic cell death, augmented levels of damaged DNA and increased levels of γ-H2AX, a protein associated with DNA double-strand breaks, was observed after THC and CBD treatment, with the THC-CBD combination having higher effects. Also, CBD-THC combination reduced MM cells migration by down-regulating expression of the chemokine receptor CXCR4 and CD147 plasma membrane glycoprotein, reduced expression of the β5i subunit (the subunit of immuno-proteasome with which the immuno-proteasome inhibitor Carfilzomib create irreversible adducts) and acted in synergy with Carfilzomib to increase MM cell death and inhibit cell migration. In summary, CBD combined with THC and with Carfilzomib seems to exert strong anti-myeloma activities.

Barbado et al. in 2017 studied the anti-myeloma effect of many cannabinoid derivatives *in vitro* and *in vivo*, in particular the cannabinoid agonist WIN-55 and four highly selective CB2 agonists PGN-6, -17, -34 and -72.[53] The compounds significantly reduced the viability of all MM cell lines. WIN-55 reduced cell viability also in myeloma plasma cells obtained from MM patients, while viability of normal healthy cells, including hematopoietic precursor cells, remains unaffected. The cytotoxicity resulted apoptotic-dependent, since WIN-55 induced active cleaved forms of caspase-3 and decreased pro-caspase-9, -8 and -2, increased expression of pro-apoptotic proteins Bax and Bak, and decreased anti-apoptotic Bcl-xL and Mcl-1. Moreover, WIN-55 upregulated p-JNK and p-Erk1/2, downregulated p-p38-MAPK, and Akt was short-term activated and long-

term downregulated. WIN-55 increased levels of ceramides, a mechanism involved in the induction of apoptosis too. The cytotoxic and proapoptotic effect of WIN-55 was CB2-dependent, since CB2 antagonists inhibited these effects. WIN-55 acted synergistically with dexamethasone and melphalan to reduce cell viability, overcoming resistance to melphalan. Finally, WIN-55 significantly suppressed tumour growth *in vivo* in preclinical model of human MM xenograft mice.

In 2020 Garofalo et al. analysed the antitumour effect of CBD and Cytokine-induced killer (CIK) cells on MM cell line.[54] CIK cells are a heterogeneous population of polyclonal T lymphocytes obtained *ex vivo* from PBMCs, that express $CD3^+$ and $CD56^+$, common markers of T lymphocytes and natural killer cells. CIK cells are mainly used in haematological patients who suffer relapse after allogeneic transplantation. They found that CB2 receptor is highly expressed on CIK cells and on MM cells. CBD decreased the viability of MM cells and can have a protective role for CIK cells. CBD also inhibited the cytotoxic activity of CIK cells against MM at high concentrations, so it is suggested the usage of the lower concentrations in combination with CIK cells.

In 2021 Mannino et al. evaluated the anticancer effect of β-caryophyllene (BCP), a sesquiterpene extracted from Cannabis and CB2 receptor agonist, on MM cells.[55] BCP reduced MM cells viability, but did not affect normal B lymphoblasts. BCP induced apoptosis through activation of Bax and caspase-3, and reduction of Bcl-2. BCP regulated AKT, Wnt1, and β-catenin expression, suggesting that CB2 receptor stimulation may decrease cancer cell proliferation by modulating Wnt/β-catenin signalling. Cell cycle was modulated and, in particular, cyclin D1 and CDK 4/6 were reduced. These effects were counteracted by AM630, a CB2 receptor antagonist, thus suggesting that BCP acts through CB2 receptor.

To summarize, work investigating the pre-clinical effect of cannabinoids on MM cell *in vitro*, found that cannabinoids reduced tumour cell growth, blocking cell cycle and inducing cell death, while were not cytotoxic on non-tumoral cells. Moreover, they synergised with chemotherapeutic drugs in inducing cytotoxicity, overcoming drug-resistance. The only *in vivo* study showed that a synthetic cannabinoid agonist suppressed tumour growth in MM mouse model.[36,51-55] (Figure 12). Obviously, the studies are a low number, and more preclinical studies and clinical trials are needed to test the efficacy and safety of introducing cannabinoids into MM treatment regimens. One trial was initially proposed in 2018, but the recruitment for the trial never occurred.[50]

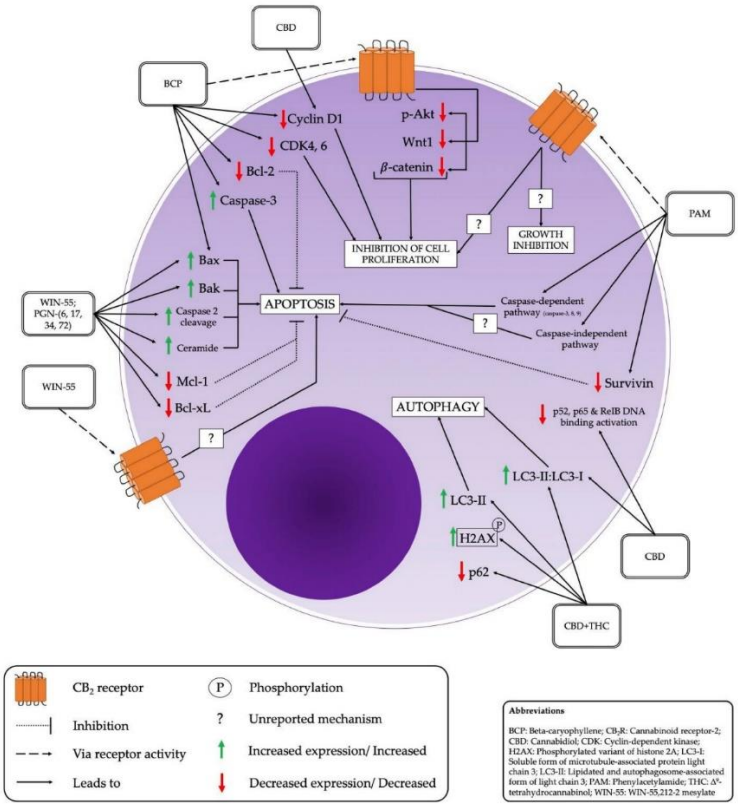


Figure 12. Effect of cannabinoids on MM cells and associated pathways.[50]

1.4.5. Effect of cannabinoids on bone disease

Scientific literature also reported the beneficial effect of cannabinoids against bone disease, one of the main complications of MM. Indeed, the ECS contributes to regulate bone metabolism and thus it plays a role in maintaining the bone strength and combating bone diseases.[56] Accordingly, eCBs and their receptors have been shown to be expressed in bone. For example, CB1, CB2 and TRPV1 have been identified in human osteoclasts, GPR55 was found to be expressed in human osteoblasts and osteoclasts, AEA and 2-AG were found in human osteoclasts- and osteoclasts-like cell, DAGL NAPE-phospholipase D, FAAH and MAGL are expressed in osteoblasts and osteoclasts.[56] Moreover, studies showed that cannabinoids regulate osteoclasts, osteoblasts and adipocytes *in vitro* and *in vivo*.[56] Some evidences about the favourable effect of eCBs in bone disease are that CB2 receptor agonists attenuated sarcoma-induced pain, reduced cancer-induced osteolytic destruction and prevented the occurrence of pathological bone fracture.[57] Moreover, it was found that CBD can improve fracture healing and is involved in collagen cross-linking and stabilization.[58] Additionally, MAGL inhibitor JZL184 reduced osteolytic bone metastasis in mouse models of breast and prostate cancers, and inhibited skeletal tumour growth and metastasis in models of osteosarcoma.[59] Also, stimulatory effects on osteoclast induced by an activator of GPR55, were attenuated by the GPR55 antagonist CBD *in vitro*, and CBD reduced bone resorption *in vivo* in mice via modulation of GPR55 signalling.[60]

1.4.6. Clinical trials of cannabinoids for cancer treatment

The first clinical trial of cannabinoids as cancer treatment was published in 2006 and was a pilot phase I trial involving 9 patients with recurrent glioblastoma multiforme who previously failed standard therapy (surgery and radiotherapy). The trial demonstrated that intracranially administration of THC was safe and reduced tumour cell proliferation in two of nine patients.[61]

A clinical trial involving patients with recurrent prostate cancer, showed that Epidiolex was safe and tolerable.[62]

In 2021 results of a phase 2 randomised clinical trial assessed the tolerability of two different ratios of medicinal cannabis (two standardised and quality-assured specially formulated oil-based organic whole plant extracts of cannabis based on a 1:1 and 4:1 ratio of THC:CBD) in patients with high grade gliomas. The trial enrolled 88 patients and results showed that the cannabis products were safe, well tolerated, and can provide symptomatic relief to patients, improving their quality of life.[63]

In 2021 the results of a phase 1b randomised, placebo-controlled trial of nabiximols in combination with dose-intense TMZ in patients with recurrent glioblastoma were published. 33% of nabiximols+TMZ- and placebo+TMZ-treated patients were progression free for 6 months. In the 21 patients enrolled, survival at 1 year was 83% for nabiximols+TMZ-treated patients (n=12) and 44% for placebo+TMZ-treated patients (n=9). Moreover, overall survival at 2 years was of 50% for patients treated with nabiximols and 22% for those treated with placebo. Additionally, Nabiximols had acceptable safety and tolerability and did not interact with TMZ pharmacokinetic.[38] (Figure 13)

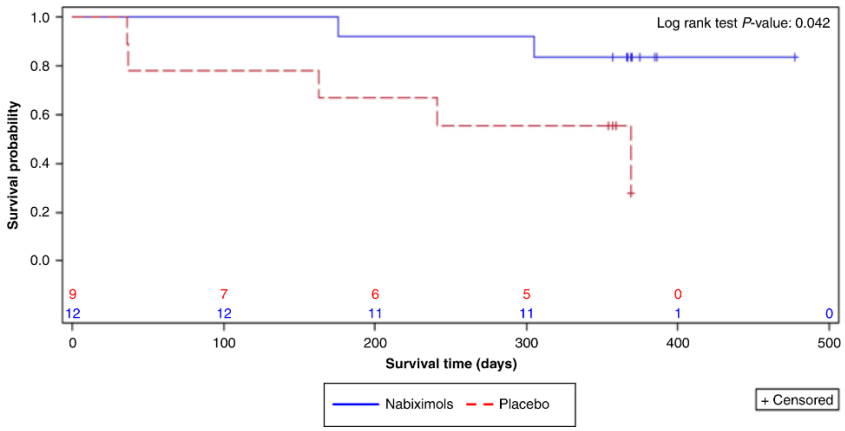


Figure 13. Kaplan–Meier survival curves (randomised safety analysis set).[38]

2. Aim of the study

The aim of the study was to investigate the potential anticancer effect of the minor cannabinoids CBG, CBC, CBN and CBDV *in vitro* in three MM cell lines and the effect of CBN *in vivo* in a murine model of MM.

3. Materials and methods

3.1. Cell lines

U266, RPMI8226 (RPMI) and SKO-007 (SKO) MM cell lines were purchased from ATCC (LGC Standards, Milan, Italy) and cultured in RPMI1640 medium (Lonza, Milan, Italy) supplemented with 10% foetal bovine serum (FBS), 2 mM L- glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 1 mM sodium pyruvate. Human osteoblast cell line (HuOB) was purchased from from InSCREENex GmbH (Braunschweig, Germany) (Braunschweig, Germany) and cultured in DMEM glucose high medium (EuroClone, Milan, Italy) supplemented with 100 IU/mL penicillin, 100 mg streptomycin, 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and 2 mM L glutamine. All the cell lines were maintained at 37°C with 5% CO_2 and 95% humidity. Monocyte THP-1 cell line was purchased from Istituto Fondazione di Oncologia Molecolare (IFOM, Rome, Italy), and cultured in RPMI1640 medium (Lonza, Milan, IT) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.05 mM β-mercaptoethanol and maintained at 37°C with 5% CO2 and 95% humidity. THP-1 cells were used to create osteoclasts. THP-1 cells were seeded at a density of 2.5 x 10^4 cells/well in a 96 well plate with PMA 100ng/mL to differentiate in macrophages. After 3 days, RANK-L 66 ng/mL and M-CSF 33 ng/mL were added and changed every 3-4 days to differentiate cells in osteoclasts. After 14 days, Tartrate-resistant Acid Phosphatase (TRAP) staining was performed using Leukocyte Acid Phosphatase kit (Sigma-Aldrich, Milan, Italy) to identify osteoclasts (cells containing wine-red particles (TRAP-positive) and multinucleated).

3.2. Reagents

Pure CBG, CBC, CBN and CBDV were purchased by Cayman Chemicals (Ann Arbor, Michigan, USA). Compounds were dissolved in ethanol 70% at 50 mM, aliquots were stored at -20°C and each aliquot was used one time.

3.3. Cell viability assay

Cells were seeded at a density of 3 x 10^4 cells/mL 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. After 72 hours, cell viability was assessed by adding 0.8 mg/mL of 3-[4,5-dimethylthiazol- 2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, Milan, Italy) to the media. After 3 h, the plates were centrifuged or not, depending on the cell line, the supernatant was removed, and the pellet of salt crystals was solubilized with dimethyl sulfoxide (DMSO) 100 µL/well. The absorbance of the samples against a background control was measured at 570 nm using an ELISA reader microliter plate (BioTek Instruments, Winooski, VT, USA). All experiments were repeated three times.

3.4. Cell death assay

Annexin V-FITC and PI staining followed by FACS analysis was used to evaluate cell death. Cells were seeded at a density of 3 x 10^4 cells/mL in 6-well plates and after one day of incubation treatments were added. 48 hours post-treatment, cells were stained with 5 µL of Annexin V FITC (Vinci Biochem, Vinci, Italy) for 10 min at room temperature, washed once with binding buffer (10 mM N- (2- Hydroxyethyl) piperazine-N0-2-ethanesulfonic acid [HEPES]/sodium hydroxide, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), then stained with 20 µg/mL PI (Sigma-Aldrich, Milan, Italy) and analysed on a FACScan flow cytometer using CellQuest software (BD Biosciences, San Jose, CA, USA). All experiments were repeated three times.

3.5. Western blot analysis

Cell 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 and H_2O). They were separated on a SDS polyacrylamide gel, transferred onto Hybond-C extra membranes (GE Healthcare, Chicago, IL, USA), blocked with 5% low-fat dry milk in phosphate-buffered saline 0.1% Tween 20, immunoblotted with mouse anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH, 1:1000, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-phospho-histone H2AX (Ser139) (γ-H2AX, 1:1000, Cell Signaling Technology, Danvers, MA, USA), mouse anti-CB1 (1:500, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-CB2 (1:200, Cayman Chemical, Ellsworth, MI, USA), mouse anti-TRPV1 (1:200, Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-TRPV2 (1:200, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-TRPV3 (0.5 µg/mL, Boster Biological Technology, Pleasanton, CA, USA), rabbit anti-TRPV4 (1:500, Assay Biotechnology Company, Fremont, CA, USA), mouse anti-TRPA1 (1:1000, Abnova, Taipei City, Taiwan), rabbit anti-TRPM8 (0.5 µg/mL, Boster Biological Technology, Pleasanton, CA, USA) Abs overnight or 1 h according to manufacturer's protocol and then incubated with their respective HRP-conjugated anti-rabbit or anti-mouse (1:2000, Cell Signaling Technology, Danvers, MA, USA) Abs for 1 hour. Peroxidase activity was visualized with the LiteAblot®PLUS or TURBO (EuroClone, Milan, Italy) kit and densitometric analysis was carried out by a Chemidoc using the Quantity One software version 4.6 (Bio-Rad, Milan, Italy). All experiments were repeated three times.

3.6. Comet assay

Cells were seeded in a 6 well plate at a density of 3 × 10^4 cells/well and treated. After 24 hours, the comet assay was performed under alkaline conditions following the ABCAM protocol. Briefly, the cells were resuspended in 1 x PBS and added to 75 µL of molten (37 °C) 0.5% low-melting-point agarose gel to achieve a cell concentration of 1 × 10^5 cells/mL. The agarose was pipetted onto the comet slides. Slides were stored in the dark at 4 °C for 10 min before adding pre-chilled lysis buffer for 45 min at 4 °C in the dark. The slides were immersed in freshly prepared alkaline solution (0.25 M NaOH containing 0.1 µM EDTA, pH 12.6) for

30 min at the same conditions. Slides were then removed and washed twice with TBE buffer for 5 min. Gel electrophoresis was performed at 1 V/cm (length of the electrophoretic chamber) for 20 min (running amperage 3–5 mA with the distance between the two electrodes of 25 cm). The comet slides were washed with 70% ethanol for 5 min and air-dried for 1 h at room temperature. A 100 µL of diluted SYBR Green solution was placed onto each dried agarose circle. The slides were then read with a fluorescence microscope (Leica, Buccinasco, Italy). Images were analysed with ImageJ software version 1.54i (National Institutes of Health, Bethesda MD, USA).

3.7. Cell invasion assay

The invasion assay was performed using the Corning® BioCoat™ Matrigel® Invasion Chamber (Corning, NY, USA), whose inserts are pre-coated with extracellular matrix (ECM) proteins. 1 x 10^4 HuOB cells were seeded in the bottom chamber. The day after, MM cells at a density of 2.5 x 10^4 cells/well were pre-treated with 1 µM calcein-AM (Life Technologies, Monza, Italy) for 30 min and then transferred inside the invasion chamber. Treatments were added in the upper part for 24 h. One well without HuOB was used as negative control. Images of migrated cells in three randomly selected fields were captured and evaluated under fluorescent microscopy (LeitzFluovert FU, Leica Microsystems, Wetzlar, Germany).

3.8. Bone resorption assay

THP-1 cells were seeded in a 96 well plate on bovine bone slices (Boneslices.com, Jelling, Denmark) and differentiated in osteoclasts as previous described. After 7 days, treatments in new media were added every 3-4 days (day 7, 10, 14). The supernatants were collected (day 10, 14) and the release of the C-terminal type I collagen fragments was evaluated by ELISA (Human Cross-linked C-telopeptides of Type I Collagen, CICP ELISA Kit, Novatein Biosciences, Woburn, MA, USA) to quantify the bone resorption.

3.9. Treatment on a xenograft model of multiple myeloma

The effect of CBN was tested *in vivo* on a xenograft model of MM, derived from the inoculation of U266 MM cells on mice. All the procedures involving the animals were conducted by MTTlab Srl (Trieste, Italy) according to the guidelines of Ministry of Health (DDL 116 of 21/2/1992 and subsequent amendments), to the Guide for the Care and Use of Laboratory Animals, Department of Health and Human Services publication no. 86-23 (National Institutes of Health, Bethesda, MD, 1985) and to the approved experimental protocol procedure (Authorization n°625/2021-PR released in accordance with article 31 D.lsg 26/2014). 10 female B-NDG mice (NOD-Prkdcscid IL2rgtm1/Bcgen) were furnished 11 weeks old by Envigo Italy. Animal were maintained under a daily 12 h photoperiod in controlled cabinet. Following a period of acclimatation, 5x10^6 U266 MM cells were subcutaneous inoculated on the hip of the mice. Treatments started when tumours were palpable, 10 days after inoculation (day 0). Animals were divided into 2 groups (n=5 per group): 1) control (CTRL) received ethanol 70% 50 µL; 2) CBN received cannabinol 15 mg/kg. Treatments were administered

subcutaneously every 3 days for 3 weeks, for a total of 7 treatments (day 0, 3, 6, 9, 12, 15, 18). The body weight of the animals was measured before the starting of the treatment and every 3 days. At the end-point, mice were sacrificed and macroscopic necroscopy was performed. Moreover, all the tumours were explanted, weighted, along with liver, spleen and pancreas.

3.10. Statistical analysis

Statistical analysis was performed with GraphPad Prism 9.0.1(128) software (GraphPad Software, San Diego, CA, USA). The data presented represent the mean with standard deviation (SD) of 3 independent experiments. Statistical analysis was performed with GraphPad Prism and One-way ANOVA followed by Dunnett's or Tukey multiple comparison post-test was used for *in vitro* analysis, while Mann-Whitney was used for *in vivo* studies.

4. Results

4.1. Expression of the main cannabinoid target receptors in human MM cell lines.

Firstly, it was analysed the expression of the main cannabinoid target receptors in the cell lines studied, to assess if phytocannabinoids could act on them. So, the protein expression of CB1, CB2, TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8 was investigated by western blot. Results showed that all the receptors are expressed at different levels in the three MM cell lines (Figure 14).

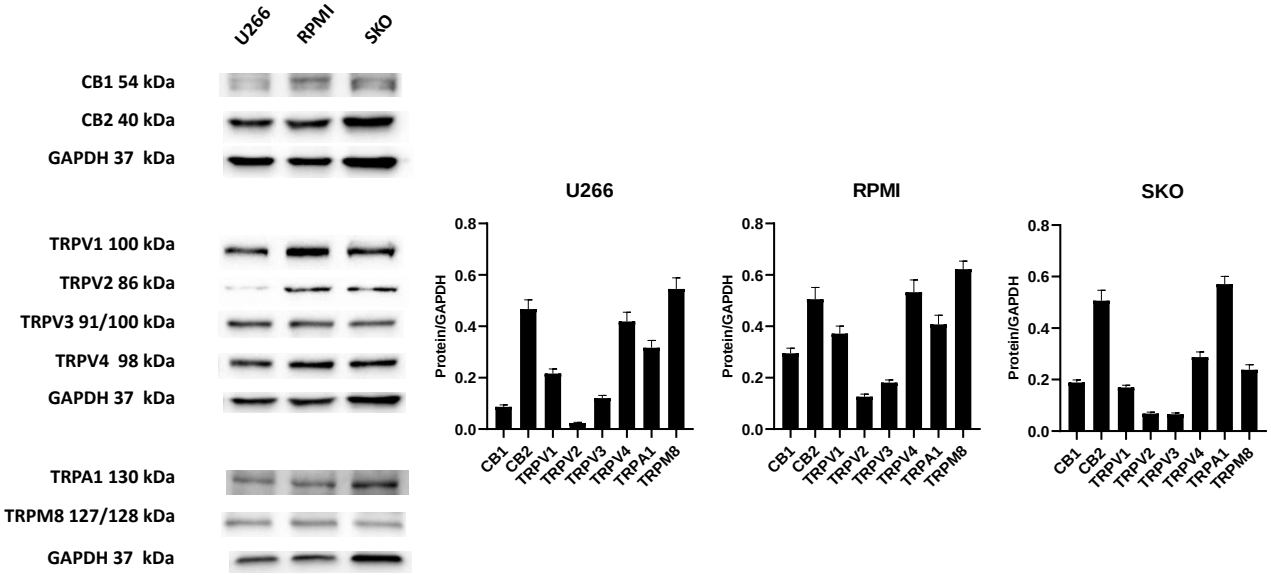


Figure 14. Expression of the main cannabinoid target receptors in MM cell lines. The expression of CB1, CB2, TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8 was determined by western blot and their densitometric values were normalized to GAPDH used as loading control. Blots are representative of one of three separate experiments. Statistics are the mean ± SD of three separate experiments.

4.2. CBG, CBC, CBN and CBDV induced cell growth inhibition in human MM cell lines

The effect of CBG, CBC, CBN and CBDV on cell viability on three MM lines was evaluated by MTT assay. Cells were treated with vehicle (VHC) or different doses of phytocannabinoids, up to 100 μM. Results show that all of them reduced MM cell viability in a dose-dependent manner (Figure 15). As demonstrated by IC_{50} value, CBN and CBDV were the most efficacious in reducing cell viability, followed by CBG and, finally, by CBC, that was the least effective (Table 1).

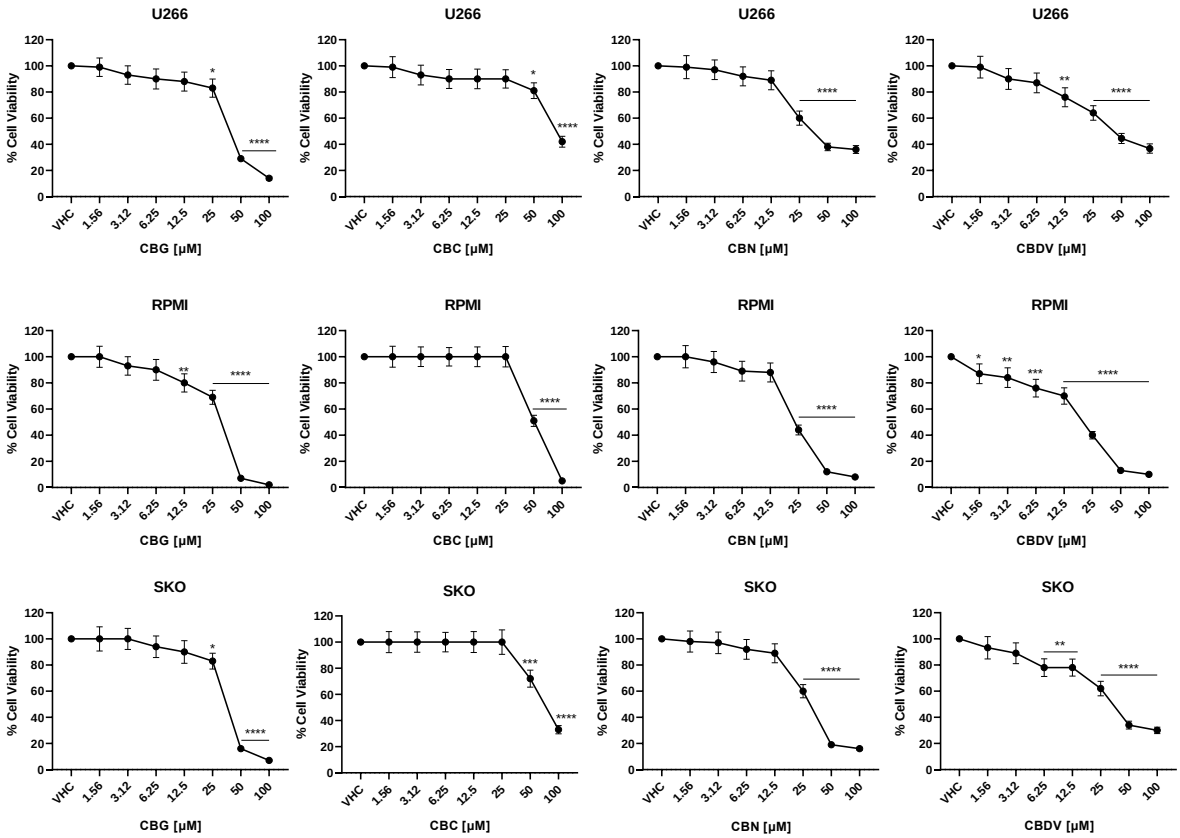


Figure 15. CBG, CBC, CBN and CBDV effect on cell viability of MM cell lines U266, RPMI and SKO. Cell viability was determined by MTT assay. Data shown are expressed as the mean ± SD of three separate experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs VHC.

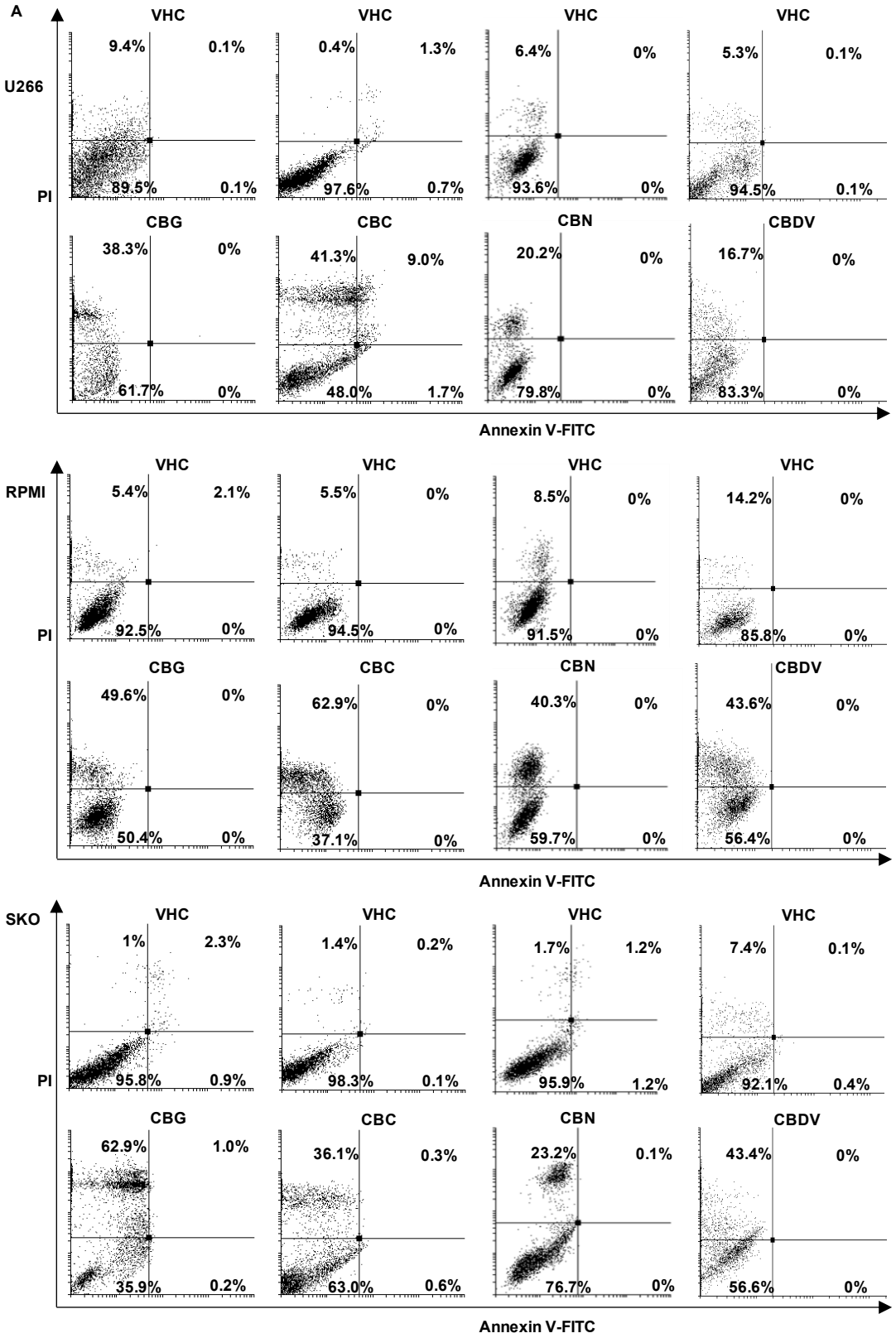
Phytocannabinoid	IC_{50} [μM]		
	U266	RPMI	SKO
CBG	35.91 ± 3.12	35.02 ± 2.97	31.36 ± 2.70
CBC	78.80 ± 6.28	69.70 ± 5.75	49.60 ± 4.68
CBN	26.51 ± 1.04	28.50 ± 2.53	23.64 ± 1.91
CBDV	26.71 ± 2.17	26.52 ± 2.39	21.68 ± 2.01

Table 1. IC_{50} of CBG, CBC, CBN and CBDV in MM cell lines. The values are calculated as the mean ± SD of three separate experiments.

4.3. CBG, CBC, CBN and CBDV induced cell death in human MM cell lines

To better investigate the MM cell line growth inhibition by the four phytocannabinoids, Annexin-V/PI staining followed by flow cytometry was used to evaluate cell death. Cells were treated with vehicle or the IC_{50} dose for each phytocannabinoid and after 48 h the cell death assay was performed. Results show that CBG, CBC, CBN and CBDV induced necrotic cell death, as seen from the increased in PI fluorescence (Figure 16 A, B) also supported by western blot analysis and comet assay. Indeed, the expression of γ-H2AX protein, marker of

DNA damage, increased (Figure 16 C) and DNA fragmentation was observed (Figure 16 D) following CBG, CBC, CBN and CBDV treatment.



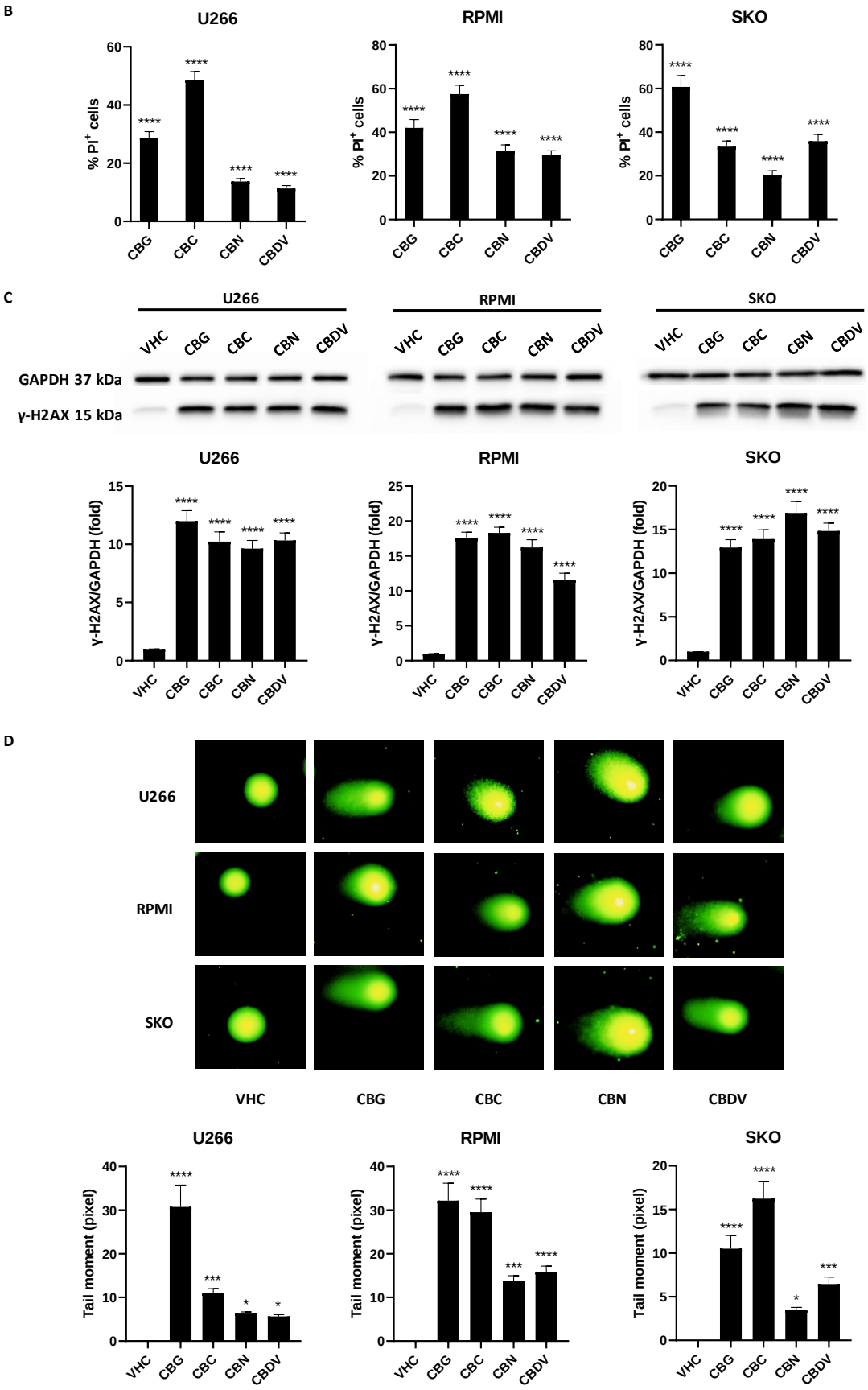

B
U266
RPMI
SKO
% PI+ cells
CBG
CBC
CBN
CBDV
C
GAPDH 37 kDa
γ-H2AX 15 kDa
VHC
γ-H2AX/GAPDH (fold)
D
Tail moment (pixel)

Figure 16. CBG, CBC, CBN and CBDV effect on cell death in MM cell lines. MM cells were treated for 48 h with CBG, CBC, CBN or CBDV. (A, B) Cell death was determined by Annexin V-FITC/PI staining and cytofluorimetric analysis. A) Histograms are representative of three experiments. B) Percentage of PI^+ cells compared to VHC. Data shown are expressed as mean ± SD of three separate experiments. (C) Effect of cannabinoids in the modulation of γ-H2AX protein on MM cell lines treated as above described. The expression of γ-H2AX was determined with western blot analysis. γ-H2AX densitometric values were normalized to GAPDH used as loading control. Images are representative of one of three separate experiments. Folds (mean ± SD of three experiments) are changes respect to vehicle. (D) Comet assay on MM cell lines treated with CBG, CBC, CBN and CBDV was used to evaluate DNA fragmentation. Images are representative of one of three separate experiments. Magnification 40x. Histograms are the mean ± SD of values of tail moment of three experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs VHC.

4.4. Expression of the main cannabinoid target receptors in human bone cell line

Then, it was analysed the presence of the main cannabinoids target receptors in bone cell line, too. The protein expression of CB1, CB2, TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8 was investigated by western blot in HuOB cell line. Results show that all the receptors investigated are present, at different levels, in HuOB (Figure 17), with the exception of TRPV1.

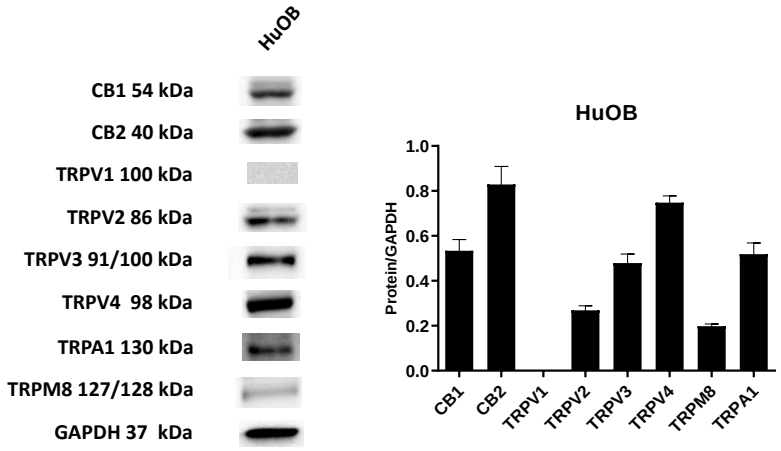


Figure 17. Expression of the main cannabinoids target receptors in HuOB cell line. The expression of CB1, CB2, TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8 was determined with western blot and their densitometric values were normalized to GAPDH used as loading control. Images are representative of one of three separate experiments. Blots are the mean ± SD of three separate experiments.

4.5. Effect of CBG, CBC, CBN and CBDV in human bone cell line

Since bone lesions are one of the main complications in MM patients, caused by cancer cells infiltrating the bone marrow, it was investigated the effect of phytocannabinoids in interfering with the bone-MM cells interaction. So, first of all, it was investigated the effect of different doses of CBG, CBC, CBN and CBDV in reducing cell viability of human osteoblasts (HuOB). Results show that all the phytocannabinoids reduced cell viability in a dose-dependent manner (Figure 18) and the IC_{50} are showed in Table 2. The doses used for bone-

MM cell interaction were 12.5 µM for CBG, CBC, CBN, and 6.25 for CBDV, since were not cytotoxic and HuOB cell viability was almost comparable with that of MM cell lines.

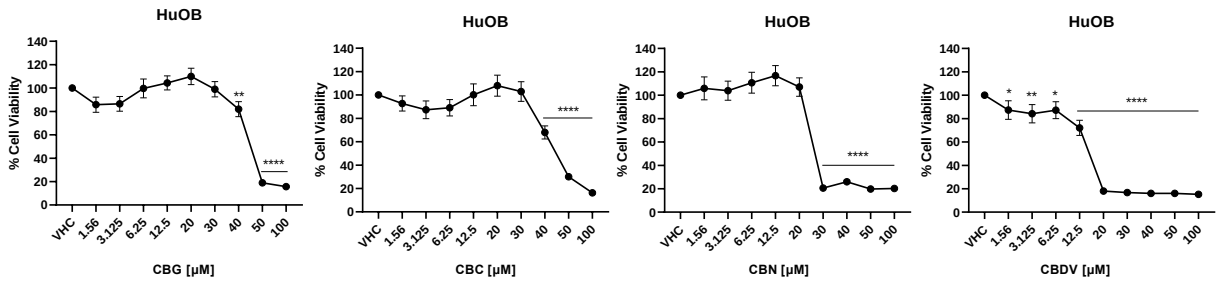


Figure 18. CBG, CBC, CBN and CBDV effect on HuOB cell line viability. (A) Cell viability was determined by MTT assay. Data shown are expressed as the mean ± SD of three separate experiments. *p<0.05, **p<0.01, ****p<0.0001 vs VHC.

Phytocannabinoid	IC_{50} [µM]
	HuOB
CBG	44.83 ± 4.03
CBC	42.41 ± 3.86
CBN	29.03 ± 2.31
CBDV	14.27 ± 1.25

Table 2. IC_{50} of CBG, CBC, CBN and CBDV in HuOB cell line. The values are calculated as the mean ± SD of three separate experiments.

4.6. CBG, CBC, CBN and CBDV reduce the invasion of MM cells toward HuOB cells

To assess if phytocannabinoids modulate the bone-MM interaction, it was evaluated the invasion of MM cells toward HuOB cells. So, an invasion assay was performed using ECM coated transwells. HuOB were plated on the bottom chamber, while MM cells, pre-treated with calcein, were plated on the upper chamber. Treatments (CBG 12.5 µM, CBC 12.5 µM, CBN 12.5µM, CBDV 6.25 µM) were added on the upper chamber. Results show that after 24 h in absence of HuOB in the lower chamber, invasion did not occur, while the presence of HuOB acted as chemoattractant, thus can be considered a positive control. In fact, it was already found that osteoblasts promote migration and invasion of myeloma cells.[10] The treatments with the four phytocannabinoids reduced the number of both MM cells that invaded the ECM coated membrane, but in particular CBG and CBN were more effective in reducing the invading MM cells (Figure 19 A, B).

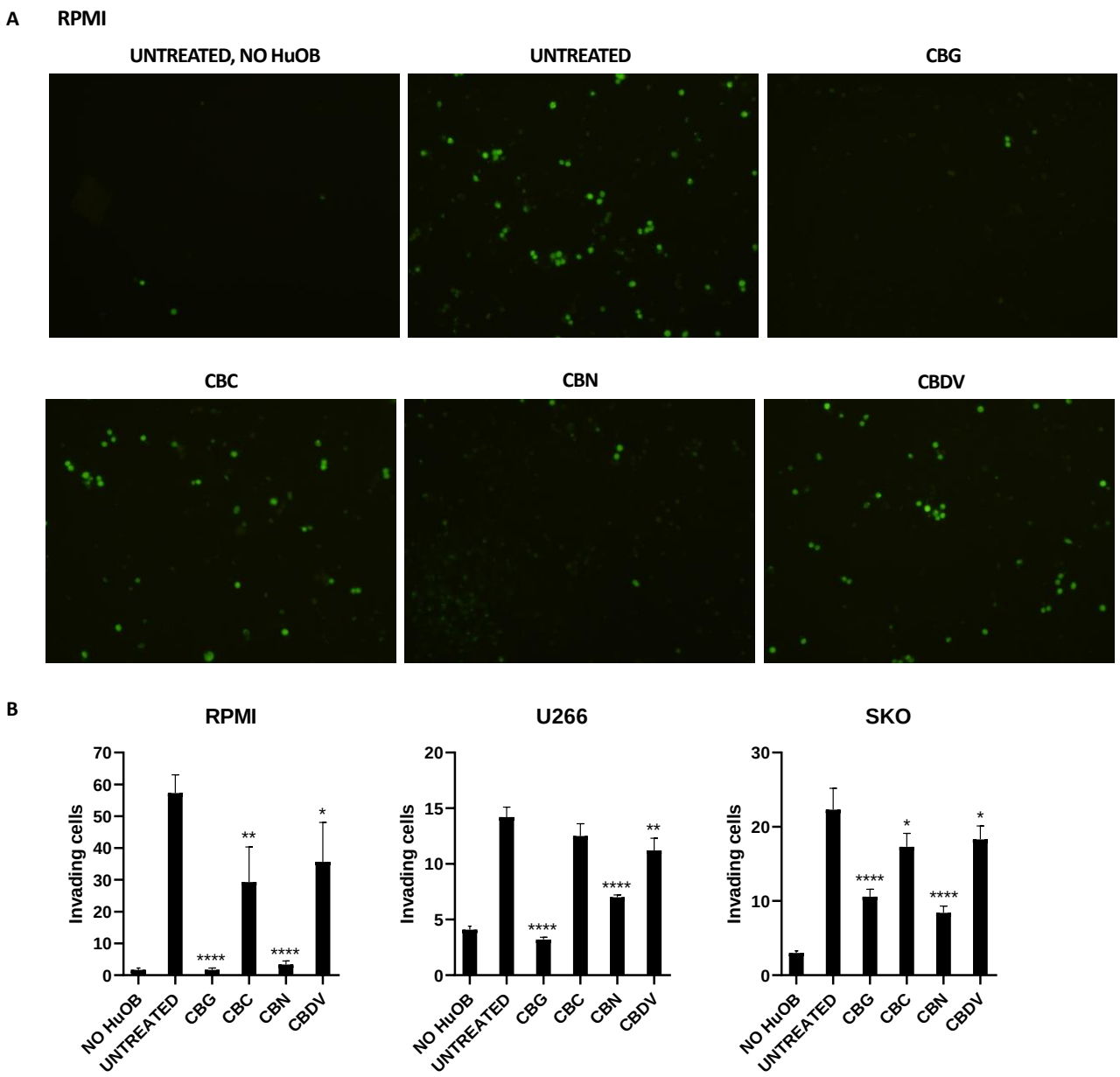


Figure 19. MM cell invading the ECM covered membrane toward HuOB cells, after treatment with CBG, CBC, CBN and CBDV. (A) Representative image of calcein stained RPMI cells invading the ECM coated membrane. Three random fields were observed under a fluorescence microscope. Magnification 10x. (B) The number of invading cells represent the mean ± SD of three separated experiments. *p<0.05, **p<0.01, ****p<0.0001 vs UNTREATED.

4.7. CBG, CBC, CBN and CBDV reduce the bone resorption

The following step was to investigate if phytocannabinoids can also modulate the bone resorption. In fact, lytic bone lesions are between the main clinical manifestation of MM, and are due to the over activation of osteoclast cells. So, we performed a bone resorption assay, evaluating by ELISA assay the release of C-terminal type I collagen fragments from bovine bone slices, by osteoclasts. In fact, N-terminal and C-terminal cross-linked telopeptides of type I collagen are breakdown products of osteolysis, used as biomarkers.[9] Briefly, THP-1 cells

were seeded in a 96 well plate on bovine bone slices and differentiated in osteoclasts (Figure 20 A). After 7 days, treatments (CBG 12.5 µM, CBC 12.5 µM, CBN 12.5 µM, CBDV 6.25 µM) were added every 3-4 days. The supernatants were collected at day 10 and 14 and the release of the C-terminal type I collagen fragments was evaluated by ELISA. Results in Figure 20 B show that all the phytocannabinoids reduced the release of these fragments, already after 10 days, meaning a reduction of bone resorption. In particular, CBG and CBN, were the most effective.

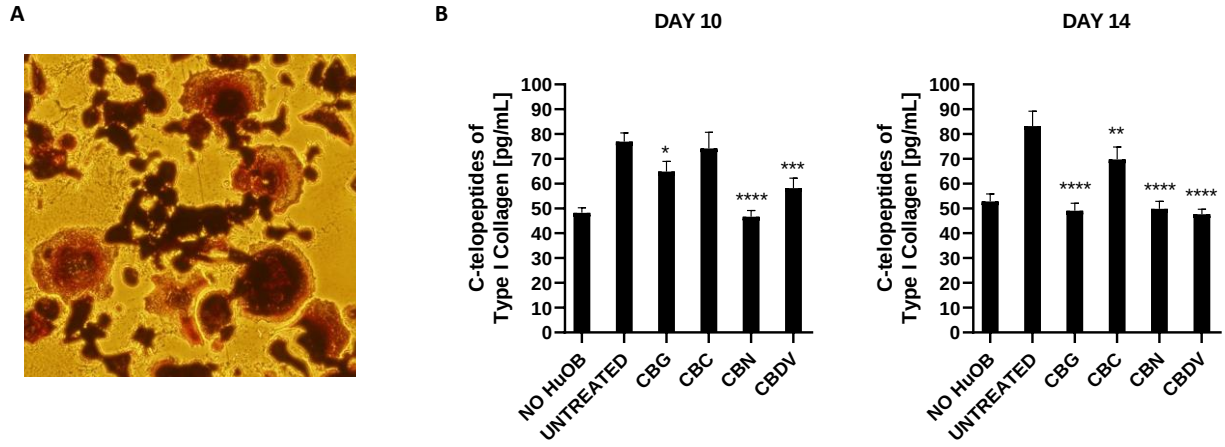


Figure 20. Effect of CBG, CBC, CBN and CBDV on bone resorption. (A) Representative images of osteoclasts differentiated from THP-1 cells (TRAP-positive). Magnification 20x. (B) Effect of CBG, CBC, CBN and CBDV on modulating the release of C-terminal type I collagen fragments, from bovine bone slices, by osteoclasts. The release of C-terminal type I collagen fragments was evaluated by ELISA. Results are the mean ± SD of three experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs UNTREATED.

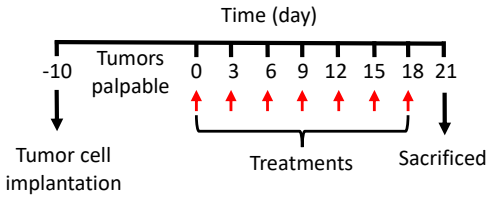
4.8. CBN reduced tumour mass in a xenograft model of MM

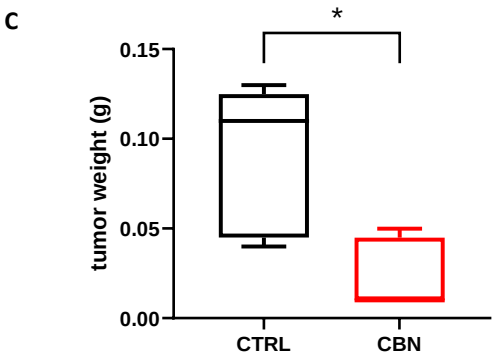
For a preliminary study, a xenograft model of MM was used to evaluate the anticancer effect of CBN which proved to be the most effective in the previous experiments regarding inhibition of MM cell growth, cell invasion and bone resorption. The xenograft model of MM was obtained by the subcutaneous inoculation of U266 MM cells on mice. When tumors were palpable, 10 days after inoculation (day 0), animals were treated by subcutaneous injection every 3 days for 3 weeks, with ethanol 70% 50 µL (control group, CTRL) or CBN 15 mg/kg (group CBN) (Figure 21 A, B). During the treatment, the body weight of the animals was measured every 3 days (Figure 21 D). At the end-point mice were sacrificed and macroscopic necroscopy was performed. All the tumors were explanted and weighted (Figure 21 C), along with liver, spleen and pancreas. Results showed that, after 3 weeks of treatment, a significant reduction of tumor weight was observed in mice treated with CBN, respect to the control group (Figure 21 C). For a toxicological evaluation, the body weight of the animals was measured every 3 days during the treatment. Results showed that the body weight of animals in CBN group was similar to the CTRL group (Figure 21 D) and the difference between the initial and final body

weight of animals of CBN group was not statistically different to CTRL group (Table 3). Also, the weight of liver, spleen and pancreas of the CBN group was not statistically different from the CTRL group (Table 3).

A

Group	Number of animals	Treatment
CTRL	5	Ethanol 70% 50 µL
CBN	5	CBN 15 mg/kg





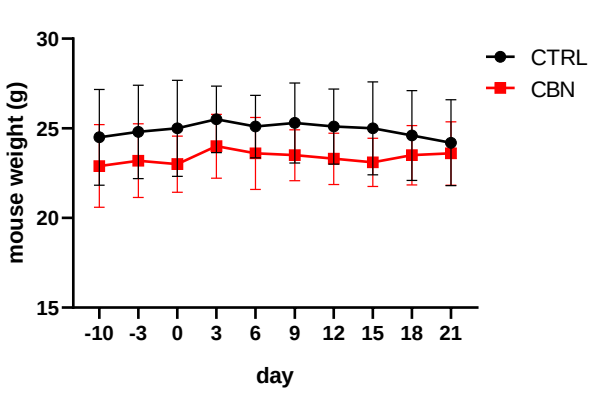


Figure 21. CBN effect on a xenograft model of MM. A) Groups of animals and treatments received. B) Experimental design of animal treatments. When tumours became palpable, animals were treated subcutaneously every 3 days, for 3 weeks. C) Tumor weight at the end of treatments. The graph shows the mean ± SD from five animals for group. *p<0.05. D) Body weight of the mice during the treatment. The graph shows the mean ± SD from five animals for group.

Group	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Spleen weight (g)	Pancreas weight (g)
CTRL	25 ± 2.68	24.2 ± 2.39	1.03 ± 0.02	0.06 ± 0.02	0.14 ± 0.02
CBN	23 ± 1.60	23.6 ± 1.80	1.07 ± 0.10	0.10 ± 0.03	0.13 ± 0.07

Table 3. Initial and final body weight of mice; liver, spleen and pancreas weight at the end of treatment. Results are expressed as the mean ± SD from five animals for group.

4.9. Combination of CBG, CBC, CBN, CBDV increased the inhibition of cell growth respect to single treatment in human MM cell lines

After the results obtained with phytocannabinoids used in single treatment, and in particular with CBN, the effect of phytocannabinoids on MM cell viability was tested in double, triple and quadruple combinations, by MTT assay. For double combinations, the doses of 12.5 and 25 µM were selected, respectively one non cytotoxic dose and one effective on cell viability, except for CBC that were two non-cytotoxic doses. For the triple and quadruple combinations, only the lower concentration, 12.5 µM, was tested. The viabilities are shown in Table 4. Results show that some phytocannabinoid combinations induced a significative higher cell growth inhibition, respect to the single administration.

Further experiments will be done to better analyse the effect of these combinations, both in MM and osteoblast cells.

Phytocannabinoid	Viability % ± SD		
ALONE 12.5 µM	**U266**	**RPMI**	**SKO**
CBG	97.4 ± 6.5	99.3 ± 8.2	105.4 ± 4.4
CBC	106.2 ± 3.5	104.9 ± 3.6	96.5 ± 6.7
CBN	89.6 ± 6.6	102.6 ± 7.4	89.2 ± 5.9
CBDV	88.6 ± 7.2	70.2 ± 5.3	82.1 ± 5.2
ALONE 25µM			
CBG	87.7 ± 5.8	69.1 ± 5.5	84.6 ± 5.5
CBC	101.2 ± 7.2	98.3 ± 5.1	94.3 ± 6.3
CBN	61.1 ± 4.8	81.1 ± 7.7	60.6 ± 3.8
CBDV	76.2 ± 6.5	40.2 ± 2.4	54.6 ± 4.4
DOUBLE COMBINATIONS 12.5 µM each one			
CBG + CBC	86.1 ± 7.3	72.4 ± 6.1 *#	89 ± 7.9
CBG + CBN	100 ± 8.8	88.8 ± 7.5	96.1 ± 8.4
CBG + CBDV	86.2 ± 7.3	65.8 ± 4.8 *§	74.5 ± 6.2 *
CBC + CBN	92 ± 8.1	87.5 ± 5.8 #	76.4 ± 6.3 #
CBC + CBDV	93.6 ± 8.8	60 ± 5.3 #§	76.1 ± 6.8 #
CBN + CBDV	93.2 ± 8.2	68.6 ± 5.5 ç§	78.3 ± 6.4
DOUBLE COMBINATIONS 25 µM each one			
CBG + CBC	51 ± 4.2 * #	8.8 ± 0.6 *#	45 ± 3.2 *#
CBG + CBN	74.7 ± 6.3	15.3 ± 0.9 *ç	64.3 ± 5.4 *
CBG + CBDV	40.8 ± 3.1 *§	15.8 ± 0.8 *§	39.4 ± 2.9 *§
CBC + CBN	72.7 ± 6.5 #	16.8 ± 0.7 #ç	57.8 ± 4.6 #
CBC + CBDV	59.5 ± 4.7 #§	27.7 ± 1.9 #§	53.3 ± 4.1 #
CBN + CBDV	58.1 ± 4.9 §	16.8 ± 0.9 ç§	52.4 ± 4.3
TRIPLE COMBINATIONS 12.5 µM each one			
CBG + CBC + CBN	86.5 ± 7.3 #	69.5 ± 5.8 * # ç	70.0 ± 6.1 * # ç
CBG + CBC + CBDV	84.5 ± 7.2 #	50.0 ± 4.1 * # §	67.8 ± 5.8 * # §
CBG + CBN + CBDV	67.0 ± 5.6 *ç §	34.6 ± 2.5 *ç §	44.6 ± 3.5 *ç §
CBC + CBN + CBDV	79.8 ± 6.9 #	43.8 ± 3.8 # ç	60.1 ± 5.2 # ç
QUADRUPLE COMBINATIONS 12.5 µM each one			
CBG + CBC + CBN + CBDV	59.9 ± 4.7 * # ç §	25.2 ± 1.7 * # ç §	30.5 ± 2.2 * # ç §

Table 4. Viability of double, triple and quadruple combination of CBG, CBC, CBN and CBDV in MM cell lines, and viabilities of single treatment as reference. Cell viability was determined by MTT assay. Results are expressed as the mean ± SD of three separate experiments. *$p<0.05$ vs CBG alone; # $p<0.05$ vs CBC alone; ç $p<0.05$ vs CBN alone; § $p<0.05$ vs CBDV alone.

5. Discussion

Despite many treatment options available, there is a need for new treatments for MM patients who became refractory to all options.[1,4] Cannabinoids are used in cancer patients who receive chemotherapy and radiotherapy for their palliative properties, like analgesic, antinauseant, antiemetic and antidepressant properties, but they are also demonstrating direct anticancer effect [13,25] *in vitro*, in *in vivo* animal models and in clinical trials.[14,30] In MM, it was already studied the effect of CBD, THC, the synthetic cannabinoid agonists WIN-55, PGN-6, -17, -34 and -72, the inverse agonist of CB2 phenylacetylamide (PMA), and β-caryophyllene, which showed promising anticancer effects *in vitro* and, WIN-55, in *in vivo* animal models.[50] Here, we investigated the anticancer effect of four minor phytocannabinoids, CBG, CBC, CBN and CBDV *in vitro* in three MM cell lines and the effect of CBN *in vivo* in a xenograft murine MM model.

Firstly, we evaluated the expression of the cannabinoid receptors CB1 and CB2, and the cannabinoid-like receptors TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, TRPM8, in the U266, RPMI and SKO MM cell line, to assess if phytocannabinoids could interact with these cells. We found that all the receptor were expressed in all the three cell lines, at different levels. Feng et al. found that CB2 receptor was highly expressed in human MM cell lines RPMI, U266, MM.1S, H929 and in primary $CD138^+$ cells from MM patients, while CB1 was not expressed in MM cell lines U266 and MM.1S, in line with our three cell lines that express high levels of CB2 receptor, respect to CB1 that was low expressed.[52] Barbado et al. found that CB2 receptor was express in MM cell lines, in particular was more expressed in the most sensitive cell lines MM1.R and RPMI, and in B and T lymphocyte, while was less expressed in the most resistant cell lines MM1.S and U266, and in hematopoietic stem cells.[53] Garofalo et al. found a high expression of CB2 in MM cell lines KMS-12 PE and U-266.[54] The high expression of CB2 receptor in MM cell lines is in line with the general know that CB2 receptor is present mainly in immune cells, while CB1 receptors mainly in the CNS.[17,18] Our laboratory previous found that RPMI and U266 cell did not express TRPV2 at appreciable levels [51] and here we found that RPMI, U266 and SKO express TRPV2 at very low levels.

Regarding the cytotoxicity of the minor phytocannabinoids investigated in this study, we found that they inhibited MM cell growth, in a dose dependent manner. As we can see from the IC_{50}, CBN and CBDV were the most efficacious in reduce cell viability, followed by CBG and in the end by CBC, that was the less cytotoxic. This was obtained in all the three MM cell lines, and RPMI cells resulted the most sensitive cell line. This four minor phytocannabinoids CBG, CBC, CBN and CBDV are less studied compared to THC and CBD, anyway there is evidence for their effect in reducing cancer cell growth in many cancer models. CBG reduced viability of human pancreatic ductal adenocarcinoma cells,[39] breast cancer cells,[40,41,42] oral epithelioid carcinoma cells,[43] colorectal cancer cells,[42,44] gastric adenocarcinoma cells,[42] cholangiocarcinoma cells,[45] glioblastoma cells and GSCs,[46] mesothelioma cell [47] and prostate carcinoma cells.[42,48] CBC reduced growth of human breast cancer cells,[42] mesothelioma cells,[47] colorectal cancer cells,[42,44] gastric adenocarcinoma cells,[42] prostate carcinoma cells,[42,48] and rat glioma cells and basophilic leukaemia cells.[42] CBN reduced viability of human breast cancer cells,[40] mesothelioma cells [47] and prostate carcinoma cells.[48] CBDV blocked cancer cell

growth of human mesothelioma cells [47] colon carcinoma cells [44] and prostate carcinoma cells.[48] In line with our results of rank of potency, CBN was more efficacious then CBG in human breast cancer cells [40] and CBC was less potent than CBG and CBDV in reducing cell viability of human colorectal cancer cells.[44] Anyway, the rank of potency of these phytocannabinoids can be different in cells of different tumor models, and also in cell lines of the same tumour, as observed in other works.[47,48] As far as studies on MM are concerned, no one evaluated the effect of CBG, CBC, CBN and CBDV, but other cannabinoids induced an inhibition of MM cancer cell growth, in particular CBD, THC, WIN-55, PGN-6, -17, -34 and -72, PMA and BCP.[36,51-55]

We demonstrated that the inhibition of MM cells growth was associated with induction of necrotic cell death by CBG, CBC, CBN, CBDV, supported by an increased expression of γ-H2AX protein, marker of DNA damage, and DNA fragmentation. In the literature, however, the main mechanism linked to cannabinoid-induced death appears to be apoptosis or even autophagy, as in the case for the most studied CBD.[34,35] The few reports with CBG indicated that it induces autophagy and subsequent apoptotic cell death in human pancreatic ductal adenocarcinoma cells,[39] apoptosis in human mesothelioma cell lines,[47] cholangiocarcinoma cells,[45] glioblastoma and glioma stem cells,[46] human colorectal cell lines.[44] Moreover, the combination of CBG, CBN, CBD and THC induced apoptosis in human breast cancer cell lines.[40] Regarding instead reports on MM cell lines, it was found that PMA, WIN-55 and BCP induced apoptosis,[52,53,55] while CBD triggered mitochondrial and ROS-dependent necrosis,[36,51] in line with our results.

In MM, bone disease is due to myeloma cells infiltrating the bone marrow and inducing excessive bone destruction.[1,10] The ECS has been shown to regulate bone metabolism, playing a role in maintaining the bones strength and combating bone diseases.[56] Here, we found that CBG, CBC, CBN and CBDV reduced the invasion of MM cells toward HuOB cells, but in particular CBG and CBN were the most effective. Moreover, CBG, CBC, CBN and CBDV reduced the bone slices resorption by osteoclast, with CBG and CBN being the most potent also in this model. In accordance, studies showed that cannabinoids can regulate osteoclasts, osteoblasts and adipocytes *in vitro* and *in vivo* [56] and in particular CB2 receptor agonists reduced cancer-induced osteolytic destruction.[57] MAGL inhibitor JZL184 reduced osteolytic bone metastasis in mouse models of breast and prostate cancers [59] and CBD attenuated stimulatory effects on osteoclast induced by an activator of GPR55, and reduced bone resorption *in vivo* in mice via modulation of GPR55 signalling.[60] ECBs and their receptors showed to be expressed in the bone [56] and here we found that human osteoblasts express all the main cannabinoids target receptors, apart from TRPV1.

Moreover, we found that CBN reduced tumour mass in a xenograft murine model of MM. CBN was selected after the previous results obtained, in which CBN was the most effective considering cytotoxicity on MM cell lines, the effect on modulating MM cell invasion and bone resorption. Many studies in years found that cannabinoids reduced tumour growth *in vivo* in animal models. CBD inhibited tumor growth for example in xenograft mice models of breast cancer,[42,65] lung cancer,[66] in orthotopic mouse model of glioma,[67] in xenograft mouse model of colorectal cancer, where increased the effect of Oxaliplatin.[68] CBG *in vivo* effect was studied in two *in vivo* models: it decreased tumour growth in a mouse model of melanoma [49] and in a xenograft mouse

model of colon adenocarcinoma.[44] About MM, one work found that WIN-55 significantly suppressed tumour growth *in vivo* in a xenograft MM mouse model.[53]

In the end, we found that some double combinations, most of the triple combinations, and the quadruple combination of the phytocannabinoids induced a significative higher cell growth inhibition, respect to the phytocannabinoid alone. In accordance, it was found that CBG combined with CBD had additive effect in reducing the viability of primary glioblastoma cells and glioma stem cells.[46] Moreover, Schoeman et al. found that between two-cannabinoid combinations of CBD, CBN, CBG and THC in human breast cancer cell lines, synergistic interaction was observed for THC:CBD, while the majority of the combinations were antagonistic. Also, they found that four-cannabinoid combinations at different ratios yielded highly cytotoxic growth inhibition.[40]

6. Conclusions

Together, our results suggest that CBG, CBC, CBN and CBDV can be promising anticancer agents for MM, due to their cytotoxic effects on MM cell lines and, for CBN, in an *in vivo* xenograft mouse model of MM. Moreover, data support also their beneficial effect on the bone in terms of reduction of MM cells invasion toward the bone and bone resorption (mainly CBG and CBN). Obviously, further studies are necessary to better understand the mechanism of action of these phytocannabinoids, and to analyse also CBG, CBC and CBDV effect *in vivo*.

7. Funding

The cost of experiments was partially granted by Entourage Biosciences (Richmond, BC, Canada).

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Appendix

1. Participation in other projects

“Ozone therapy, cannabis and pancreatic cancer”, project granted by Fondazione Maria Guarino Amor Onlus.

“Cannabinoids and endometrial cancer”.

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

“Valutazioni di formulazioni di Cannabis terapeutica nella malattia di Crohn”, project granted by ASUR and Regione Marche.

Projects in collaboration with prof. Maggi Filippo, prof. Bonacucina Giulia and prof. Sagratini Gianni laboratories.

2. Conferences and seminars

Poster presentation at the 29th EACR (European Association for Cancer Reasearch) congress. Poster title: “Cannabigerol in vitro effect on human pancreatic ductal adenocarcinoma”. Torino, 2023.

Poster presentation at the Cannabinoid Conference 2022. Poster title: “TRPV2 correlates with endometrial cancer aggressiveness and its activation by Cannabidiol induces cytotoxicity and improves chemosensitivity”. Basilea, 2022.

Poster presentation at the 28th EACR congress. Poster title: “TRPV2 correlates with endometrial cancer aggressiveness and its activation improves chemosensitivity”. Siviglia, 2022.

Speaker at the conference “Cancro e dolore: ossigeno ozono, cannabis e terapie integrate”. Presentation title: “Fitocannabinoidi “minori” in modelli preclinici di tumore al pancreas”. Napoli, 2022.

Speaker at the 2nd “International Webinar on Cancer Research and Therapeutics”. Abstract title: **“**Expression and biological function of PD-1 ligands in Endometrial Cancer”. Webinar, 2022.

Speaker at the congress "Alimenti e nutraceutici: Salute e prevenzione attraverso il cibo". Abstract title: **“**Hemp extract safety for dermatological application”. Università di Camerino (webinar), 2021.

3. Publications

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

Angeloni, S., Marinelli, O., **Aguzzi, C**. et al. Anti-inflammatory and antioxidant activities of ginsenosides and ginseng root extract to counteract obesity. Eur Food Res Technol (2023). https://doi.org/10.1007/s00217-023-04371-1

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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.

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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.

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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.