Cannabinoids inhibit energetic metabolism and induce AMPK-dependent autophagy in pancreatic cancer cells

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Abstract
ABSTRACT

The anti-tumoral effects of cannabinoids have been described in different tumor systems, but their mechanism of action remains unclear. The aim of this thesis was to unravel key pathways mediating cannabinoid dependent inhibition of pancreatic cancer cell growth through the analysis of energetic metabolism. Panc1 cells treated with two synthetic cannabinoids, GW and ACPA, showed elevated AMPK activation induced by a ROS-dependent increase of AMP:ATP ratio. ROS promoted nuclear translocation of GAPDH, which was further amplified by AMPK, thereby attenuating glycolysis. Furthermore, ROS determined the accumulation of NADH, suggestive of a blockage in the respiratory chain, which in turn inhibited the Krebs cycle. Concomitantly, inhibition of Akt/c-Myc pathway led to a decreased activity of pyruvate kinase isoform M2 (PKM2), further down-regulating glycolysis, and glutamine uptake. Altogether, these alterations of cancer cell metabolism mediated by cannabinoids resulted in a strong induction of autophagy and inhibition of cell growth.
Introduction
INTRODUCTION

1. Pancreatic adenocarcinoma

Pancreatic adenocarcinoma (PDAC) is one of the most aggressive human malignancies and is the 4th cause of death for cancer in the United States of America, although it represents only 2-3% of all cancers (1).

PDAC is associated with only a few known demographic and environmental risk factors and a handful of autosomal dominant genetic conditions. Multiple studies have established advanced age, smoking, and long-standing chronic pancreatitis as clear risk factors; diabetes and obesity also appear to confer increased risk. Increased risk has also been documented in relatives of PDAC patients, and it is estimated that 10% of PDAC cases are associated with an inherited predisposition based on familial clustering (2).

Many advances have been made over the past two decades in the characterization of the molecular alterations that take place in pancreatic cancer, especially concerning oncogenes, growth factors, and tumor suppressor genes.

1.1 Oncogene activation in PDAC

Genes that increase their biologic activity, as a result of mutation, are termed oncogenes. The oncogene most commonly detected in human cancers is Ras. Not surprisingly, Ras is also the most important oncogene identified to date in pancreatic cancer. The Ras gene comprises 3 families, H-Ras, K-Ras, and N-Ras. Of these, the K-Ras family is responsible for almost all of the pancreatic cancer mutations, with mutations in the other families occurring only rarely. Studies indicate that K-Ras, which is located on chromosome 12p13, is mutated in up to 95% of pancreatic adenocarcinomas. These mutations, thought to be an early event in the pathogenesis of pancreatic carcinoma, are point mutations and result in a constitutively active GTP-bound product that
promotes increased signal transduction and uncontrolled growth (3).

A key downstream target of the Ras family is phosphoinositide 3-kinase (PI3K), the enzyme responsible for generation of 3-phosphorylated phosphoinositides and activation of the protein kinase Akt (4). Activation of Akt represents a biological indicator of the aggressiveness of pancreatic cancer (5), and preclinical studies have shown that generic inhibitors of PI3K, such as wormannin and LY294002, induce apoptosis and inhibit tumor growth of pancreatic cancer xenografts (6). PI3K signaling also involves mammalian target of rapamycin (mTOR), a downstream target of Akt, and activation of mTOR has been observed in approximately 75% of PDACs (7). Therefore, inhibition of mTOR is an interesting target for therapy, and for this purpose there are currently FDA-approved inhibitors on the market. Although the exact role of the PI3K/Akt/mTOR pathway in pancreatic cancer remains to be elucidated, signaling of this pathway was shown to inhibit apoptosis, and inhibition of this pathway caused an increased sensitivity to gemcitabine (8, 9).

Like many other types of cancer, pancreatic cancer also shows high frequencies of overexpression and/or amplification of the c-Myc oncogene. It has been shown that 43.5% of primary tumors and 31.6% of metastases overexpress c-Myc (10) and simultaneous amplification of activated K-Ras and c-Myc has been found in both primary tumor and lymph node metastasis, suggesting that c-Myc may collaborate with other oncogenes to promote development and progression of pancreatic cancer (11).

The FGF (fibroblast growth factor) family consists of 19 homologous polypeptide growth factors that participate in essential cell functions, including cell differentiation during tissue repair, mitogenesis, and angiogenesis. FGF-1-5 and 7 have been found to be overexpressed in certain human pancreatic cancer cell lines. Furthermore, most human pancreatic cancers have been shown to overexpress FGFR-1β, which in turn activates various cascades, like PI3K, the Ras, Raf, and MAP kinases. Furthermore, it is also known that several ligands for the FGF receptor such as EGF, tumor growth factor-β (TGF-β), and heparin binding EGF-like growth factor (HB-EGF) are also
overexpressed in pancreatic cancer. It has been proposed that this receptor-ligand system plays a role in pancreatic carcinogenesis via autocrine and paracrine mechanisms. Other growth factors may play a role in pancreatic cancer, including insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) (3).

1.2 Tumor suppressor gene inhibition in PDAC

Inactivation of specific genes, called tumor suppressor genes, results in the elimination of vital regulators of cell proliferation allowing for uncontrolled growth. A growing number of tumor suppressor genes have been identified in the pathogenesis of pancreatic cancer, like p53, members of the INK4 family, and DPC4/SMAD4.

The p53 tumor suppressor gene is located on chromosome 17p. It encodes a 53-kDa nuclear phosphoprotein that acts as a transcription factor capable of modulating the expression of an array of genes involved in critical functions, including cell cycle regulation/arrest, apoptosis, differentiation, DNA surveillance, and repair. p53 is thought to be mutated anywhere between 40% and 100% of cases and it constitutes the most commonly mutated tumor suppressor gene in pancreatic cancer.

The p16 tumor suppressor gene was the first member to be identified in the INK4 family of cyclin-dependent kinase (CDK) inhibitors. It is located on chromosome 9p and has been implicated in a variety of tumors, including pancreatic cancer. The p16 gene product normally binds to the cyclin CDK4 complex and prevents it from phosphorylating the retinoblastoma protein (RB). In its nonphosphorylated state, the RB protein arrests the cell cycle at the G1/S checkpoint. Loss of p16 activity results in the lack of inhibition at the level of the cyclin-CDK4 complex and allows for uncontrolled growth. Studies suggest that p16 activity is lost in about 40% of pancreatic cancers by homozygous deletion.

Survival signalings driven by Akt are counteracted by PTEN, a PI3K antagonist, which de-
phosphorilates the 3-phosphorylated phosphoinositides (PIP3) into PIP2, thus inhibiting Akt pathway. In fact, PI3K and Akt are overexpressed in a variety of cancers and it has been shown that PTEN is frequently deleted in advanced tumors (12). These alterations lead to a constitutively active survival-signaling pathway that enhances the insensitivity of tumor cells to apoptosis induction.

Around 5% of pancreatic cancers show somatic inactivating mutations in LKB1, one of the major AMPK activators, and levels of LKB1 protein expression are decreased in around 20% of human PDAC (13). AMPK is a metabolic sensor and its activation results in a decrease in ATP-consuming and an increase in ATP-producing processes. Conditional LKB1 deletion in mice pancreatic epithelium demonstrated defects in acinar cell polarity, cytoskeletal organization and loss of tight junctions concomitantly with an inactivation of the AMPK/MARK/SAD family kinases. Mice rapidly develop pancreatic serous cystadenomas, but LKB1 loss alone is not sufficient to drive formation of PDAC (14) and remains still unknown how LKB1 could influence metabolic processes in PDAC.

2. Chemotherapy approach

Up to now, despite decades of efforts at elucidating molecular pathways involved in initiation and progression of PDAC and at identifying effective therapies, the prognosis of pancreatic adenocarcinoma has not improved. At diagnosis, still less than 20% of patients are candidates for surgery with curative intent (15).

Standard treatments for advanced disease and for adjuvant and neo-adjuvant regimens include radiotherapy and/or chemotherapy. Radiotherapy has been shown to have some utility for regional confined cancers, but it is often too toxic for tissues surrounding the neoplasia. Monotherapy with gemcitabine (GEM) has been the standard treatment during the last decade, although it has a response rate of less than 20% (16).
The cytosine analogue GEM is an S-phase nucleoside that is currently considered to be the single agent of choice in advanced pancreatic cancer.

GEM undergoes phosphorylation by deoxycytidine kinase to difluorodeoxycytidine di- then tri-phosphate. GEM diphosphate inhibits ribonucleotide reductase, which is the primary enzyme involved in the formation of deoxycytidine monophosphate, a natural substrate in DNA replication. This allows the incorporation of GEM triphosphate nucleotides into the DNA chain during replication. GEM permits one more nucleotide to pair before termination of the replication process. This means that the GEM nucleotide is less susceptible to excision repair by exonuclease enzymes, making DNA repair more difficult (masked termination).

GEM has shown a 5–11% response rate for advanced pancreatic cancer, with a median survival rate of 5.7–6.3 months in Phase I and II studies. In the pivotal Phase III trial the median survival was increased from 4.4 months using bolus i.v. 5-FU (63 patients) to 5.7 months with GEM (63 patients), and the one-year survival was increased from 2% to 18%, respectively (17). Many clinical trials have failed to demonstrate an improvement in overall survival with the addition of different drugs to GEM, including cetuximab and bevacizumab. Nevertheless, some modest but interesting advances have been provided by drug combination therapies such as GEM-erlotinib, GEM-capcitabine, and GEM plus a platinum salt (15). Now, more than 250 clinical trials are in the recruiting, active, terminated or completed phase for PDAC treatment with GEM in combination with other potential therapies (http://www.clinicaltrials.gov).

3. Metabolism regulation and Warburg effect

Over the past 25 years, the “oncogene revolution” was at the base of many studies, and nowadays it is clear that there are thousands of point mutations, translocations, amplifications and deletions that may contribute to cancer development (18). Moreover, it is becoming clear that many of the key oncogenic signalling pathways converge to adapt tumour cell metabolism in order to support cell
growth and survival, and these metabolism alterations nowadays should be considered a crucial hallmark of cancer (19). Multiple molecular mechanisms, both intrinsic and extrinsic, converge to alter core cellular metabolism and provide support for the three basic needs of dividing cells: i) rapid ATP generation to maintain energy status, ii) increased biosynthesis of macromolecules, and iii) tightened maintenance of appropriate cellular redox status (Fig. 1) (19).

**Fig. 1 Determinants of the tumour metabolic phenotype.** The metabolic phenotype of tumour cells is controlled by intrinsic genetic mutations and external responses to the tumour microenvironment. Abnormal microenvironmental conditions such as hypoxia, low pH and/or nutrient deprivation elicit responses from tumour cells, including autophagy, which further affect metabolic activity. These adaptations optimize tumour cell metabolism for proliferation by providing appropriate levels of energy in the form of ATP, biosynthetic capacity and the maintenance of balanced redox status (19).

The link between cancer and altered metabolism is not new, in fact in the early 1900s, Otto Warburg described that ATP, normally required to maintain physiologic cellular processes, is used by proliferating tumour cells also to generate the energy required to support rapid cell division. Furthermore, tumour cells reprogramme their metabolic pathways and evade the checkpoint controls that would normally block proliferation under stressful metabolic conditions. This characteristic is called “Warburg effect”, which is a shift from ATP generation through oxidative phosphorylation to ATP generation through glycolysis, even under normal oxygen concentrations, and for this reason it has been called “aerobic glycolysis” (Fig. 2) (19). As a result, unlike most normal cells, many transformed cells obtain a substantial amount of their energy from aerobic
glycolysis, converting most incoming glucose to lactate rather than metabolizing it in the mitochondria through oxidative phosphorylation (20, 21). This shift therefore implies that tumour cells implement an abnormally high rate of glucose uptake to meet their increased energy, biosynthesis and redox needs.

**Fig. 2 Molecular mechanisms driving the Warburg effect.** Relative to normal cells (part a) the shift to aerobic glycolysis in tumour cells (part b) is driven by multiple oncogenic signalling pathways. The dashed lines indicate loss of p53 function (19).

There are different hypotheses behind the biological explanation of the Warburg effect, for example the idea that aerobic glycolysis has the capacity to generate ATP at a higher rate than oxidative
phosphorylation and so would be advantageous as long as glucose supplies are not limited. Alternatively, it has been proposed that glycolytic metabolism arises as an adaptation to hypoxic conditions during the early vascular phase of tumor development, as it allows for ATP production in the absence of oxygen. Adaptation to the resulting acidic microenvironment that is caused by excess lactate production may further drive the evolution of the glycolytic phenotype (22, 23). Finally, the most recently hypothesis proposed that aerobic glycolysis provides a biosynthetic advantage for tumor cells, and that a high flux of substrate through glycolysis allows for effective shunting of carbon to key subsidiary biosynthetic pathways (20, 21).

4. Autophagy
Autophagy is a genetically programmed catabolic process that degrades proteins and organelles as a mechanism in response to stress, such as adverse environments, deprivation of nutrients or growth factors. It is a highly conserved intracellular degradation pathway by which bulk cytoplasm and superfluous or damaged organelles are enveloped by double membrane structures termed autophagosomes (24, 25). The contents of these autophagosomes are degraded after fusion with lysosomes, which are then called autophagolysosomes. Autophagy can also occur under basal conditions, called basal autophagy, which maintains cellular homeostasis, plays a role in development (26), defence against microbial infections (27) and clearance of misfolded proteins. So far, more than 30 different autophagy-related genes (Atg) have been identified in yeast and most of them have mammalian homologs that participate in autophagy or in an autophagy-related process (28). Although the suppression of the mammalian target of rapamycin (mTOR) is one of the major signaling pathways regulating autophagy, this process can also be activated independently of mTOR by various stimuli, like increasing hypoxia, reactive oxygen species (ROS), endoplasmatic reticulum stress, starvation, and other (Fig. 3) (29).
During the genesis of cancer, tumour cells experience various forms of intracellular and extracellular stress. This hostile situation results in a damage of cellular proteins, in the production of ROS and in the replication of cells with heritable DNA damage. Tumour cells must therefore utilise homeostatic mechanisms in order to maintain sufficient energy and integrity in order to survive. In fact, at the early stage of carcinogenesis, autophagy inhibits inflammation and promotes genomic stability acting as a tumour-suppressing mechanism. In an established tumour, autophagy may protect cancer cells from various forms of cellular stress, including those determined by chemotherapeutic agents and ionizing radiation. Nevertheless, under certain circumstances, autophagy mediates the therapeutic effects of some anticancer agents. This apparent paradox is probably related to the amplitude of the phenomenon, which may determine the die-or-survive decision of autophagy (30).
5. Cannabinoids

Cannabis has been used as a medicine over many centuries and for many different purposes (31). However, the endocannabinoid signaling system has only recently been considered a potential therapeutic target (32) and individual cannabinoid receptor agonists made their first entry into the clinic less than 30 years ago (31).

At the present time, three general types of cannabinoids have been described: i) phytocannabinoids, which occur uniquely in the cannabis plant, ii) endogenous cannabinoids, which are produced in the bodies of humans and other animals, and iii) synthetic cannabinoids, which have a similar structure to the natural or endogenous ones and are produced in laboratory.

Phytocannabinoids, also called natural cannabinoids, herbal cannabinoids, and classical cannabinoids, are only known to be present in significant quantity in the cannabis plant, and are concentrated in a viscous resin that is produced in glandular structures known as trichomes. All the natural cannabinoids are derived from their respective 2-carboxylic acids (2-COOH) by decarboxylation (catalyzed by heat, light, or alkaline conditions). At least 70 cannabinoids have been isolated from the cannabis plant. Tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) are the most prevalent natural cannabinoids and have received the most study. Endocannabinoids are endogenous lipid signaling molecules. They are generated in the cell membrane from phospholipid precursors and possess cannabimimetic properties because they bind and activate one or more cannabinoid receptor subtypes. Endocannabinoids are implicated in different physiological and pathological functions (regulation of food intake, immunomodulation, inflammation, analgesia, cancer, addictive behavior, epilepsy and others). The two best-studied endocannabinoids isolated to date are arachidonylethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG). AEA is hydrolyzed by the enzyme fatty-acid amide hydrolase (FAAH) whereas 2-AG is degraded by the enzyme monoacylglycerol lipase (MGL).

Synthetic cannabinoids are particularly useful in experiments to determine the relationship between the structure and activity of cannabinoid compounds, by making systematic/incremental
modifications of cannabinoid molecules. Laboratory synthesis of cannabinoids is often based on the structure of herbal cannabinoids, and a large number of analogues have been produced and tested. Untill some years ago, it has been thought that cannabinoids produce their effects only through the activation of two G protein-coupled receptors, identified as CB1 and CB2 receptors. Nowdays, more and more papers are demonstrating that cannabinoids can also act through the dimerization of CB1 or CB2 with other receptors, like transient receptor potential vanilloid type 1 (TRPV1) (33), or via a receptor independent pathway (33, 34), even if their mechanism of action remains not completely clear.

5.1 Cannabinoids as medicines
Medicines that exploit cannabinoids are already in the clinic for their potential therapeutic effect, that include: relief of pain, inhibition of angiogenesis, relief from various symptoms, management of anxiety disorders, glaucoma, some gastrointestinal disorders, atherosclerosis, and tardive dyskinesia (31). Cannabinoids have been also successfully used in the treatment of some of the side effects that often accompany cancer, such as nausea and vomiting, weight loss, lack of appetite and pain. Exemples of cannabinoid medicines are Cesamet® (nabilone), Marinol® (dronabinol), and Sativex® (THC and cannabidiol). The first two of these medicines can be prescribed to reduce chemotherapy-induced nausea and vomiting. Marinol® can also be prescribed to stimulate appetite, while Sativex® is prescribed for the symptomatic relief of neurophatic pain in adults with multiple sclerosis and as an adjunctive analgesic treatment for adult patients with advanced cancer (31).

5.2 Antitumoral action of cannabinoids
Although cannabinoids are used in the palliative treatment of cancer, they are not yet used as a treatment for tumor progression itself. However, the first study to show that cannabinoids possess anti-tumor effects was reported by Munson et al. in 1975. They demonstrated that administration of $\Delta^9$-THC, $\Delta^8$-THC and cannabinol inhibited the growth of Lewis lung adenocarcinoma cell growth
in vitro, and in vivo after oral administration to mice. Since then, cannabinoids have been shown to have anti-proliferative, anti-metastatic, anti-angiogenic and pro-apoptotic effects in various cancer types (lung, glioma, thyroid, lymphoma, skin, pancreas, uterus, breast, and prostate carcinoma) using both in vitro and in vivo models (35).

Although with some exceptions, cannabinoids have been shown to inhibit cancer cell proliferation (36) and autophagy has been demonstrated to mediate this process or to be itself a death mechanism (36, 37). Recent findings have also shown that the ER stress-evoked upregulation of the p8/telomere repeat binding factor 3 (TRB3) pathway induced autophagy via inhibition of the Akt/mTOR axis and that activation of autophagy promoted the apoptotic death of tumor cells (37). Moreover, Vara et al. recently showed that THC and JWH-015 not only regulated TRB3 and Akt, but also induced the activation of adenosine monophosphate kinase (AMPK) in hepatocellular carcinoma (38), with the concomitant inhibition of mTOR and induction of autophagy.

Although some information about the mechanism of action of cannabinoids have been implemented by the literature, the signal transduction pathway of cannabinoid is unclear. There is still the need for a more extensive research into the dose-response relationships as well as the mechanisms elicited by the specific cannabinoids in order for them to be further developed into potential cancer treatments (35).

5.3 Combined treatment gemcitabine-cannabinoid in PDAC

Recently, we demonstrated that the chemotherapeutic agent GEM induces a strong synergistic effect in combination with the three different synthetic cannabinoids GW, ACPA, and rimonabant (SR1) (36). In this study, we showed that GEM induced both CB1 and CB2 receptors by an NF-κB-dependent mechanism and that its association with cannabinoids synergistically inhibited pancreatic adenocarcinoma cell growth and increased ROS induction by single treatments. The antiproliferative synergism was prevented by the radical scavenger N-acetyl-L-cysteine and by the specific NF-κB inhibitor BAY 11-7085, demonstrating that the induction of ROS by
GEM/cannabinoids and of NF-κB by GEM was required for this effect. In addition, we reported that neither apoptotic nor cytostatic mechanisms were responsible for the synergistic cell growth inhibition, which was strictly associated with the enhancement of endoplasmic reticulum stress and autophagic cell death. Noteworthy, the antiproliferative synergism was stronger in GEM-resistant pancreatic cancer cell lines compared with GEM-sensitive pancreatic cancer cell lines and the combined treatment strongly inhibited also the growth of human pancreatic tumor cells xenografted in nude mice without apparent toxic effects.

These findings supported the potential use of cannabinoids also in combination with other drugs in order to inhibit cell growth in human pancreatic cancer cells and in other tumoral models.
Aim of the thesis
Pancreatic adenocarcinoma is one of the most aggressive and devastating human malignancies with a death-to-incidence ratio of 0.99 (39). At diagnosis, less than 20% of patients are candidates for surgery with curative intent (15). Monotherapy with gemcitabine (GEM) has been the standard treatment during the last decade, although it has a response rate of less than 20% (16). For this reason, it is very important to identify new molecules able to selectively inhibit pancreatic cancer cells proliferation. Cannabinoids have been shown to have anti-proliferative, anti-metastatic, anti-angiogenic and pro-apoptotic effects in various cancer types using both in vitro and in vivo models (35), and recently we demonstrated that combination of cannabinoids with GEM synergistically inhibit pancreatic cancer cell lines proliferation through autophagy-induction (36). Since little is known about cannabinoid mechanism of action and since cannabinoids have promising features as potential anti-cancer drugs, the aim of this thesis was to analyse the effect of two synthetic cannabinoids, GW and ACPA, to unravel the key pathways mediating the induction of autophagy by cannabinoids in pancreatic cancer cells.
Materials and Methods
MATERIALS AND METHODS

All the metabolomic analysis, metabolite extractions, rapid resolution reverse-phase HPLC, ESI mass spectrometry, and metabolite data elaborations were performed in collaboration with Prof. Lello Zolla, University of Viterbo, Italy.

Materials
ACPA was obtained from Cayman Chemicals (Inalco, Milan, Italy); GW405833 hydrochloride (1-(2,3-dichlorobenzoyl)-2-methyl-3-(2-(1-morpholine)ethyl)-5-methoxyindole), and NAC (N-acetyl-L-cysteine) were obtained from Sigma (Milan, Italy); Compound C (C.C.) was obtained from Calbiochem. Acetonitrile, formic acid, and HPLC-grade water were purchased from Sigma Aldrich (Milano, Italy). Standards (equal or greater than 98% chemical purity) D-glucose 6-phosphate, fructose 6-phosphate, D-fructose 1,6 biphosphate, glyceraldehyde 3-phosphate, phosphoenolpyruvic acid, L-lactic acid, α-ketoglutaric acid, L-malic acid, succinic acid, ATP, NADH, NADPH, 6-phosphogluconolactic acid, L-glutamic acid, glutamine, reduced glutathione, oxidized glutathione were purchased from Sigma Aldrich (Milan).

Cell culture
Panc1 cell line was grown in RPMI 1640 supplemented with 2 mM glutamine, 10% FBS, and 50 mg/ml gentamicin sulfate (BioWhittaker, Lonza, Bergamo, Italy), and incubated at 37°C with 5% CO₂.

Immunoblot analysis
Cells (1.2 x 10⁶ cells/dish 10 cm) were treated as reported in the figures and then lysated with RIPA buffer for total protein extract. Protein concentration was measured with the Bradford protein assay
reagent (Pierce) using bovine serum albumin as a standard. Forty µg of protein extracts were electrophoresed through a SDS–polyacrylamide gel and electroblotted onto polyvinylidene difluoride membranes (Millipore). Membranes were then incubated for 1 h at room temperature with blocking solution (5% low-fat milk in 100 mMTris pH 7.5, 0.9% NaCl, 0.1% Tween 20) and probed overnight at 4°C with the primary monoclonal antibodies (1:1000 in blocking solution). Light chain protein-II (LC-3II), AMPK, phospho-AMPK Thr172, p70S6K, phospho-p70S6K Thr389, Akt, phospho-Akt Ser473, and PDHK were obtained from Cell Signaling, HIF-1 alpha antibody from Novus Biologicals, and α-tubulin antibody from Oncogene. Horseradish peroxidase-conjugated secondary antibodies IgG (1:8000 in blocking solution; Upstate Biotechnology) were used to detect specific proteins. Immunodetection was carried out using chemiluminescent substrates (Amersham Pharmacia Biotech) and recorded using HyperfilmECL (Amersham Pharmacia Biotech). The bands for total and phosphorylated proteins were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units using the public domain NIH Image software (http://rsb.info.nih.gov/nih-image/) and then normalized with α-tubulin expression. Quantifications were obtained as fold induction relative to controls and, for phosphorylated proteins, quantifications represent the ratio phosphorylated/total protein.

Analysis of metabolite uptake and release

Cells (1.2 x 10^6/dish 10 cm) were treated for 12 h with 200 µM GW or ACPA. The cell culture medium was collected and analysed with Bioprofile Flex (Nova Biomedical).

Immunofluorescence analysis

Cells (1.6 x 10^4) were grown on coverslips and treated for 12 h with 200 µM GW or ACPA and 20 µM C.C. or 20 mM NAC. GAPDH antibody was obtained from Cell Signaling. Cells were incubated with rabbit GAPDH-antibody (1:100) at RT for 90 min and then incubated with Alexa Fluor 488 anti-rabbit IgG antibody (1:500) at RT for 60 min. To assess nuclear morphology, cells
were incubated with HOECHST for 2 min at RT. Fluorescence was visualized using excitation/emission wavelengths of 488/520 nm (green) and 350/460 nm (blue) for GAPDH and HOECHST, respectively. Cells were examined using TCS-SP5 Leica confocal microscope at 40× and 63x magnification.

**PKM2 activity assay**

One µg of total protein extract obtained from Panc1 cells treated for 12 and 24 h with 200 µM GW or ACPA, was used to analysed PKM2 activity as previously reported (40).

**c-Myc activity assay**

Cells (4 x 10⁵ cells/dish 6 cm) were treated for 1, 12 or 24 h with 200 µM GW or ACPA and then were lysed with nuclear extract kit (Active Motif) for nuclear and citosolic protein extracts. Five µg of the nuclear extract was used to measured c-Myc activity through ELISA assay (Active Motif, TransAM, c-Myc).

**Transfection experiments**

Cells (2.5x10⁵ cells/dish 6 cm) were transfected with the pcDNA5/FRT expression vector containing the human AMPK gamma-2 subunit wt (γ2wt) or R531G mutated (γ2R531G) using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's directions. Cells were incubated for 72 h and then treated with 200 µM GW or ACPA for 12 h, to evaluate the role of AMP production on AMPK induction. Transfection efficiency was assessed by cytofluorimetric analysis and ranged between 56%. The expression vectors for the AMPK γ2wt and mutant γ2R531G subunit were kindly provided by Dr. Hawley (University of Dundee, Scotland, United Kingdom).
Statistical analysis

ANOVA (post hoc Bonferroni) and graphical presentations were performed by GraphPad Prism 5. P values of (*) p<0.05, (**) p<0.01, or (***) p<0.001 are indicated on the figures.
Results
RESULTS

Cannabinoid-induced autophagy depends on AMPK activation

We previously demonstrated that arachidonoyl cyclopropamide (ACPA) or GW405833 (GW), two synthetic cannabinoid ligands specific for CB1 and CB2, respectively, are able to induce ROS-mediated autophagy in pancreatic adenocarcinoma cell lines (36). To more deeply investigate the molecular mechanisms of this effect, we performed in Panc1 cells treated with ACPA or GW, kinetic analyses of p-AMPK and of the p-p70S6K, HIF-1 alpha, and PDHK downstream targets of mTORC1, a known autophagy inhibitor. As shown in Figure 4, after one hour of treatment with cannabinoids, the increase of AMPK phosphorylation already occurred, with a further extension of the effect up to 24 h (Fig. 4A). It is worthy to note that the AMPK total protein levels decreased after 12 and 24 h treatment. The ratio phospho-p70S6K/p70S6K decreased after 1 h treatment and at a larger extent at 12 and 24 h (Fig. 4B), although the total protein form also decreased after treatment with cannabinoids. Instead, HIF-1α (Fig. 4C) showed a reduction of the expression starting at 12 h treatment and PDHK (Fig. 4D) at 12 h and 24 h for GW and ACPA, respectively.
Fig. 4 Effect of cannabinoids on key metabolic proteins. Western blot analyses of (A) phospho-AMPK, (B) phospho-p70S6K, (C) HIF-1 alpha, and (D) pyruvate dehydrogenase kinase (PDHK) were performed after treatment of Panc1 cells for 1, 12 and 24 h with 200 µM GW or ACPA. The bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units, as described in Materials and Methods. The value of α-tubulin was used as a normalizing factor and quantifications represent the ratio phosphorylated/total protein. Quantification values are the means of three independent experiments (±SD). Statistical analysis: (*) p<0.05, (**) p<0.01, and (***) p<0.001.

These results strongly suggested that AMPK could be involved in autophagy induction by cannabinoids in pancreatic adenocarcinoma cells. To further examine the role of AMPK in this effect, we treated cells with cannabinoids in the absence or presence of compound C (C.C.), an AMPK inhibitor (41), and we analysed AMPK phosphorylation as control (Fig. 5A) and the regulation of the autophagy marker phosphoethanolaminated LC3-II (Fig. 5B).
Fig. 5 Effect of C.C. on AMPK activation and LC3-II induction. Western blot analysis of (A) phospho-AMPK and (B) LC3-II after treatment of Panc1 cells for 12 h with 200 µM GW or ACPA and 20 µM C.C.. The bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units, as described in Materials and Methods. The value of α-tubulin was used as a normalizing factor and phospho-AMPK quantifications represent the ratio phosphorylated/total protein. Values are the means of three independent experiments (±SD). Values are the means of three independent experiments (±SD). Statistical analysis: (**) p<0.01, and (***) p<0.001.

As shown in Figure 5, C.C. prevented AMPK phosphorylation and autophagy induction by cannabinoids providing a strong support to the dependence of cannabinoid-induced autophagy on AMPK activation.

To rule out non-specific effect of C.C. and to characterize upstream events of AMPK activation by cannabinoids, we performed transient transfection of Panc1 cells using a plasmid coding for the mutated γ2 subunit of AMPK (γ2R531G), which is unable to bind AMP. The results reported in Figure 6A and B show that the overexpression of the mutant γ2R531G AMPK significantly decreased both p70S6K phosphorylation inhibition and LC3-II induction by cannabinoids, while the wild type AMPK (γ2wt) had no effect on those cannabinoid activities.
Fig. 6 Role of AMPK on cannabinoid-induced autophagy. Western blot analysis of (A) p-p70S6K and (B) LC3-II after treatment of Panc1 cells for 12 h with 200 µM GW or ACPA, in presence or absence of the expression vectors for the AMPK wt or mutant γ2 subunit. The bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units, as described in Materials and Methods. The value of α-tubulin was used as a normalizing factor. Values are the means of three independent experiments (±SD). (C) AMP/ATP ratio was measured after 12 h treatment with 200 µM GW or ACPA, in the absence or presence of a pre-treatment for 1 h with 20 mM NAC, as reported in Materials and Methods. Values are the means of three independent experiments (±SD). Statistical analysis: (*) p<0.05, (**) p<0.01, and (***) p<0.001.

These findings indicated that AMPK was involved in cannabinoid-induced autophagy via an AMP-dependent mechanism. To confirm the role of AMP in this event and to verify whether it could depend on ROS production, we analysed the cellular AMP:ATP ratio following GW or ACPA treatment in the absence or presence of the radical scavenger N-acetyl-L-cysteine (NAC). As shown
in Figure 6C, the level of AMP:ATP was strongly increased by cannabinoids, while it was similar to the control upon NAC pre-treatment of the cells.

**Cannabinoids inhibit the glycolytic pathway**

To assess whether a restriction of the energetic metabolism by cannabinoids could be responsible for the enhancement of the cellular AMP level, we performed a targeted metabolomic analysis. We determined fold-change variations of concentration levels of several key metabolites from the glycolitic pathway upon GW or ACPA treatment. Figure 7A show a significant increase of glyceraldehyde 3-phosphate (G3P) and phosphoenolpyruvate (PEP) and a decrease of lactate (LACT) following 12 h treatment with GW. Instead, ACPA treatment determined only a G3P increase (Fig. 7B). To exclude the possibility that the glycolysis metabolite increase was determined by a higher glucose uptake by the cells, we measured the amount of glucose in the supernatant of treated and untreated cells and we found that glucose incorporation did not significantly change upon cannabinoid treatment (Fig. 7C).
Fig. 7 Effect of cannabinoids on glycolysis. Panc1 were treated for 12 h with (A) GW or (B) ACPA and the metabolites analysis was performed as reported in Materials and Methods. Legend: F6P, fructose-6-phosphate; G6P: glucose-6-phosphate; FDP: fructose 1,6-bisphosphate; G3P: glyceraldehyde-3-phosphate; PEP: phosphoenolpyruvate; LACT: lactate. (C) Glucose consumption was measured as reported in Materials and Method. Values are the means of three independent experiments (±SD). Values are the means of three independent experiments (±SD). Statistical analysis: (*) p<0.05.

Since the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is known to contribute to the up-regulation of autophagy by translocating to the nucleus (42), we analyzed cellular distribution of GAPDH after treatment with GW or ACPA. Figure 8 shows confocal images where GAPDH appear to be translocated into the nucleus of the cells after 12 h cannabinoid treatment. This effect was inhibited by NAC or C.C., indicating its dependence on ROS and AMPK activation.
Fig. 8 Cannabinoids induce AMPK-dependent GAPDH nuclear translocation. Representative confocal images of GAPDH translocation in Panc1 cells nuclei after 12 h treatment with 200 µM GW or ACPA in the absence or presence of a pre-treatment for 1 h with 20 mM NAC or 20 µM C.C. A 63x magnification representative image has been proposed to better visualize the cytoplasmic/nuclear distribution of GAPDH.
These data suggested that the increased level of G3P observed in the metabolomic analysis could arise from the diminished presence of GAPDH in the cytosol of cells treated with cannabinoids.

PKM2 is an embryonic isoform of pyruvate kinase (PK) re-expressed in cancer cells, which dephosphorylates PEP to pyruvate and determines a metabolic advantage for the cell by allowing the availability of phosphometabolites upstream of pyruvate as precursors for cellular syntheses (43). To assess whether PKM2 activity might be responsible for the increase of PEP level by GW, we analysed the activity of PKM2 on crude protein extracts from cells treated with cannabinoids for 12 or 24 h (Fig. 9).

**Fig. 9** Effect of cannabinoids on pyruvate kinase activity. PKM2 activity was performed after treatment of Panc1 cells for 12 or 24 h with 200 µM GW or ACPA, as reported in Materials and Methods. Values are the means of three independent experiments (±SD). Statistical analysis: (**) p<0.01, and (*** ) p<0.001.

Figure 9 shows that PKM2 activity after GW treatment significantly decreased at 12 h and even more at 24h, while it was inhibited by ACPA only after 24 h treatment. These data are consistent with the increased level of PEP observed in the metabolomic analysis after 12 h of treatment with GW (Fig. 7A). Furthermore, data not shown indicate that PEP did increase after 24 h treatment with ACPA.
Taken together, these findings suggested an impairment of the glycolytic pathway by cannabinoids that correlated with the decrease of the AMP:ATP ratio.

**Cannabinoids inhibit the Krebs cycle**

To further examine the involvement of the energetic metabolism in the induction of AMPK mediated autophagy by cannabinoids, we analysed critical metabolites of the Krebs cycle. As shown in Figure 10, only the levels of the α-ketoglutarate (Fig. 10A and B) and, at a very large extent, those of NADH (Fig. 10C) increased after cannabinoid treatments.

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**Fig. 10 Effect of cannabinoids on Krebs cycle.** Panc1 were treated for 12 h with 200 μM (A) GW or (B) ACPA and the metabolites analysis was performed as reported in Materials and Methods. Legend: KET: α-ketoglutarate; SUCC: succinate; MA: malate. (C) NADH was measured after 12 h treatment with 200 μM GW or ACPA in the presence or
absence of a pre-treatment for 1 h with 20 mM NAC, as reported in Materials and Methods. Values are the means of three independent experiments (±SD). Statistical analysis: (*) p<0.05, (**) p<0.01, and (***) p<0.001.

This result suggested the occurrence of the inhibition by NADH of the α-ketoglutarate dehydrogenase, the enzyme of the Krebs cycle most sensitive to the levels of that coenzyme, and the impairment of the oxidative phosphorylation in the increased AMP/ATP ratio determined by cannabinoids. Interestingly, NAC was able to significantly reduce the increase of NADH by GW or ACPA (Fig. 10C), suggesting the involvement of the oxidative stress in that effect. To verify whether the ROS-dependent NADH increase correlated with the activation of the pentose phosphate pathway and thus with the accumulation of the coenzyme NADPH, known to play a key role in the antioxidant response of the cell, we measured the amounts of the phosphogluconolactone and of NADPH. The levels of both compounds remained unchanged after treatment with GW or ACPA, indicating that cannabinoids were unable to activate the pentose phosphate pathway in our experimental conditions (Fig. 11).

![A](image1.png) ![B](image2.png)

**Fig. 11** Effect of cannabinoid on pentose phosphate pathway (PPP) and NADPH production. Phosphogluconolactone (PGL) and NADPH were analysed after treatment for 12 h with 200 µM (A) GW or (B) ACPA as reported in Materials and Methods. Values are the means of three independent experiments (±SD).
Cannabinoids inhibit the anaplerotic flux of the Krebs cycle from glutamine

Glutamine and glucose are the only two molecules catabolized in appreciable quantities in most mammalian cell in culture, to supply the cell with most of the carbon, nitrogen, free energy, and reducing equivalents. To assess whether regulation of glutamine catabolism was involved in the down-regulation of the energetic metabolism, we measured the levels of glutamine and glutamate in the culture medium after treatment of cells with cannabinoids. **Figure 12A** shows that glutamine incorporation was strongly reduced by both GW and ACPA, whereas glutamate release remained unchanged. The oncogene c-Myc has been described to coordinate the expression of genes involved in glutamine catabolism, including the induction of glutamine transporters (44). Furthermore, it has been shown that Akt induces the up-regulation of c-Myc and that Akt suppression inhibits c-Myc expression (45). We evaluated both Akt phosphorylation on serine 473, which is a marker of activation for this kinase, and c-Myc activity after GW or ACPA treatments. **Figure 12B** and **C** show that GW strongly inhibited Akt phosphorylation and c-Myc activity at 12 h treatment and even more at 24 h, while ACPA determined a significant decrease of both proteins only at 24 h treatment. Altogether, these results suggested that the inhibition of glutamine uptake by cannabinoids could depend, at least for GW, on the repression of glutamine transporters determined by the decrease of c-Myc activity.
Fig. 12 Effect of cannabinoids on glutamine metabolism. (A) The analyses of glutamine uptake and glutamate release were performed after treatment of Panc1 cells for 12 h with 200 µM GW or ACPA. (B) Western blot analysis of phospho-Akt was performed using whole extracts of Panc1 cells treated for 1, 12 and 24 h with 200 µM GW or ACPA. The bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units, as described in Materials and Methods. The value of α-tubulin was used as a normalizing factor and quantifications represent the ratio phosphorylated/total protein. Values are the means of three independent experiments (±SD). (C) C-Myc activity was analysed after treatment for 1, 12 and 24 h with 200 µM GW or ACPA. Values are the means of three independent experiments (±SD). Statistical analysis: (*) p<0.05, (**) p<0.01, and (***) p<0.001.

In Figure 13, we propose a model of the mechanism of autophagy induction by the cannabinoids ACPA and GW in pancreatic adenocarcinoma cells that is based on the results presented in this paper and on literature data.
Fig. 13 Schematic representation of the model describing the inhibition of energetic metabolism and the induction of AMPK-induced autophagic cell death by cannabinoids.
Discussion
DISCUSSION

AMPK has been shown to play a crucial role in cannabinoid-induced autophagy (38). We previously reported (36) that cannabinoids inhibit pancreatic cancer cell proliferation both in vitro and in vivo and autophagy has been demonstrated to mediate this process (36, 37) or to be itself a death mechanism (10). Since the anti-tumour effects of cannabinoids are beginning to be clinically assessed, following the promising preclinical data, the need to clearly establish the molecular mechanisms of cannabinoid-induced autophagy has become more critical. For this purpose the aim of this thesis was to study key pathways regulating autophagy induction in pancreatic adenocarcinoma cells treated with two synthetic cannabinoids, ACPA and GW.

It is well known that AMPK exerts an active role in autophagy by inhibiting mTORC1, the major regulator of protein synthesis and cell growth (46). In this project, we found that the phosphorylation level of p70S6K, a direct target of mTORC1, significantly decreased following cannabinoid treatment and that this event started at a very early stage, well correlating with the increase of the AMPK phosphorylation. Furthermore, we found that the decrease of HIF-1α, a transcription factor that is indirectly regulated by mTORC1 via protein stabilization, started only after 12 h of treatment and, in turn, the decrease of one of its target, PDHK, mainly after 24 h with both cannabinoids. Taken together, these data strongly suggested that the main site of autophagy regulation by cannabinoid-activated AMPK was mTORC1.

AMPK activation is generally mediated by the increase of the cellular AMP/ATP and ADP/ATP ratios that favours the binding of adenine nucleotides to the γ subunit of AMPK (47). In most of the cell types, this event is followed by Thr 172 phosphorylation by the LKB1 complex, which appears to be constitutively active (47). The Ca^{2+}/calmodulin-dependent protein kinase (CaMKKβ) has been also reported to phosphorylate AMPK at the same site in some cell types (47). However, activation by this mechanism can occur in the absence of any change in adenine nucleotide ratios, although increases in Ca^{2+} can act synergistically with increases in AMP or ADP (48). Our data
demonstrated that in cells overexpressing an AMPK containing a mutation in the \( \gamma \) subunit isoform 2 that renders the enzyme insensitive to increases in AMP or ADP, the autophagy pathway was not activated following cannabinoid treatment. This result indicated that in our experimental system the main mechanism of AMPK activation by cannabinoids depended on the alteration of adenine nucleotide ratios and suggested that LKB1 could be involved in the subsequent phosphorylation of AMPK at Thr 172.

AMPK has been described as an intracellular energy sensor and regulator (49), but is also important in maintaining intracellular homoeostasis during many kinds of stress challenges, such as oxidative stress, which has been shown to induce AMPK (50). In our previous paper and unpublished data, we demonstrated that the cannabinoids ACPA and GW were able to induce oxidative stress in pancreatic adenocarcinoma cell lines (36), which was crucial for triggering autophagic cell death in combination with gemcitabine, and that this event started 30 min after the beginning of cannabinoid treatment. Here, we reported that ROS were necessary to increase the AMP/ATP ratio, which in turn mediated the activation of AMPK by cannabinoids leading to autophagy. In agreement with our result, Shrivastava et al. have recently shown that cannabidiol induces apoptosis and autophagy by increasing the generation of ROS (34). We found that NADH cellular level strongly increased after treatment of cells with either ACPA or GW and that this increase was inhibited in the presence of the radical scavenger NAC. Taken together, these data suggested that ROS production by cannabinoids could impair the electron transport chain leading to NADH accumulation and to oxidative phosphorylation inhibition, which in turn could further increase the level of ROS. Consistent with this hypothesis, many articles have been published describing the oxidative stress as a cause of mitochondrial injury, often occurring at the level of complex I of the respiratory chain by direct oxidation of the proteins belonging to the complex (51). In correlation with the strong increase of the NADH level after cannabinoid treatment, we found a strong increase of the AMP/ATP ratio, which suggested that the oxidative phosphorylation inhibition could play a crucial
role in generating the energetic blackout of the cell. Further studies have been planned to clarify this aspect.

Our analyses of key metabolites and enzymes of the glycolysis suggested a general inhibition of the pathway after ACPA or GW treatment. Although glucose uptake by cells remained unchanged, the level of two glycolytic metabolites, G3P and PEP, significantly increased in cells treated with cannabinoids, well correlating with a decrease of the respective enzymes, GAPDH and PKM2. GAPDH has been recently reported as a key redox-sensitive protein, the activity of which is largely affected by covalent oxidative modifications at its highly reactive Cys152 (52). These modifications stimulate nuclear translocation of the enzyme and regulate the fate of the cell (53), often leading to autophagy activation by up-regulating the autophagy protein Atg12 (52). Furthermore, also AMPK has been shown to stimulate GAPDH translocation into the nuclei (54). Our data showed that both NAC and C.C. inhibited GAPDH nuclear translocation, demonstrating that this event was mediated by both ROS and AMPK.

PKM2 is the embryonic pyruvate kinase isoform almost universally re-expressed in cancer that promotes aerobic glycolysis (19, 55). It has recently been shown that c-Myc up-regulates the transcription of genes involved in the alternative splicing leading to the expression of PKM2 (19, 56). Our results showed a strict correlation between PKM2 and c-Myc activities in both ACPA and GW treated cells, suggesting that cannabinoids determined the down-regulation of PKM2 via the inhibition of c-Myc. c-Myc is a classical oncogene that promotes not only proliferation but also the production of accompanying macromolecules and antioxidants that are required for growth (19). c-Myc increases glutamine uptake by directly inducing the expression of glutamine transporters (44) and promotes the expression of the PKM2 isoform (56). Furthermore, c-Myc is up-regulated by Akt, the suppression of which inhibits c-Myc expression (45). Our kinetic analyses showed that GW inhibited Akt phosphorylation at 12 h and even more at 24 h, while ACPA at 24 h treatment. Similar results were obtained analysing c-Myc activity. These findings suggested the dependence of c-Myc down-regulation on the inhibition of Akt phosphorylation by cannabinoids. One of the major
regulator of Akt is the phosphatase PTEN, a tumour suppressor mutated in a wide range of human cancer, the activity of which has been found to increase upon endocannabinoid treatment (57).

In summary, our results demonstrated for the first time that cannabinoid-dependent autophagy induction in pancreatic adenocarcinoma cells is strictly related to the inhibition of the energetic metabolism, which, in turn, is dependent on the early production of ROS induced by the compounds, as we proposed in our model (Fig. 7). We can conclude that cannabinoids seem to counteract the Warburg Effect, which is a peculiarity of cancer cells, by inhibiting energetic metabolism and causing an energetic cellular blackout.
Bibliography


