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INDUCTION OF PSEUDONORMOXIA AS ADJUVANT THERAPEUTIC STRATEGY FOR CANCER

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ABSTRACT

Hypoxia is a hallmark of several solid tumors and it is often associated with chemotherapy resistance. The O_2 levels decrease induces the activation of Hypoxia-inducible factor 1 (HIF-1) which coordinates cellular adaptive changes and metabolic reprogramming. This heterodimeric transcriptional factor is considered as a master regulator of hypoxic cellular response and plays key roles in development, physiology and disease. Under normoxic conditions, HIF-1α subunit has a high turnover strictly regulated by the activation of prolyl hydroxylases (PHDs). At low oxygen levels, PHDs activity is inhibited and HIF-1α is stabilized and migrates into the nucleus where it bounds the HIF-1β and exerts its transcriptional activity.

Our research group has demonstrated that the lack of respiratory complex I (CI) imbalances the α-ketoglutarate (αKG)/succinate (SA) ratio towards αKG, metabolites that regulate the PHDs activity. The chronic activation of PHDs leads to HIF-1α destabilization even when O_2 is available, a condition that we termed *pseudonormoxia*, and hampered tumor growth *in vitro* and *in vivo*.

We hypothesized that pseudonormoxia may be induced by unbalancing the αKG/SA ratio mimicking a normoxic condition and leading to the block of tumor progression. In this context, the aim of this PhD project was to prove that HIF-1α destabilization and pseudonormoxia can be pharmacologically induced by increasing αKG levels using CI inhibitors (indirect approach) or cell permeable αKG ester derivatives (direct approach) in order to identify novel and safe adjuvant therapeutic agents for aggressive cancers. We screened a panel of compounds, composed by molecules that potentially may act as CI inhibitors and seven αKGlogues, for their ability to specifically destabilize HIF-1α in cancer cells, while being not toxic for their non-cancer counterpart. From these experiments, we identified four promising compounds that fit these criteria and that will be used for further investigations. The link between HIF-1α stabilization and mitochondrial function opens to new metabolic-based therapeutic approaches for cancers for which adaptation to hypoxia is a step that must be overcome to progress toward malignancy.

INTRODUCTION

Hypoxia as a drive force of solid tumor malignancy.

The maintenance of adequate oxygen levels is required to sustain physiologic cellular functions [1]. However, a reduction of oxygen availability, also known as hypoxia (0.05-5% O_2), often represents a condition that can be limited to some cells within a tissue, to certain tissue belonging to specific organs or involving the whole organism. The effect of hypoxia can be more or less severe, depending on the O_2 levels, duration and context [2]. During fetal development, hypoxia represents a positive, necessary stimulus, required for the appropriate growth and function of certain organs [3]. Indeed, a gradient in the oxygen tension across the tissue is required for the physiologic function of some organs [4]. Over the long-term, hypoxia is not usually compatible with normal cell function and therefore multicellular organisms have evolved both systemic and cellular responses to inadequate oxygen tension. In this context, the oxygen shortage is related to several pathologies, such us myocardial and cerebral ischemia, diabetes, neurodegeneration and cancer [5, 6, 7]. Indeed, a common feature of most solid tumors is the ability to adapt to low O_2 levels, even anoxia ($\leq$ 0.01% O_2) [8, 9]. The high proliferative rate of cancer cells and growing tumor mass imply that tumor cells often have restricted access to nutrients and oxygen. Most solid tumors are characterized by the presence of regions permanently or transiently subjected to hypoxia due to aberrant vascularization and a poor blood supply promoting the formation of hypoxic niches [10]. Tumor cells in these regions begin to adapt to low oxygen tension by activating several survival pathways mainly mediated by the transcriptional activation of the hypoxia-inducible factor 1 (HIF-1), which is considered the key regulator of cell hypoxic response by modulating the expression of hundreds of genes involved in glycolytic metabolism, angiogenesis and cancer cell survival [11, 12] (Figure 1). Under hypoxic conditions, cancer cells are constrained to switch their metabolism from oxidative to glycolytic and enhance their ability to generate ATP by increasing glucose uptake. In this context, HIF-1 is responsible for the up-regulated expression of glycolytic enzymes and glucose transporters (GLUTs) [13]. Active HIF-1 also promotes angiogenesis through the transcriptional induction of several pro-angiogenic factors, including the vascular endothelial growth factor (VEGF) which is considered the most potent endothelial-specific mitogen by recruiting endothelial cells into hypoxic areas and stimulating their proliferation [14]. In addition, HIF-1 promotes the migration of tumor metastasis into distant and more oxygenated tissues by activating the transforming growth factor-β3 (TGF-β3), epidermal growth factor (EGF) and others [15]. In this scenario, HIF-1 is considered as one of the main "musicians" able to orchestrate cancer cells adaptation to hypoxic environment.

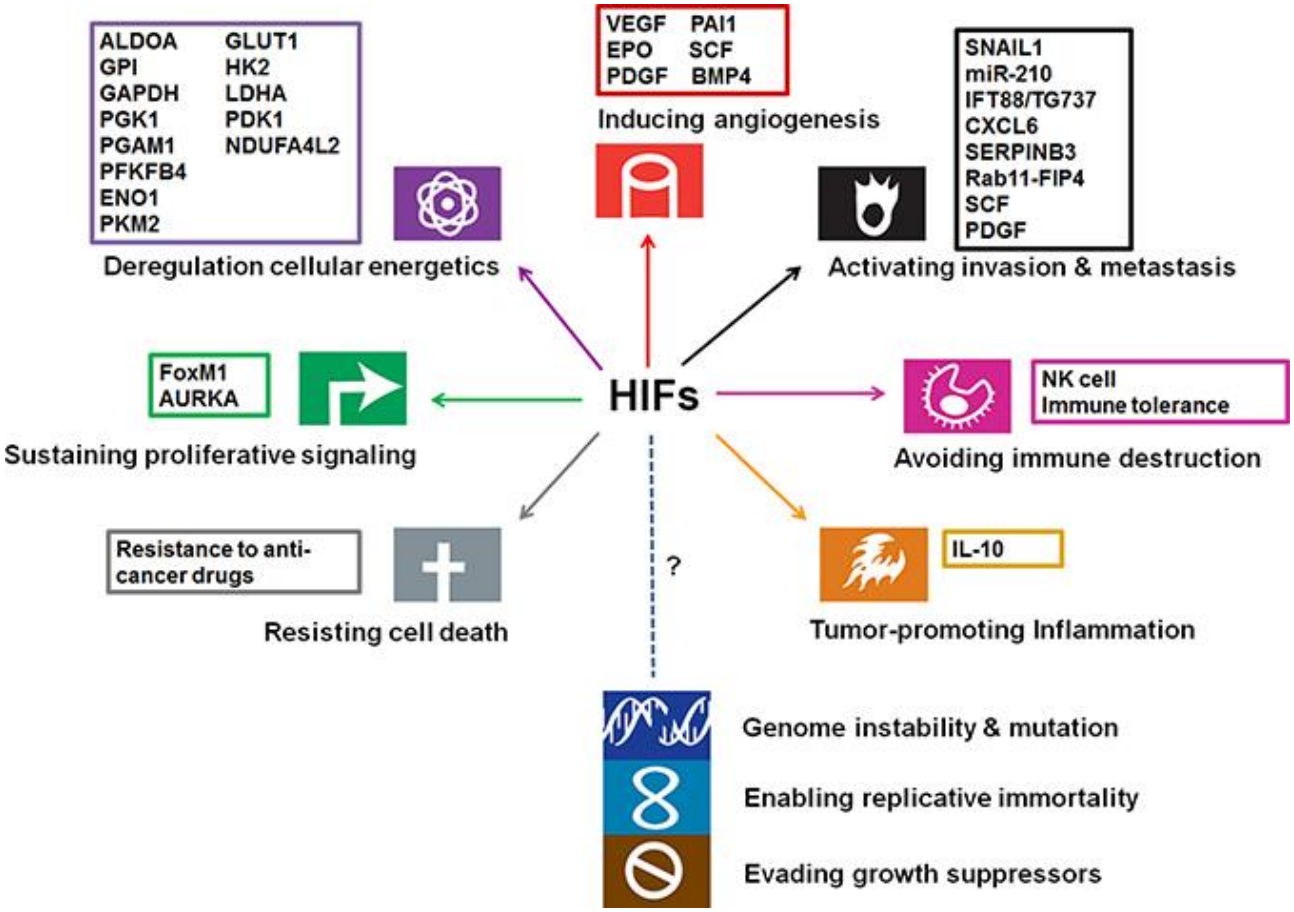


Figure 1: Involvement of HIF-1 and their targets in different aspects of cancer [16]**.** The HIF-1 activity has been implicated in promoting angiogenesis, invasion/metastasis, proliferation, glycolysis, therapeutic resistance, inflammation, and immune evasion.

HIF-1 structure and its involvement in cancer cells adaptation to hypoxia.

HIF is a heterodimeric transcription factor consisting of an oxygen sensitive α-subunit and a constitutively expressed β-subunit. In human cells has been identified three paralogs of HIF-α (HIF-1α, HIF-2α, HIF-3α) and two paralogs of the HIF-β subunit (ARNT, ARNT2). HIF-1α and HIF-2α are very similar structurally and better characterized compared to HIF-3α, which seems to inhibit HIF-1α and HIF-2α activity in a dominant-negative fashion [17]. These last two α subunits differ in their transcriptional domains and may therefore bind different co-factors and affect the expression of different genes [18]. Furthermore, HIF-1α is ubiquitously express in all cells, whereas HIF-2α and HIF-3α are preferentially expressed in specific tissue [19]. However, all HIF-α subunits can heterodimerize with both β-subunits. ARNT2 has a more limited tissue distribution but dimerizes with the same efficiency as ARNT [20].

Both α and β subunits belong to the basic Helix-Loop-Helix-Per-Arnt-Sim (bHLH-PAS) protein family [21]. The bHLH-PAS domain is necessary for the heterodimerization between α and β subunits and for the subsequent recognition and binding with Hypoxia Response Element (HRE) sequence, present in the promoters of the HIF-1 target genes [22] (Figure 2). HIF-1α contains two transactivation domains located respectively in the N-terminal and C-terminal of the protein, named N-TAD and C-TAD [23]. In particular, the C-TAD domain interacts with the transcriptional coactivators p300/CREB through the hydroxylation of asparagine 803 (Asn803), while proline 564 and 402 (Pro564 and Pro402) in the oxygen dependent degradation domain (ODDD) mediate the recognition of HIF-1α by von Hippel-Lindau tumor suppressor protein (pVHL) [24, 25, 26] (Figure 2). These prolines are essential for the regulation of HIF-1α abundance in response to changes of oxygen tension. Under normoxic conditions (21% O_2), HIF-1α is hydroxylated on Pro402 and Pro564 by prolyl hydroxylases (PHDs), which are members of the Fe(II)/α-Ketoglutarate (αKG)-dependent dioxygenase enzyme family [27]. The hydroxylation of these conserved residues triggers the VHL-mediated ubiquitination and consequent proteasomal degradation of HIF-1α subunit [28]. In parallel, Asn803 is hydroxylated by the factor inhibiting HIF-1 (FIH-1), another Fe(II)/αKG-dependent dioxygenase as PHDs, that binds to HIF-1α and inhibits its transactivation function blocking its interaction with the coactivators p300/CREB [29]. Conversely, under hypoxic condition (0.05-5% O_2), PHDs activity is inhibited preserving HIF-1α stability, which translocate into nucleus and dimerizes with HIF-1β.

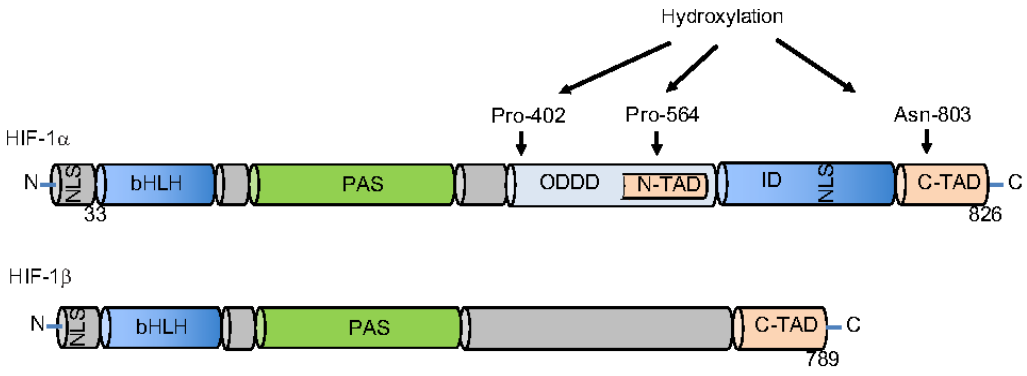


Figure 2: Domain structure of HIF-1α and HIF-1β subunits (adapted image) [30]**.** Schematic representation of HIF-1α (826 amino acids) and HIF-1β (789 amino acids). The N-terminal of both subunits consists of basic helix-loop-helix (bHLH) and Per-Arnt-Sim homology domains (PAS) that are required for heterodimerization and DNA binding, respectively. HIF-1α possess an oxygen-dependent degradation domain (ODDD) required for oxygen-dependent hydroxylation and degradation. The C-terminal region of HIF-1α contains two terminal-transactivation domains (TADs), which are separated by an inhibitory domain (ID). The transcriptional activity of the C-TAD domain is associated with the binding of transcriptional co-activators, including the p300/CREB. HIF-1α is potentially hydroxylated at two proline residues (Pro), which induces binding of von Hippel–Lindau protein (pVHL). Hydroxylation of an asparagine residue (Asn) located in the C-TAD of HIF-1α inhibits their interaction with p300/CREB. NLS, nuclear localization signal.

The stability and activity of HIF-1α is strictly regulated by PHDs (Figure 3). In humans, three different isoforms of these enzymes have been identified (PHD1, PHD2 and PHD3); isoform 2 seems to be the one mainly involved in HIF-1α regulation [31], while the other two preferentially regulate HIF-2α stability [31, 32]. PHDs catalyze the transfer of one oxygen atom from the substrate O_2 to the ketonic carbon of αKG and the second oxygen atom on the proline residues of HIF-1α, forming the hydroxyl group. These enzymes require αKG as co-substrate and Fe(II) as cofactor for their catalytic activity. In parallel to the hydroxylation reaction, αKG undergoes to decarboxylation to form succinic acid (SA) and CO_2. Fe(II) is coordinated in the catalytic site of PHD by two histidine and one aspartate residues oxidizing in Fe(III) after the interaction with PHD substrates and co-substrates. Ascorbate acts as reducing agent keeping iron in the active site in the reduced state [28] (Figure 3). Most importantly, the relatively high K_m of PHDs for O_2 is slightly above its atmospheric concentration (K_m=230-250μM), such that O_2 is rate limiting for enzymatic activity under physiological conditions [33].

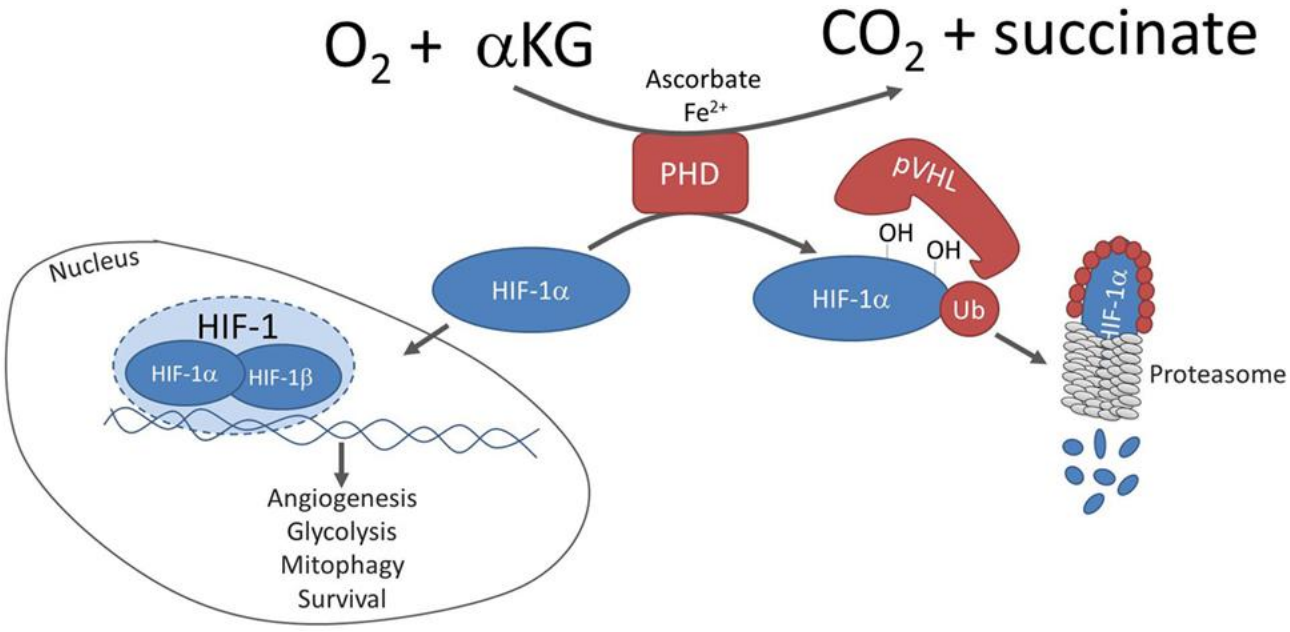


Figure 3: PHDs mediate HIF-1α stability [34]. In normoxia, HIF-1α is hydroxylated on two conserved proline residues by PHDs, triggering pVHL-mediated ubiquitination and proteasomal degradation. The hydroxylation reaction is coupled with the conversion of αKG to succinate and requires co-factors ascorbate and iron. In hypoxia, the enzymatic activity of PHDs is inhibited and HIF-1α dimerizes with the constitutively expressed HIF-1β forming the heterodimeric transcriptional factor HIF-1, which transcribes genes involved in angiogenesis, glycolytic metabolism, mitophagy, and survival.

At the same time, the K_m for αKG is ~50 μM therefore close to its physiological concentration. Thus, besides intracellular O_2 levels, even modest variation of αKG concentrations may interfere with PHDs activity leading to profound modifications of cancer cells metabolism [33, 35, 36]. In line with this, our group demonstrated that the imbalance of αKG/SA ratio, towards αKG, was related to the NADH accumulation, due to the inhibition of αKG dehydrogenase, in respiratory Complex I (CI)-deficient cells. Growing levels of αKG lead to a chronic PHDs activity and constitutive destabilization of HIF-1α even in hypoxic environment. This profound metabolic alteration induces a condition defined *pseudonormoxia* which humper tumor growth *in vitro* and *in vivo* [37, 38, 39, 40]. In this context, the use of specific CI inhibitors or the direct unbalance αKG/SA levels may be adopted as a pharmacological strategy to induce a chronic PHDs activity in HIF-1 dependent cancer cells in order to mimic a normoxic condition and to induce the arrest of tumor progression.

The effect of oncometabolites on HIF-1α stability in cancer.

Besides intracellular oxygen levels, also a great number of additional cellular players and molecular pathway may concur to regulate HIF-1α stability [34]. Concerning its PHD-mediated degradation, it was demonstrated that also modest variations of αKG concentrations may interfere with PHDs activity based on the fact that the K_m of αKG for many dioxygenases is similar to the physiological concentration of this ketoacid [35, 36]. In this scenario, several tricarboxylic acid (TCA) cycle metabolites are able to interfere with HIF-1α stability through the allosterically competition with αKG to modulate PHDs activity, independently from intracellular O_2 tension.

The accumulation of SA, fumarate and 2-hydroxyglutarate (2-HG) have been reported in multiple type of cancers harboring respectively succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase 1 and 2 (IDH1/2) mutations. Generally, increased levels of SA and fumarate occur as result of the loss of SDH and FH gene function, respectively. Conversely, for IDH, a single-allele mutation confers a gain of function producing an excess of 2-HG metabolite [41].

SDH, besides a TCA cycle enzyme, is also the complex II of mitochondrial respiratory chain. Its role consists to oxidize SA to fumarate transferring one electron to ubiquinone. It is composed by four different subunits, each of which can be mutated causing SDH loss of function [42, 43]. Conversely, FH catalyzes the hydration reaction of fumarate to malate and, as for SDH, loss of function has been identified in hereditary forms of leiomyomatosis, renal cancer and childhood encephalopathies [44]. IDH is another TCA cycle enzyme which mutations has been related to tumorigenesis [45, 46]. Three isoforms of IDH (IDH1, IDH2, IDH3) have been already identified. IDH1 and IDH2 convert isocitrate to αKG by oxidative decarboxylation, with the production of NADPH from $NADP^+$ in the cytosol and mitochondria, respectively. The TCA cycle enzyme IDH3

is structurally different from the other two isoforms and utilizes NAD^+ to produce αKG and NADH [41]. Interestingly, IDH3 has not be found mutated in any cancer until now, whereas mutations in IDH1/2 has been associated to loss of enzymatic function to decarboxylate isocitrate and, at the same time, also result in a gain of function for the NADPH-dependent reduction of αKG [41]. The altered activity of IDH1/2 causes an increased affinity for αKG leading to the utilization of NADPH in a partially reversed reaction to produce an excess of new metabolite, 2-HG [41]. The structure of 2-HG, differently from αKG, is characterized by the presence of one hydroxylic group instead of the ketonic group (Figure 4).

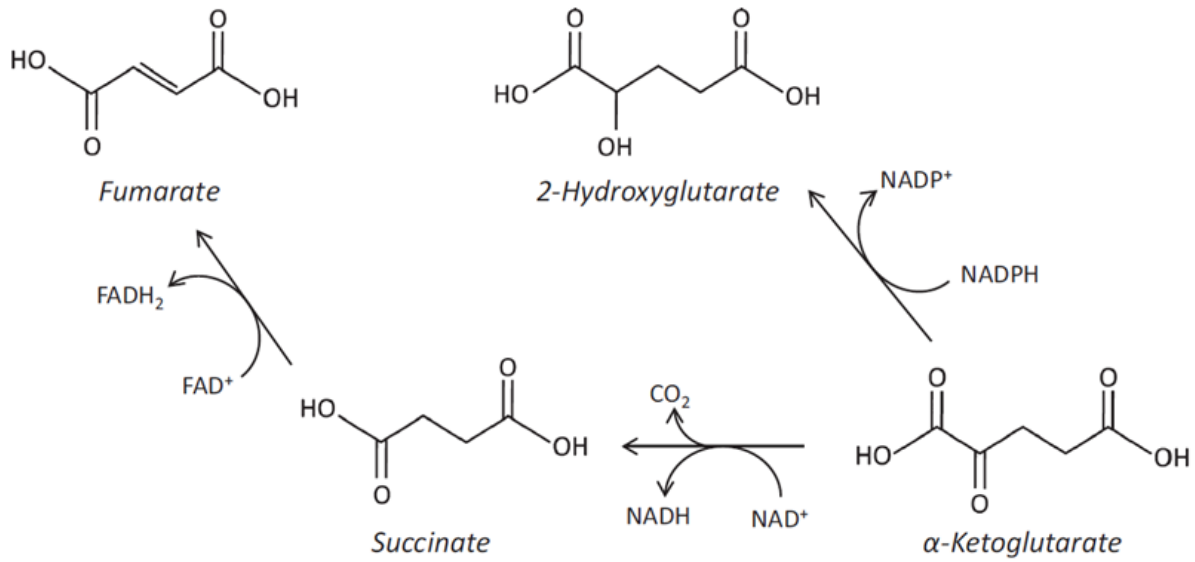


Figure 4. Similarity of oncometabolites structure [47]**.** SA, fumarate and 2-HG are closely linked, both structurally and metabolically, to αKG. SA and fumarate differ from αKG only by the presence of a hydroxyl group on C2 and the loss of C1. In addition, SA and fumarate differ from each other's only by the presence of an ethylenic bond, which may explain their similar tumorigenic effects. 2-HG differs from αKG only by the presence of a hydroxyl group instead of a ketone group.

Increased levels of SA, fumarate and 2-HG, as consequence of SDH, FH and IDH1/2, influence HIF-1α stability through the modulation of PHDs activity since they share a structurally similar with αKG (Figure 4). In particular, SA and fumarate are allosterically competitor of αKG and their intracellular higher levels chronic inhibit PHDs activity leading to the stabilization of HIF-1α and activation of downstream hypoxic pathways, even in normoxia (i.e. pseudohypoxia) [48, 49, 50]. On the other hand, increase of αKG intracellular levels is sufficient to oppose SA and fumarate effect in SDH and FH-deficient tumors leading to the constitutive HIF-1α destabilization [51, 52]. Hence, the levels of αKG, even in hypoxia, may be sufficient to foster PHDs activity and prevent the hypoxic response. On the other hand, the metabolite 2-HG probably acts as competitor inhibitor of αKG

occupying its binding site [53], but the effect exerted by the accumulation of 2-HG on PHDs activity is still debated [50]. The discovery that higher levels of these TCA cycle metabolites may play a primary role to induce metabolic alteration in cancer cells highlights their ability to act as oncogenic signaling molecules, being defined *oncometabolites* [54].

Cancer cells are often characterized by epigenetic, as well as metabolic, alterations. In fact, in many types of cancer the genome is affected by the reduction of DNA demethylation and the hypermethylation of CpG islands in specific promoter regions compared to the corresponding normal tissue. This condition is associated with worse prognosis, potentially due to a silencing of tumor suppressor genes [55]. Recent studies have highlight that *oncometabolites* may also be involved in the alteration of the epigenetic landscape of cancer cells. Increased SA levels inhibit Ten-Eleven Translocation proteins (TETs), enzymes involved in DNA demethylation, leading to CpG island hypermethylation and also causes the inhibition of Histone Lysine Demethylases (KDMs), involved in histone demethylation [56, 57]. Both TETs and KDMs belong to the αKG-dependent dioxygenase family and, for this reason, their activity may allosterically affect by SA intracellular levels. Interestingly, DNA hypermethylation is related to dedifferentiation and enhanced tumorigenic potential in SDH-deficient tumors [58, 59]. Similarly, it has been demonstrated that also fumarate and 2-HG can promote alterations in DNA and histone methylation supporting tumorigenesis [56, 58, 60]. Studies *in vitro* have revealed that in SDH-deficient tumors the exogenous administration of αKG may restore the TETs enzyme activity inhibited by SA restoring the normal cell phenotype [58]. In contrast to genetic mutations, epigenetic modifications are reversible process, offering the possibility of the introduction of new therapies for the treatment of certain tumors [61].

αKG a metabolite with pleiotropic activity.

αKG is a metabolite of TCA cycle derived from the oxidative decarboxylation of isocitrate catalyzed by IDH3 and it can be further converted to form succinyl-CoA by αKG dehydrogenase enzyme [62] (Figure 5). The NAD^+/NADH ratio plays a crucial role to determine the amount of αKG produced in mitochondria. In fact, an accumulation of NAD^+ promotes the oxidative decarboxylation of αKG to succinyl-CoA, conversely the unbalance toward NADH triggers the reductive transamination of αKG to form glutamate through glutamate dehydrogenase reaction. Glutamate formed in this reaction can be then converted in glutamine [63] (Figure 5). Moreover, αKG can be generated as a product of pyridoxal phosphate-dependent trans-amination reactions in which glutamate is a common amino donor. This TCA cycle metabolite, as others, can penetrate into the cytoplasm, where it is used as precursors for biosynthetic reactions. αKG can be transported across the inner mitochondrial membrane through the oxoglutarate carrier, also known as an

oxoglutarate/malate antiporter, and subsequently pass through channels (such as voltage-dependent anion channels) located in the outer mitochondrial membrane [64, 65].

αKG is often defined as a metabolite with pleiotropic activity since it plays a key role in multiple metabolic and cellular pathway. Cataplerotic reactions prevent an accumulation of αKG providing to the production of two amino acids, as glutamate and glutamine, that have a crucial role in many metabolic pathways. In this context, αKG as intermediate in biosynthesis of these two amino acids stimulates protein synthesis, inhibits protein degradation in muscle, constitutes an important metabolic fuel for cells of the gastrointestinal tract and determines proper functioning of organs such as kidney, intestine, liver, as well as pancreatic β-cells, neurons, and cells of the immune system [66].

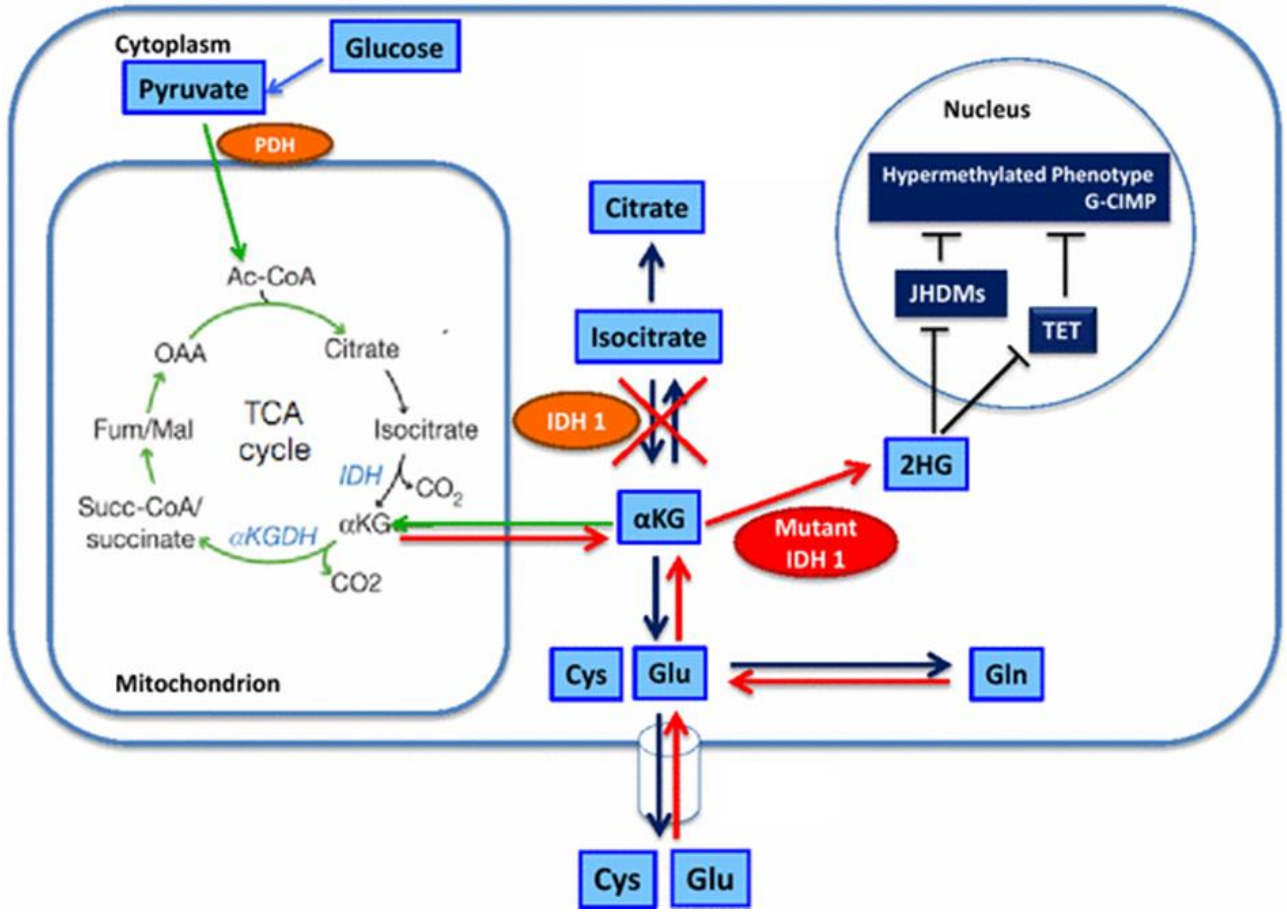


Figure 5: Overview of biochemical reactions involving αKG [67]. αKG have a central role in cellular pathways like glutaminolysis or reductive carboxylation of glutamine. Mutant IDH1 changes the dynamics of metabolic processes in the cell and leads to accumulation of 2-HG, which in turn inhibits Jumonji domain-containing histone demethylases (JHDMs) and TET leading to a hypermethylated epigenetic phenotype.

Additionally, exogenous αKG administration may affect the level of histones and DNA methylation trigging epigenetic changes. Accordingly, embryonic stem cells (ESC) are characterized by elevated αKG/SA ratio that promote histone and DNA demethylation and maintain pluripotency [68]. In *vitro* studies have shown that cell-permeable αKG directly supports ESC self-renewal, while increased levels of SA promotes differentiation suggesting the contribution of proper αKG and SA levels to the maintenance of cellular identity by modulate the epigenetic state of stem cells [68].

Moreover, αKG could also act as signaling molecule by acting as ligands for GPR99, a G protein-coupled receptor [69]. The activation of this pathway in involved in intracellular Ca^{2+} mobilization (via activation of phospholipase C), which acts as a diffusible second messenger regulating a wide range of vital cell functions, including cellular metabolism and cell growth, division and differentiation [70].

A recent study has shown that the supplementation of αKG can extend the lifespan of adult *Caenorhabditis elegans* by inhibiting ATP synthase. The inhibition by αKG of this enzyme, also known as complex V of the mitochondrial electron transport chain, is related to reduced ATP content, decreased oxygen consumption and increased autophagy in both *Caenorhabditis elegans* and mammalian cells [71]. Such moderation of ATP synthesis induced by TCA cycle metabolite may be evolved to ensure energy efficiency in response to nutrient availability. Chin et co-workers suggested that this system may be exploited to confer a dietary restriction-like state that favors maintenance over growth, and thereby delay ageing and prevent age-related diseases [71]. Interestingly, physiological increases in αKG levels have been reported in starved yeast and bacteria and in humans after physical exercise [72, 73]. In this context, the accumulation of αKG in starved condition appears to be a consequence of increased glutamine metabolism to sustain anaplerotic gluconeogenesis from amino acids catabolism [73]. These findings suggest a model in which αKG is a key metabolite mediating lifespan extension by starvation/dietary restriction.

Metabolism is closely linked with ageing. Same of the main consequences of ageing, among others, are genomic instability, telomere shortening, epigenetic changes, decreased nutrient sensing and altered metabolism [74]. Given its metabolic properties, αKG may be useful in reducing this type of disorders. Moreover, altered metabolism is also a feature of cancer cells [75]. In the last years, several evidences have shown that the mitochondrial genes encoding for Krebs cycle enzymes may function as oncogenes or tumor suppressors by influencing different cellular processes.

As already described, gene encoding SDH and FH might also act as tumor suppressor, while IDH1/2 gene products could act as oncogenes [43, 44, 49, 76, 41]. Thus, mutations in these enzyme support cancer progression by inhibiting the activity of αKG-dependent dioxygenase enzymes, such as PHDs, TET, and KDM, and in this way they participate in the pathogenesis of many cancers. In this

scenario, exogenous administration of αKG could be used for the reactivation of αKG-dependent dioxygenase family proteins and to reverse metabolic and epigenetic alterations in cancer cells.

Anti-cancer property of exogenous αKG.

It is demonstrated that exogenous administration of αKG alone or in combination with chemotherapeutic agent exhibits anti-tumor activity by inhibiting the HIF-1α subunit expression. This is related to the inhibition of tube formation in *in vitro* angiogenesis models and with a reduction of tumor growth and angiogenesis in tumor tissues transplanted in mice [77, 78]. In these studies, the inhibition of HIF-1α expression, promoted by increased αKG levels, induces a reduction of *Vegf* gene promoter activity and, consequently, inhibits VEGF and erythropoietin transcription [77]. Furthermore, others experimental data support the possibility to use αKG in treatment of neoplastic diseases in which Krebs cycle enzyme defects cause the chronic activation of HIF-1 even under normoxic condition (pseudohypoxia). Brière and colleagues have demonstrated that administration of exogenous αKG prevents the translocation of HIF-1 into nucleus of fibroblasts carrying mutation in SDHA gene [79]. In another study, it is shown that the competitive inhibition of PHDs by SA or fumarate may be reversed by increasing the cellular level of αKG in SDH- and FH-deficient cancer cells opposing to hypoxia mediated activation of HIF-1α [51]. Since αKG is a hydrophilic metabolite, to allow the ketoacid to efficiently penetrate and accumulate into cells MacKenzie and colleagues have synthetized cell-permeating αKG derivatives, i.e. αKG esters with increased hydrophobicity named octyl-αKG and 1-trifluoromethyl benzyl-αKG (TαKG) which were converted to the free αKG form by cytoplasmic diesterases (Figure 6). These derivatives, by restoring PHDs activity in such tumors, not only induced HIF-1α destabilization, but also enhanced glycolysis and cell death [51]. A subsequent study has shown that TαKG derivative was able to penetrate and destabilize HIF-1α by promoting PHDs activity also in human colon cancer spheroids models, although this compound only provided a transient increase of intracellular αKG levels [52]. Thus, in the same work, Tennant and colleagues ethylated the free carboxylic group of TαKG to form a diester ETαKG (1-trifluoromethylbenzyl, 5-ethyl-αKG) to make it more stable in aqueous solution, with a reduced degradation observed after 2 hours [52]. Intriguingly, the treatment of SDH-deficient xenograft tumors with diester form of αKG can reactivate PHDs and destabilize HIF-1α *in vivo* [52].

Overall, treatment with exogenous αKG may represent an explorable metabolic approach to humper tumor growth by inducing a pseudonormoxic state in hypoxic cancer cells. Indeed, the strategy to target the PHDs could potentially affect multiple aspect of hypoxic cancer cells, as glycolytic metabolism, angiogenesis, increased aggressiveness, by reversing a greater number of pro-tumorigenic hypoxic phenotypes. In this scenario, the use of αKG analogues will progressively

support the development of new adjuvant therapeutic strategies to treat human HIF-dependent aggressive cancers.

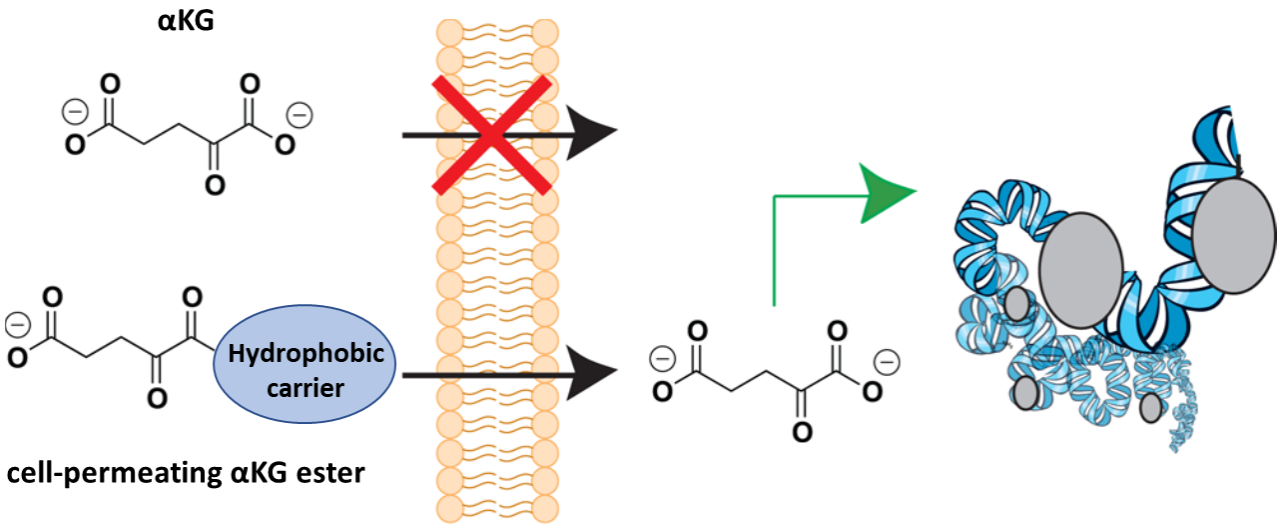


Figure 6: The αKG can be conjugate to different hydrophobic carriers to increase its permeability across the plasma membrane (adapted image) [80]**.** Cell-permeating αKG esters allow the ketoacid to cross the plasma membrane. Once in the cytosol, cellular diesterasis hydrolyze the ester bound releasing αKG that accumulate in the cell.

The inhibition of respiratory CI activity as indirect approach to increase endogenous αKG levels.

The abundance of αKG in the cell is also dependent to αKG dehydrogenase activity. Indeed, αKG can be decarboxylated to form succinyl-Coenzyme A (succinyl-CoA) and CO_2 reducing the pyridine nucleotide NAD^+ to NADH. Besides αKG, the enzymatic reaction requires also CoA as substrate and thiamine pyrophosphate as a cofactor. The αKG dehydrogenase represents a key control point of TCA cycle since it has a downstream regulatory effect on OXPHOS activity and ATP production by controlling the amount of available reducing equivalents (such as NAD^+/NADH) generated by the Krebs cycle [81]. As one of the products of αKG decarboxylation reaction, accumulation of NADH may inhibit αKG dehydrogenase activity leading to growing intracellular αKG levels [82]. In this scenario, the αKG dehydrogenase function is strictly interconnected with respiratory CI activity. The latter is the first and larger enzyme of mitochondrial respiratory chain and catalyzes NADH oxidation to generate the electrochemical gradient by feeding the electron transport chain to generate ATP, making NADH an essential substrate for oxidative metabolism [83, 84, 85]. The impairment of CI function is related to the unbalance of NAD^+/NADH ratio, which in turn contributes to inhibit αKG dehydrogenase activity increasing αKG endogenous levels [37, 38, 40, 81]. In this light, inhibited CI activity may indirectly promote the induction of pseudonormoxia in HIF-dependent cancer cells.

The impairment of respiratory CI activity modulates different facets of cancer progression.

Respiratory CI, also known as NADH:ubiquinone oxidoreductase, is the first component of electron transport chain and can be imagined as a hard-working machine that promotes the oxidation of NADH, produced through the TCA cycle, and simultaneously the proton translocation across the inner membrane, contributing to the generation of the mitochondrial membrane potential needed for ATP production [85] (Figure 7). CI has a characteristic L-shaped structure that consists of one hydrophobic harm embedded into the inner membrane and a hydrophilic arm facing mitochondrial matrix [86]. It is possible to distinguish three functional and structural modules: the N-module responsible for the NADH binding and oxidation, the Q-module which allows the electron transfer to ubiquinone and the P-module involved into proton pumping in the mitochondrial intermembrane space [87]. CI is composed by 44 subunits, seven of which are codified by the mitochondrial DNA (mtDNA), while others 37 subunits are encoded by the nuclear DNA (nDNA) and subsequently imported into mitochondria. All subunits must be assembled through a coordinated and finely regulated process in order to form the functional enzyme. Several chaperon proteins take part to this process and are necessary for the correct assembly [88]. Considering the crucial role of respiratory CI

and the complexity of its structure and function, it is not surprising that alterations in its activity are involved is several pathological processes, including cancer.

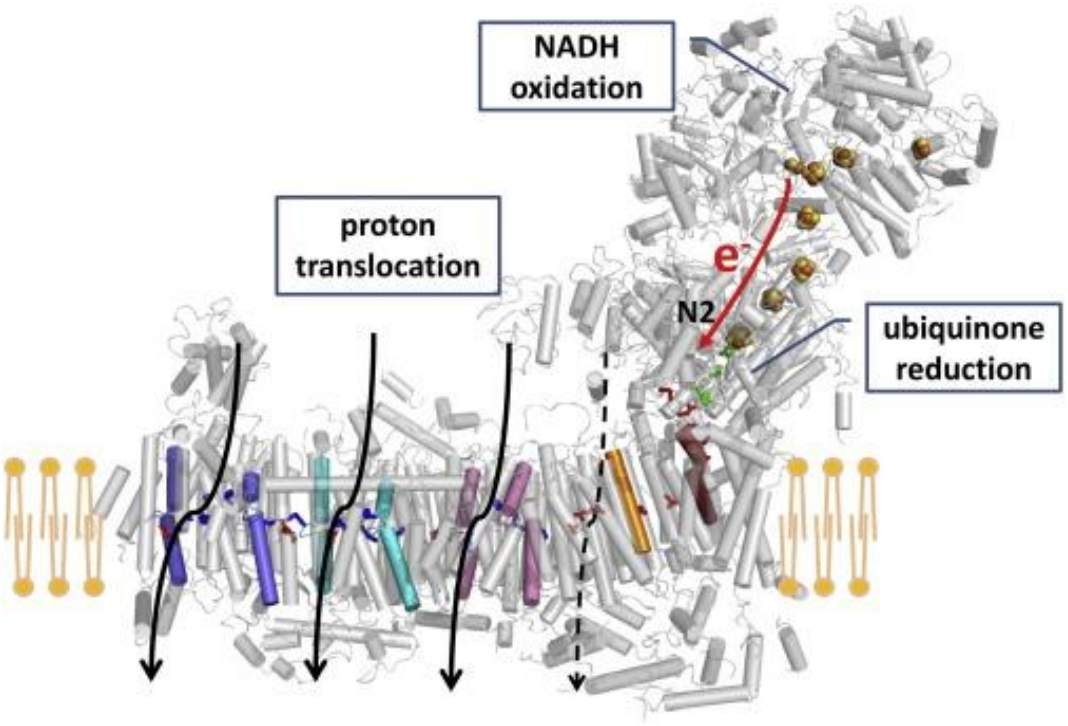


Figure 7: CI structure and enzymatic activity [89]**.** This enzyme promotes the transport of electrons from NADH to Coenzyme Q (ubiquinone) and proton transport through the inner mitochondrial membrane. This process allows 4 protons to be pumped from the mitochondrial matrix into the intermembrane space. CI shows a peculiar L-shaped structure which consists of one hydrophobic harm embedded into the inner membrane and a hydrophilic arm facing mitochondrial matrix. It is composed of three functional and structural modules: the N-module, Q-module and P-module responsible for the NADH binding and oxidation, the ubiquinone reduction and the proton translocation in the mitochondrial intermembrane space, respectively.

Several studies have highlighted the necessity of mitochondrial respiratory function to promote tumorigenesis, metastasis migration and escape from dormancy of cancer cells [90, 91, 92]. For many years, a growing number of works reported altered expression or mutations in both nDNA and mtDNA-encoded CI subunits in different types of tumors and their role in the modulation of tumor progression has been widely debated. Now it is known that impairment of CI activity may plays double-edged role in cancer since the severity of dysfunction strongly influences tumor progression, metastasis formation and therapy resistance [93]. Mutations that completely abolish CI activity or assembly prevent tumor progression, while mild CI dysfunction may increase intracellular ROS levels stimulating cancer cell proliferation and invasiveness [93].

The double edge role of CI subunits mutations in cancer.

Mutations and altered expression of both mitochondrial and nuclear encoded CI subunits may concur to impair the activity of this enzyme in cancer cells. However, in different tumors has been reported the modified expression of several nDNA-encoded CI subunits, rather than the occurrence of specific mutation since such genetic alterations could be potential transmitted to every daughter cell leading to a profound and generalized metabolic alteration [94, 93]. Recent works reported the correlation between poor prognosis in oncological patients with reduced levels of several nDNA-encoded CI subunits [95, 96, 20, 97, 98]. In this context, the evaluation of these gene expression levels could represent a prognostic marker for tumor outcome at the moment of diagnosis. Conversely, others have shown that a reduction of nuclear encoded CI subunit might promote different aspect of tumor progression by increasing mitochondrial ROS levels and leading to the activation of the Akt/mTOR pathway, which in turn promotes the epithelial-mesenchymal transition (EMT) [99, 100] or enhancing the metastatic potential of cancer cells [101]. Although a growing number of works reported the existence of a correlation between altered expression of nDNA-encoded CI subunits and their influence on different aspect of cancer biology, only few of these studies investigate in deep the molecular mechanism behind this relation.

Conversely, the contribution of mutations in mtDNA genes encoding for CI subunits in different aspect of tumor biology, such as tumor progression, metastasis formation, metabolic adaptation and therapy resistance, is better characterized since they have been frequently found in several tumors [94, 93]. The occurrence of somatic mtDNA mutations in cancer is mainly due to the peculiarities of this genome. Human mtDNA is a double-stranded circular molecule mostly coding and somatic mutations may occur and accumulate since the mitochondrial DNA polymerase (POLG) is error-prone [102, 103, 104]. Furthermore, mammalian cells typically contain from hundreds to thousands copies of mtDNA that can replicate independently of nDNA [105]. During cell division, mitochondria are randomly distributed to daughter cells indicating that the mutation load can range with time in somatic cells [106]. Given the mtDNA polyploid nature, a condition of homoplasmy is established when all copies of this DNA (wild-type or mutant) are all identical to each other, conversely the coexistence of different mitochondrial genome in the same cells is a condition known as heteroplasmy (Figure 8). Whether a mtDNA mutation occurs, about 70-90% of mtDNA must be affected, depends of the mutation type and the energy requirements of the affected cell, in order to observe the phenotype (threshold effect) [105, 102, 103, 107] (Figure 8). Thus, the phenotypic manifestation of mtDNA mutation appears dynamic and variable due to mitotic segregation and threshold effect.

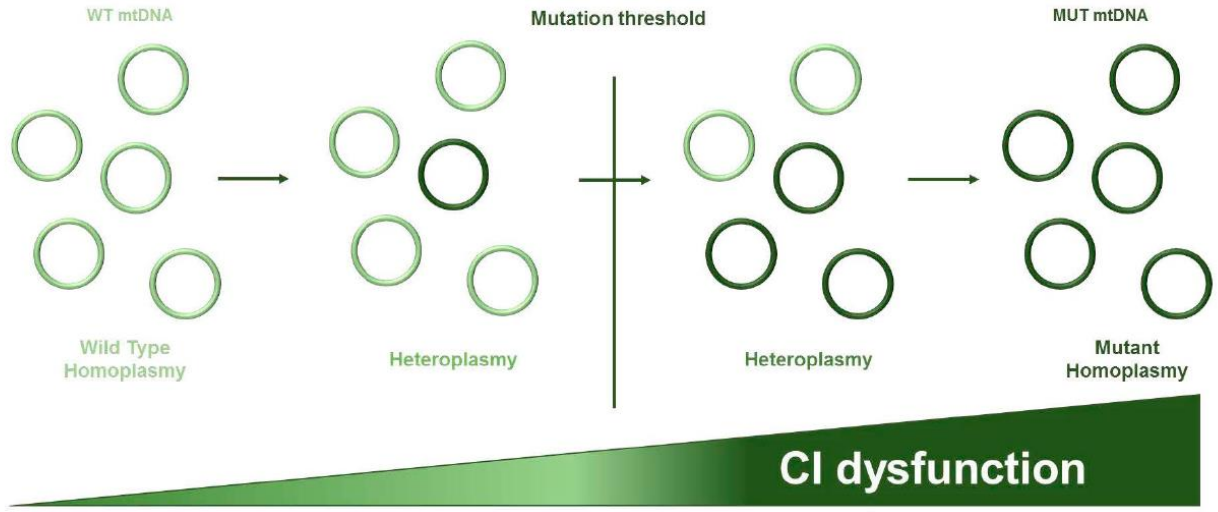


Figure 8: Threshold effect [93]**.** The condition of homoplasmy occurs when all mitochondrial DNA (mtDNA) molecules are identical (wild type (WT) or mutant (MUT)), while heteroplasmy implies the coexistence of different mitogenomes. Hence, to exert their functional effect mtDNA mutations must be accumulated and surpass a specific mutation threshold that depends on the mutation type and the tissue or cell affected.

It has been demonstrated that cancer cells are often characterized by the occurrence of heteroplasmic mutations, that may depend to the pressure of the tumor microenvironment, since the impairment of mitochondrial function may confer a selective advantage to cancer cells [108, 109]. Furthermore, mostly neutral missense mutations are gradually shifted towards homoplasmy over time in tumors [104]. Conversely, severe and disruptive homoplasmic mutations are negatively selected by cancer cells underlining the importance of mitochondria in cancer cells [104].

The effect of CI-encoded mtDNA mutations subunits on tumor progression strictly depends on the severity of the biochemical defects induced and on the mutant load (Figure 9) [40, 93]. In this context, missense mutations or heteroplasmic disruptive mutations causing mild CI deficiency have been related with an increase in mitochondrial ROS production by conferring a growth advantage and stimulating aerobic glycolysis through the accumulation of HIF-1α in cancer cells [110, 111]. Similarly, nonsense and missense *MT-ND6* mutations and heteroplasmic frameshift m.12417insA/*MT-ND5* mutation, correlated with lower activity of CI, are able to enhance aggressiveness of cancer cells by inducing over-production of ROS and promoting the activation of Akt pathway [112, 108, 113]. However, when the mutant load is close to homoplasmy, the same mutations induce a severe CI dysfunction that hampers tumorigenic potential promoting apoptosis of tumor cells underlining that the impact on tumor progression mainly depends on the threshold effect and on its bioenergetic consequences [108, 113]. In line, our group has demonstrated that homoplasmic mutation m.3571insC/*MT-ND1* impairs CI structure and activity leading to profound metabolic changes, including imbalance of

αKG/SA levels and chronic destabilization of HIF-1α hampering tumor growth *in vitro* and *in vivo* [37, 38, 40]. Interestingly, the same mutations when present in heteroplasmic state did not affect tumor progression [37, 38, 39, 40]. This study represents a classical example of *oncojanus* nature of CI-encoded gene mutations that may display a different impact on tumorigenesis as consequence of their severity and their effect on the metabolic status of cancer cells.

Further, some mtDNA mutations in CI genes are involved in metastasis formation and chemotherapy resistance, as they may promote oxidative stress, as consequence of reduced CI activity, enhancing cancer cells invasiveness and metastatic potential [114, 115, 116]. However, these data are in contrast with others works that highlight the importance of functional or enhanced OXPHOS activity to support metastasis formation [90, 117, 118]. In this light, the contribution of mtDNA mutations to foster the metastatic potential need to be better investigate.

Conversely, growing evidence underline the importance of mitochondrial contribution in tumor cells response to chemotherapy treatments. In this regard, we have recently demonstrated the involvement of two mtDNA mutations, one of which affect respiratory CI, in cisplatin resistance of gynecological origin cancer cells. The occurrence of these genetic alterations reduces proliferative and tumorigenic potential, in term of migratory and invasive capacity. At the same time, mitochondrial deficiency induces also a damage to cytoskeleton, in particular to filamentous tubulin, which is the main target of paclitaxel. It is also demonstrated that such mutations are positively selected and became homoplasmic leading cancer cells to acquire a resistant phenotype to chemotherapy treatment [119]. These data are supported by a previously work, in which it is shown the insurgence of paclitaxel resistant mass in a patient with ovarian cancer harboring homoplasmic disruptive m.10875T>C/*MT-ND4* mutation after anti-cancer treatment. Beside the resistance to chemotherapy, the occurrence of mtDNA mutation resulted in a more quiescent, non-invasive, and low-proliferative neoplasm [120]. This finding emphasizes once again the *oncojanus* nature of mtDNA mutations in CI genes.

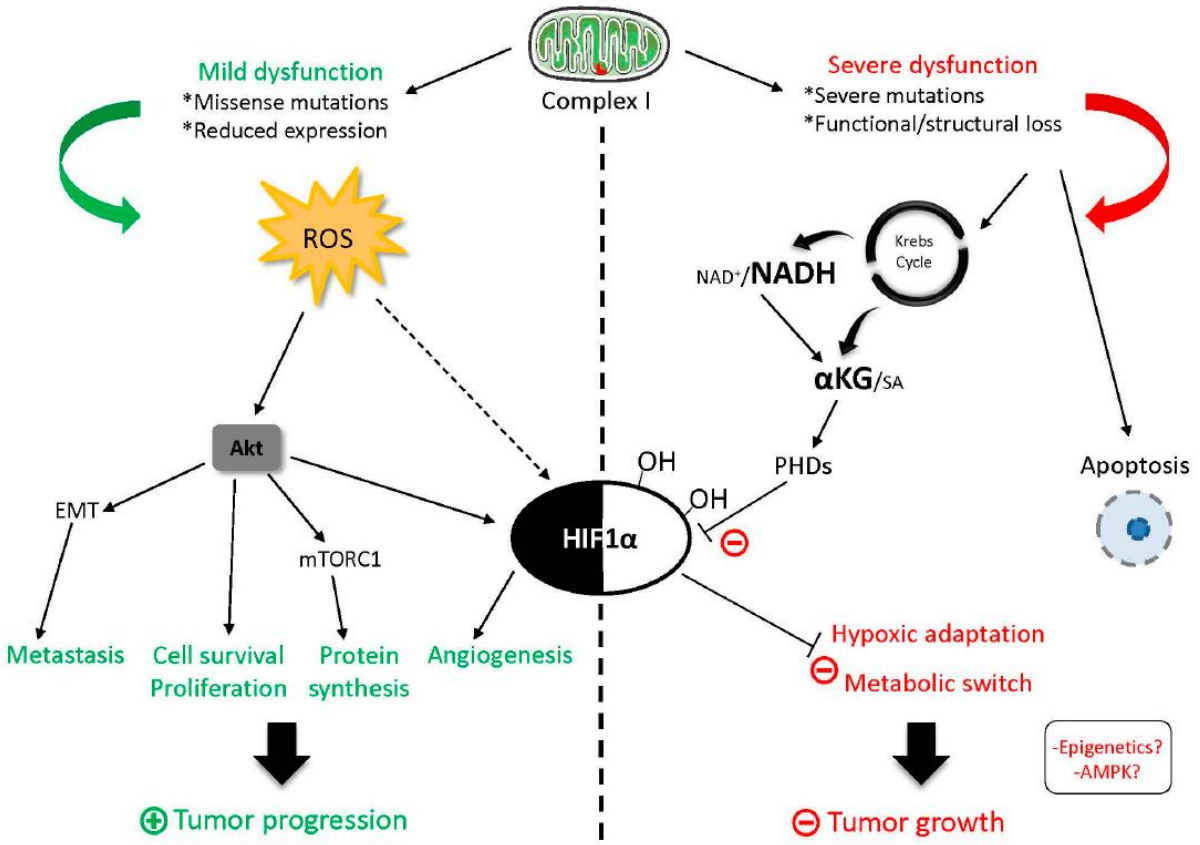


Figure 9: The double edge effect of mutations in CI encoding genes on tumorigenesis [93]**.** Mild alterations of CI activity induce an increase of mitochondrial ROS production leading to the consequent activation of Akt/mTORC1 pathway which in turn sustain cancer cells proliferation and survival, EMT and HIF-1α stabilization. On the other hand, severe CI dysfunction are related to the intracellular accumulation of NADH and αKG, which destabilizes HIF-1α even under hypoxic condition preventing the hypoxic adaptation of cancer cells and promoting the arrest of tumor growth.

The potential role of CI inhibitors as novel adjuvant anti-cancer strategy.

In line with what described above, it is plausible to consider the use of specific inhibitors that severely affect CI function as adjuvant treatment for oncological patients. At the same time, we have to take in consideration the crucial role played by respiratory CI also in the energy production, metabolic pathway and oxidative stress homeostasis in non-cancer cells [121, 122, 123]. In this regard, the difficulty is the development of specific CI inhibitors suitable for the employment as anti-cancer agents, without inducing a metabolic catastrophe in non-cancer cells.

Rotenone, piericidin A and capsaicin are considered as classical CI inhibitors and their administration decreases the tumorigenic potential of several cancer cell lines under metabolic stress condition [124, 125, 126] (Table 1). Nevertheless, rotenone and piericidin A favor an increase in ROS production blocking electron transfer from Fe-S clusters to the ubiquinone and therefore their use is excluded to therapeutic application [127].

Recently, several works have highlighted the emergent role of anti-diabetic biguanides metformin and phenformin as potential anti-cancer CI inhibitors. Phenformin is not clinically used since its higher risk of lactic acidosis incidence, a possible consequence of mitochondrial CI inhibition [128]. Conversely, metformin is the first line therapy for diabetes mellitus II and epidemiological studies revealed that patients treated with this drug have reduced risk to develop cancer and present with a better prognosis [129, 130, 131]. Growing evidences suggested that metformin reduce cancer cell proliferation through the specific inhibition of respiratory CI activity [132, 133, 134], although the molecular mechanism has not been completely elucidated. However, it is demonstrated that metformin treatment induces an increase of glucose consumption and lactate production and, at the same time, decreases mitochondrial glucose oxidation in cancer cells [135, 136]. Furthermore, its anti-proliferative effect results enhanced under glucose starvation [135, 133], which is a feature commonly shared by most of specific CI inhibitors. In line, novel small molecules (BAY 87-2243 and AG311) that humper tumor growth by inhibiting CI activity shown the same ability of metformin to reduce cancer cell viability mainly under limited glucose concentration indicating that cancer cells rely exclusively on glycolysis to survive after CI inhibition [137, 138, 139]. A common feature of this new class of CI inhibitors is to induce a profound energetic crisis in cancer cells which, in turn, induces the phosphorylation and the activation of energetic stress sensor adenosine monophosphate (AMP)-activated kinase (AMPK). AMPK activation, firstly triggered by CI inhibition, concurs to the anti-tumorigenic effect through the inhibition of mTORC1 signaling and subsequently promoting the cell cycle arrest [140, 138, 139]. However, this mechanism cannot exclusively explain the anti-proliferative effect of CI inhibitors since different types of tumors, unable to activate AMPK, are still susceptible to these drugs [140]. Further, CI defective cells activate AMPK signaling probably to allow mitochondrial biogenesis and to trigger metabolic adaptation, but this kinase does not concur to the arrest of cancer cell proliferation [40]. Conversely, lack of CI activity caused by disruptive mutations or exposure to drugs (metformin, BAY 87-2243 and AG311) destabilizes HIF-1α preventing hypoxic adaptation of cancer cells [133, 137, 139]. The inactivity of respiratory CI may cause a reduction of intracellular oxygen consumption leading to a condition of intracellular normoxia even when the extracellular oxygen tension is low [141]. Moreover, CI inhibition may promote the activation of PHDs, even in hypoxia, as consequence of increasing αKG intracellular levels leading to a chronic hydroxylation and degradation of HIF-1α (*pseudonormoxia*) [38, 39, 40]. In line, Griss and co-workers have demonstrated that metformin treatment greatly reduces the anaplerotic flux of both glucose and glutamine into the TCA cycle, leading to a significant reduction of all metabolites of the TCA cycle, except αKG [142]. The increased αKG/SA ratio may thus prevent HIF-1α stabilization and consequent activity. Furthermore, unlike the majority of CI inhibitors, metformin

administration is not correlated with an increase of mitochondrial ROS levels [143, 133, 144], which are known to stimulate HIF-1α transcription in an Akt-mediated fashion independently from oxygen levels [34].

During the past years, many efforts have been made to identify small molecules that inhibit HIF-1α signaling through different mechanisms, but these inhibitors are often cytotoxic or not specific. The link between HIF1α stabilization and mitochondrial function opens to new metabolic-based therapeutic approaches for those cancers for which adaptation to hypoxia is a step that must be overcome to progress toward malignancy. In this frame, the inhibition of CI activity may be used as a strategy to induce a profound energetic and metabolic crisis in cancer cells. Indeed, trough the unbalance of NAD^+/NADH ratio, which in turn affects TCA fluxes and metabolites levels including αKG, the lack of CI activity is associated to a constitutive destabilization of HIF-1α [38, 39, 40]. In this way, cancer cells are unable to enhance the glycolytic machinery during hypoxia and display a lower tumorigenic potential *in vitro* and *in vivo* [39].

Inhibitor	Mechanism of action	Cellular model	References
Rotenone	Induces cell death under starvation *in vitro* Increases ROS production *in vitro*	Breast cancer cells, bovine heart mitochondria	125, 127
Piericid A	Induces cell death under starvation *in vitro* Increases ROS production *in vitro*	Breast, pancreatic and lung cancer cells, bovine heart mitochondria	125
Capsaicin	Induces cell death under starvation *in vitro*	Breast cancer cells	125
Metformin	Induces cell death under starvation *in vitro* Promotes AMPK phosphorylation Induces HIF-1α destabilization under hypoxic condition	Colon,lung, breast, cervical, osteosarcoma, oral carcinoma cancer cells	133, 134, 140, 145
Phenformin	Induces cell death under starvation *in vitro* Promotes AMPK phosphorylation Induces HIF-1α destabilization under hypoxic condition	Colon and breast cancer cells and xenografts	133, 146, 147, 148
BAY 87-2243	Induces cell death under starvation *in vitro* Decreases tumor growth *in vivo* Promotes AMPK phosphorylation Induces HIF-1α destabilization under hypoxic condition Increases intracellular ROS levels	Lung cancer and melanoma cells and xenografts	137, 138
AG311	Induces cell death under starvation *in vitro* Decreases tumor growth *in vivo* Promotes AMPK phosphorylation Induces HIF-1α destabilization under hypoxic condition Induces mitochondrial superoxide production	Breast cancer cells and xenografts	139, 149
Fenofibrate	Induces cell death *in vitro*	Glioblastoma cells	150
JCI-20679	Induces cell death *in vitro*	A panel of 39 human cancer cell lines	151
Celastrol	Promotes ROS production and apoptosis *in vitro*	Lung cancer and hepatocellular carcinoma cells	152
Kalkitoxin	Induces cell death *in vitro* Induces HIF-1α destabilization under hypoxic condition	Neuroblastoma, breast and colon cancer cells	153
Lehualide B	Induces cell death *in vitro*	Multiple myeloma cells	154

Table 1: Respiratory CI inhibitors with anti-cancer proprieties [93].

AIM

Solid tumors are often characterized by hypoxic areas hardly attainable and susceptible to cancer therapies. For this reason, these types of cancers result to be very aggressive and associated with poor prognosis. Under hypoxic condition, several adaptive changes and metabolic reprogramming are promoted and coordinated at transcriptional level by HIF-1. This transcriptional factor is considered the master regulator of hypoxic cellular response and plays key roles in development, physiology and disease, including cancer. In this context, the idea to develop a pharmacological approach able to reduce or inhibit HIF-1 activity in hypoxic cancer regions could represent a strategy to prevent an adverse outcome in tumor patients. Despite efforts in the past years, no agents directly inhibiting HIF-1 have been approved for treating cancer patients due to safety or limited therapeutic efficacy. A few inhibitors interfere directly with its mRNA or protein, the dimerization of α and β subunits, or the interaction of HIF with its co-activators, but most of them are indirect inhibitors or have other activities.

HIF-1 is a heterodimeric protein constituted by two different subunits. HIF-1α turn-over and stability is strictly regulated by intracellular O_2 levels and PHDs activity, while HIF-1β is constitutively expressed. Besides intracellular O_2 levels, also the abundance of TCA metabolites αKG and SA results to be crucial to allosterically regulate PHDs activity. Being one of the products of HIF-1α hydroxylation reaction, an increase of SA levels chronically stabilizes HIF-1α by inhibiting PHDs. On the contrary, increasing αKG intracellular levels enhance the PHDs affinity for O_2 and feed their hydroxylation reaction preventing HIF-1α stabilization, even in hypoxia.

Our research group has previously demonstrated that cancer cells characterized by severe mtDNA mutations that impair CI assembly and activity displayed an increase of αKG/SA ratio, likely due to the inhibition of αKG dehydrogenase resulting from an increase of NADH intracellular levels. High concentrations of αKG promote the activation of PHDs and consequent chronic HIF-1α destabilization, even when O_2 is available, hampering tumor growth *in vitro* and *in vivo*. This condition was termed *pseudonormoxia*.

Based on these findings, we hypothesized that *pseudonormoxia* can be induced by unbalancing the αKG/SA ratio that mimicking a normoxic condition prevents the hypoxia adaptation. Thus, the main goal of this project was to identify novel compounds able to promote PHDs activity triggering HIF-1α destabilization and preventing tumor growth and progression. We pursued this objective with two different pharmacological approaches. On one hand, we used mitochondrial CI inhibitors to indirectly promote the increase of αKG intracellular levels. We attempted to identify new molecules able to act as specific CI inhibitors without exerting cytotoxic effect on non-cancer cells

in order to be considered safe and suitable as therapeutic agents. In parallel, we developed a direct approach to induce a *pseudonormoxic* state by increasing αKG intracellular levels using membrane-permeating ester derivatives of this TCA metabolite.

In this thesis, we exploited the destabilization of HIF-1α as a potential target for a new possible adjuvant cancer therapy. This metabolic-based approach can be envisioned as a strategy for all those types of cancer in which hypoxic adaptation and metabolic reprogramming are necessary for tumor progression toward malignancy.

EXPERIMENTAL PROCEDURES

Cell cultures

TPC-1 (human papillary thyroid cancer cells) and Nthy-ori 3.1 (NTHY 3.1) (non-tumoral thyroid follicular epithelial cells) were cultured in RPMI 1640 Medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. HK-2 (non-tumoral renal proximal tubule epithelial cells) and UOK-257 (human renal carcinoma cells) were cultured in Dulbecco's modified Eagle medium (DMEM) High Glucose, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Osteosarcoma 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cell models were generated using NDUFS3-targeted zinc finger nuclease technology. A clonal selection and genotyping, after the transfection, were performed to identify cells with frameshift NDFUS3 mutations. Heterozygous clones were first expanded and then a second transfection with NDUFS3 zinc finger pool was performed in order to select homozygous frameshift NDUFS3 mutants and homozygous wild-type (WT) revertants used as control in all experiments performed in this work. Cells obtained with this method were cultured in Dulbecco's modified Eagle medium (DMEM) High Glucose, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL uridine.

All these cell lines were maintained in an incubator with a humidified atmosphere at 5% CO_2 and 37°C. Experiments in hypoxia were performed using the Invivo2 300 (Baker Ruskinn) incubator, set up at 5% CO_2, 37°C and 1% O_2. Where indicated, cells were incubated with 150 µM of desferoxamine (DFO) for 16 hours under normoxic condition and with dimethyl-2-oxoglutarate 2mM under hypoxic condition.

Sulforhodamine B (SRB) viability assay

Cells were seeded in 96-well plates ($1x10^3$ cells/well for TPC-1, $2x10^3$ cell/well for NTHY 3.1 and UOK-257 cells, $3x10^3$ cell/well for HK-2, $2x10^3$ cell/well for 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cells). For additional treatments, cells were incubated 24 hours after seeding with different medium for the times and using concentrations indicated in legends of figures.

At the end of incubations time, cells were fixed to the well with 50% trichloroacetic acid dissolved in the grow medium for 1 hour at 4° C, washed 5 times with water and dried at room temperature. Cells still attached to the well were incubated for 30 minutes with SRB 0.4% diluted in 1% acetic acid at room temperature. SRB is an anionic dye, amino-xanthene, which forms electrostatic complexes with the basic protein residues, in weak acid conditions. Subsequently, wells were

washed 4 times with 1% acetic acid to remove excess dye. The dye bound to the cells was finally solubilized with 10mM Tris (pH 10.5) and the relative absorbance of SRB was detected using plate reader VICTOR^3 (1420 Multilabel Counter-PerkinElmer, Turku, Finland) at the wavelength of 560 nm.

Colony formation assay

The ability of single cancer cell to grow forming a colony was evaluating by seeding 250 cells/well (TPC-1 and UOK-257 cells) or 500 cells/well (143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cells) in a 6-well plate in their growth medium. After seeding, cells were incubated at 37° C in a humidified 5% CO_2 atmosphere. To assess the anti-tumorigenic potential of compounds to test, treatments were effectuated 24 hours after seeding. Colonies were fixed after 7 or 10 days, for TPC-1 and UOK-257, 143B respectively, using 50% trichloroacetic acid and incubating cells at 4°C for 1 hour. Then colonies were washed 5 times with water, dried, stained with SRB 0.4% diluted in 1% acetic acid for 30 minutes at room temperature and destained washing 4 times cells with 1% acetic acid. Image were acquired using Gel Logic 1500 Imaging System (Kodak, Ronchester, NY, USA) and the number of colonies quantified using ImageJ software. The dye bound to the cells was solubilized with 10mM Tris (pH 10.5) and the relative absorbance of SRB was detected using plate reader VICTOR^3 (1420 Multilabel Counter-PerkinElmer, Turku, Finland) at the wavelength of 560 nm.

Mitochondria isolation

Enriched mitochondrial fractions were obtained from cells seeded into 10 cm dish and after 24 hours incubated with KH-compounds for 72 hours using the concentrations shown below. At the end of incubation time, cells were trypsinized and mitoplast were obtained resuspending cellular pellet ($10x10^6$ cells) with cold PBS (154 mM NaCl, 1 mM KH_2PO_4, 3 mM Na_2HPO_4) and adding digitonin (final concentration 50 µg/ml) for 1 minute. The reaction was blocked by diluting 2,5-fold the suspension volume using cold PBS. The digitonin treatment may be repeated until at least 90% of cells were permeabilized, as detected at the microscope using Eritrosine B. After that, cells were centrifuged at 13000 rpm for 15 minutes at 4°C and the pellet was stored at -80°C. Protein content was quantified according to Bradford [155].

Clear Native Page

Mitoplasts were suspended in mitochondrial buffer (750 mM 6-aminocaproic acid; 50 mM BisTris, pH 7.0) and solubilized by adding 2% n-Dodecyl β-D-maltoside (DDM), kept in ice for 10 minutes and centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatant was recovered, and mitochondrial proteins quantified using Bradford [155].

CN-Sample Buffer (0.1% Red Ponceaux and 50% Glycerol) was added to 50 µg of mitochondrial protein sample and loaded onto a native gel constituted by a gradient of polyacrylamide from 4% to 12%, made with a gradient building machinery and a peristaltic pump (Delta-Pump). The electrophoresis was performed at 80V using Anode Buffer (25 mM Imidazole, pH 7) and cathode buffer (50 mM Tricine, 7.5 mM Imidazole, 0.02% DDM, 0.05% Sodium Deoxycolate pH 7).

CI-In Gel Activity

After electrophoresis, gel was incubated in a solution containing 2 mM Tris-HCl, 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 0.02% NADH for 15 minutes at room temperature, in the dark. The detection of purple bands on the gel revealed the enzymatic function of CI in a single complex or in supercomplexes and the colorimetric reaction was due to reduction of MTT to tetrazolium salts by NADH oxidation through CI. Image were acquired using Gel Logic 1500 Imaging System (Kodak, Ronchester, NY, USA).

Total lysates

Cells were seeded in 6-well plate and after 24 hours were incubated with 200µM of αKGlogues for different time points. At the endo of incubation time, cells were trypsinized and the pellet was washed once with PBS and centrifugated at 13000 rpm for 1 minute at 4°C. Then, the pellet was resuspended in RIPA buffer (50mM TrisHCl pH7.4, 150mM NaCl, 1% SDS, 1% Triton, 1mM EDTA pH7.6) supplemented with protein and phosphatase inhibitors and incubated for 15 minutes in ice. The lysate was frozen and thawed twice and centrifugate at 13000 rpm for 15 minutes at 4°C. The supernatant was recovered, and the protein content quantified using Bradford's method [155].

SDS-PAGE and Western Blot

Total lysates, obtained as described above, were added with Laemmli sample buffer 5X (62,8mM Tris pH 6.8, 12,5% glycerol, 2% SDS, 0,7M 2- mercaptoethanol, 0,1% bromophenol blue) and boiled for 5 minutes to induce complete protein denaturation. Denaturing SDS-PAGE Electrophoresis was carried out at 100V at room temperature in Sodium Dodecyl Sulphate (SDS) running buffer (250mM Tris, 2M glycine, 40mM SDS). At the end of the run, proteins were transferred onto a nitrocellulose membrane 0.45mm in full-wet system in Western Transfer Buffer (25mM Tris pH 8,3, 93mM glycine, 20% methanol) at 100V for 1 hour at room temperature using Bio-Rad Mini Trans-Blot Cell system. After, the membrane was incubated in powdered milk solved in TBS 1X-Tween 0.05% (25mM Tris-HCl pH 7.5, 137mM NaCl, 0,05% Tween) for 1 hour at room temperature and incubated with primary antibodies using following dilutions/conditions: anti-HIF1a (GeneTex GTX127309) 1:2000, 2 hours at room temperature; anti-β-Actin (Sigma-Aldrich cat# SAB5500001) 1:5000, 2 hours at room temperature; anti-NDUFS3 (Abcam cat# ab110246)

1:1000 2 hours at room temperature; anti-NDUFA9 (InVitrogen cat# ab14713) 1:1000 2 hours at room temperature; anti-NDUFB8 (Abcam cat# ab110242) 1:1000 2 hours at room temperature; anti-SDHA (InVitrogen cat# ab459200) 1:10000, 2 hours at room temperature; anti-VDAC (Abcam cat# ab154856) 1:1000, 2 hours at room temperature.

Membranes were washed 3 times with TBS 1X-Tween 0.05% and incubated with the proper secondary antibody (Jackson ImmunoResearch Laboratories Anti-Mouse cat# 115035003 and Anti-Rabbit cat# 211032171) diluted 1:5000/1 hour at room temperature. After further 3 wash in TBS 1X-Tween 0.05% and chemiluminescence was obtained by ECL. Images were acquired using Gel Logic 1500 Imaging System (Kodak, Ronchester, NY, USA). The densitometric analysis was performed using ImageJ software.

KH-compounds

KH-compounds were produced by Khondrion, a Dutch pharmaceutical company located in Nijmegen, The Netherlands. Cells were treated with these molecules for different time points using concentrations indicated by Khondrion and reported in the table below (Table 2).

Compound	Structure	Concentration
KH407	undisclosed	300µM
KH412	undisclosed	300µM
KH426	undisclosed	300µM
KH474	undisclosed	300µM
KH0B	undisclosed	10µM
KH0C	undisclosed	10µM

Table 2: In the panel were reported the name of KH- compounds and relative concentration used for all experiments performed in this work. The structure is still undisclosed.

αKGlogues

To increase αKG permeability across the plasma membrane, several carriers, characterized by different hydrophobic indices, were conjugated to αKG through an ester bound. In viability and colony formation assays, cells were treated for different time points with 2µM, 20µM and 200µM of αKGlogues. Whereas, cells were treated with 200µM to obtain total lysates to perform western blot analysis.

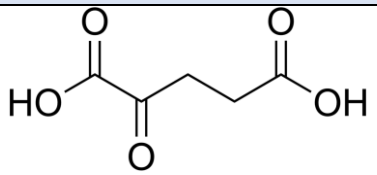
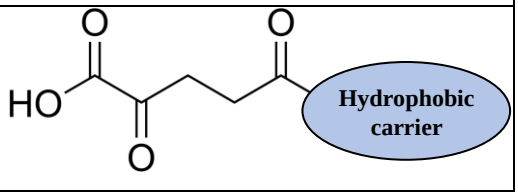
Compound	Structure
αKG	
αKGlogue	

Table 3: schematic representation of αKG and αKGlogue structure.

RESULTS

CI inhibitors: an indirect approach to induce a pseudonormoxic state in cancer cells.

During these years, a growing number of papers have highlighted the crucial role of respiratory CI in cancer. Functional defects caused by mutations or altered expression levels of its subunits lead to opposite effect in the modulation of tumorigenic potential [93]. Our research group has demonstrated that CI-deficient cells are characterized by profound metabolic alterations, including the imbalance of αKG/SA ratio, which are related with the cancer cells growth arrest both *in vitro* and *in vivo*. We reported that increased αKG intracellular levels allosterically promote PHDs activity boosting the continuous hydroxylation and degradation of HIF-1α and preventing hypoxic adaptation [37, 38, 39, 40]. Therefore, the use of specific CI inhibitors may be adopted as a pharmacological strategy to induce a pseudonormoxic state in HIF-1 dependent cancer cells. In this context, several compounds have been recently identified as CI inhibitors with a potential anti-proliferative activity on cancer cells and, interestingly, most of them shared the ability to target the same pathways identified through the ablation of CI by genetic studies, including adaptation to hypoxia. These findings support our hypothesis that the inhibition of CI activity is disadvantageous for tumor progression and opens the door to new possible therapeutic approaches for solid tumors, as mitochondrial function and adaptation to hypoxia are pivotal for tumor progression.

The specific anti-cancer effect of KH-compounds in thyroid cells does not depend on respiratory CI inhibition.

A panel of six compounds (KH) produced by Khondrion company were screened in our laboratory in order to evaluate their potential inhibitor effect on respiratory CI activity. First, we assessed whether treatment with these molecules may prevent cancer cell growth *in vitro*, without exerting a cytotoxic effect on non-cancer cells. To this purpose, thyroid-derived cells NTHY 3.1 and TPC-1 were treated for 24, 48 and 72 hours with these compounds and cell proliferation was evaluated. Based on previous screening performed by Khondrion, compounds KH407, KH412, KH426 and KH474 were tested using 300μM of concentration, while KH0B and KH0C were employed at lower concentration (10μM).

The treatment with KH407 reduced NTHY 3.1 cell proliferation ~3.5-fold compared to corresponding untreated cells after 72 hours (Figure 10A, 10G). Another compound that had shown to large influence NTHY 3.1 cell viability was KH0C. In fact, a cytostatic effect was observed treating cells with this compound (Figure 10F, 10G). Conversely, a slight but significant decrease of

cell growth was observed only after 72 hours incubating NTHY 3.1 cells with KH412 and KH0B (Figure 10B, 10E). The compounds KH474 and KH426 did not altered cell viability compared to untreated cells (Figure 10C, 10D). All KH-compounds tested exerted almost the same effect shown in thyroid normal cells in cancer cells counterpart (Figure 11). KH407 and KH0C were the most effecting compounds in reducing TPC-1 cell viability promoting a cytostatic effect (Figure 11A, 11F, 11G), while KH-412 and KH0B significant decreased cell viability at 72h (Figure 11B, 11E, 11G). Furthermore, differently from NTHY 3.1 cells, KH474 induced a reduction of TPC-1 viability after 72 hours in a significant manner, while did not affect NTHY 3.1 cell proliferation (Figure 10G, 11G).

As already said, one of the main goals of this PhD project was to identify new CI inhibitors that displayed an anti-tumorigenic potential *in vitro* but, at the same time, could be considered safe for their effect on non-cancer cells. For this reason, KH407 and KH0C were excluded from subsequent studies since they had shown a more severe impact to reduce NTHY 3.1 cell proliferation compared to other molecules tested, particularly after 72 hours of treatment (Figure 10G). The compound KH426 did not reduce TPC-1 cell proliferation; therefore, independently to its effect on NTHY 3.1 cell viability, was also not considered for further studies (Figure 10D, 10G, 11D, 11G). Conversely, TPC-1 cell growth was reduced ~2.5-fold after 72 hours of treatment with KH412 and KH0B, while the same molecules were able to decrease NTHY 3.1 viability ~1.5-fold always after 72 hours (Figure 10G, 11G). Therefore, these compounds were the most promising for further investigations. Similarly, we decided to proceed our study using also KH474 compound since it reduced significantly cell proliferation only in TPC-1 (Figure 10G, 11G).

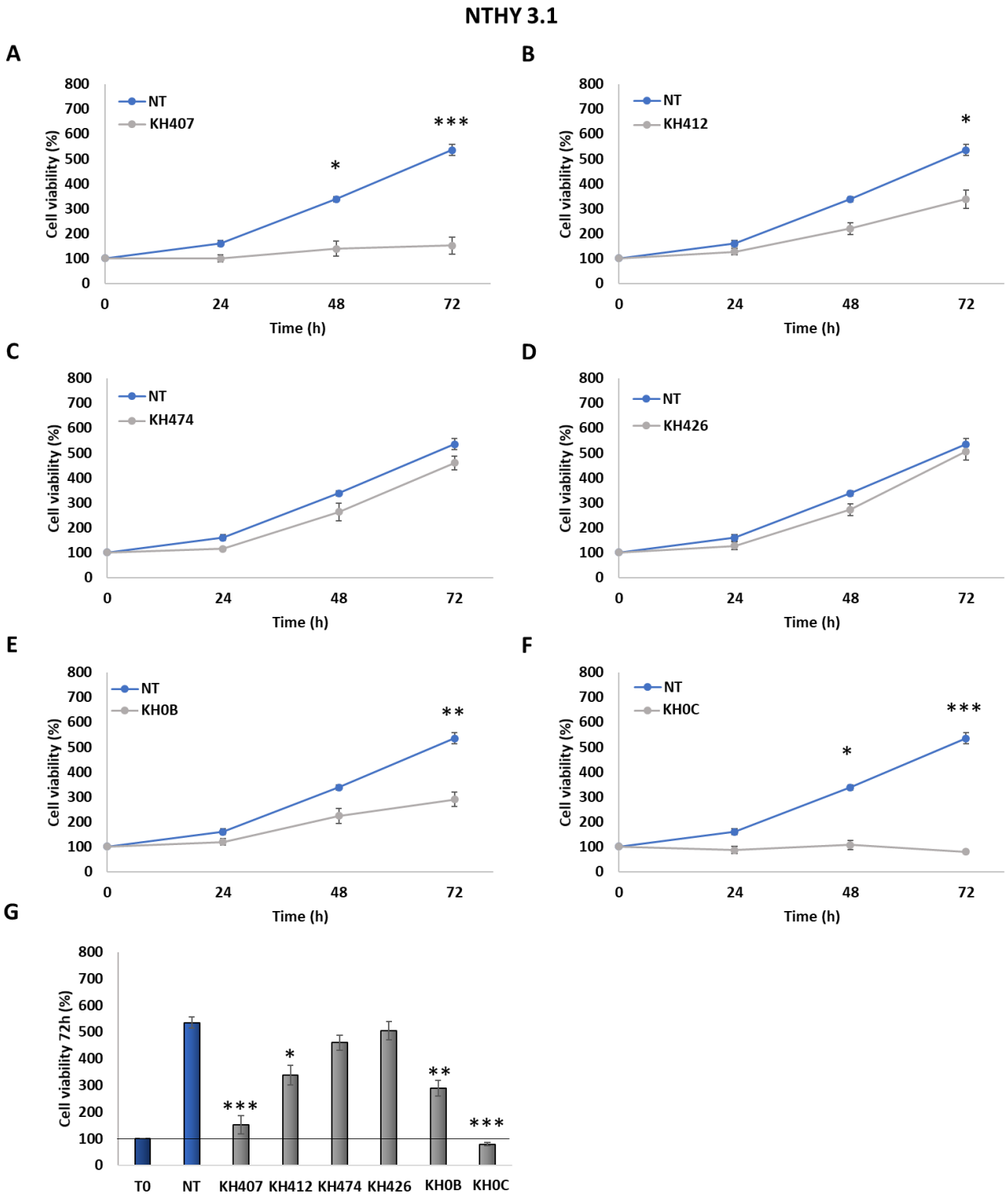


Figure 10: KH407 and KH0C induce the arrest of NTHY 3.1 cells proliferation, while other KH-compounds have a mild effect to reduce their growth. (A-F) NTHY 3.1 cells were incubated for 24, 48 and 72 hours with KH407, KH412, KH474 and KH426 (300µM) and KH0B and KH0C (10µM). Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). NT= Not-Treated cells. **(G)** Bar graph represented NTHY 3.1 cell viability after 72 hours of treatment with KH-compounds. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0. Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

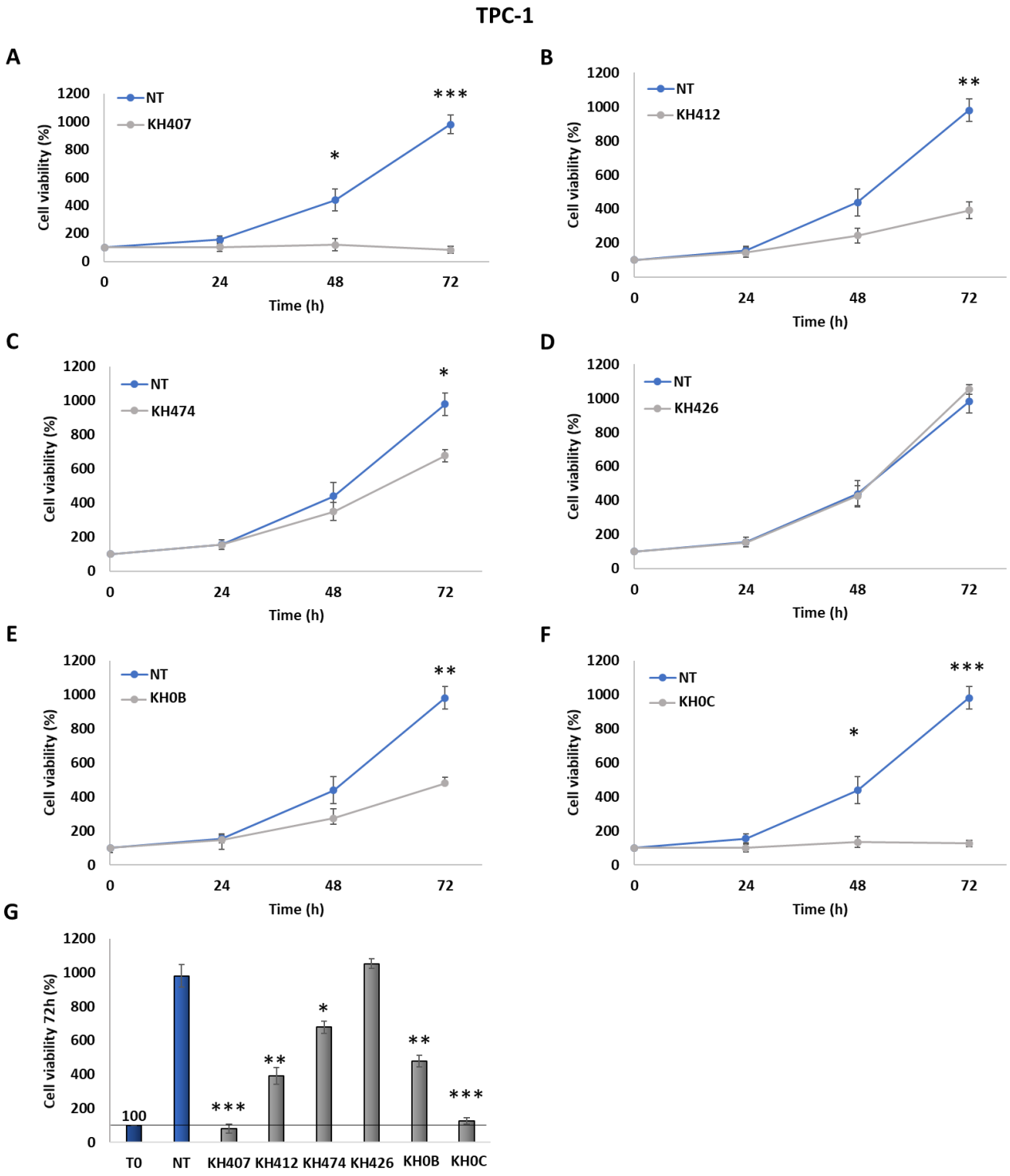


Figure 11: KH412, KH-474 and KH0B reduce TPC-1 cell growth without inducing out-side effects on NTHY3.1 cell viability. (A-F) TPC-1 cells were incubated for 24, 48 and 72 hours with KH407, KH412, KH474 and KH426 (300µM) and KH0B and KH0C (10µM). Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). NT= Not-Treated cells. **(G)** Bar graph represented TPC-1 cell viability after 72 hours of treatment with KH-compounds. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0. Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

To investigate the anti-tumorigenic potential of these three molecules, we performed a colony formation assay treating TPC-1 cells with KH-compounds previously selected. The ability to form colonies from a single cell is one of the peculiarities of cancer cells. This *in vitro* test allowed us to evaluate the ability of KH412, KH474 and KH0B compounds to inhibit cancer cell growth. In this context, the reduction in the number of colonies can derive either from the block of proliferation or from the induction of cell death. All molecules tested were able to reduce the number of colonies formed by TPC-1, with KH412 and KH0B being the more effective (Figure 12A, 12B). Thus, all three molecules may be considered as good candidates for more detailed studies of anti-tumorigenic effect *in vitro*.

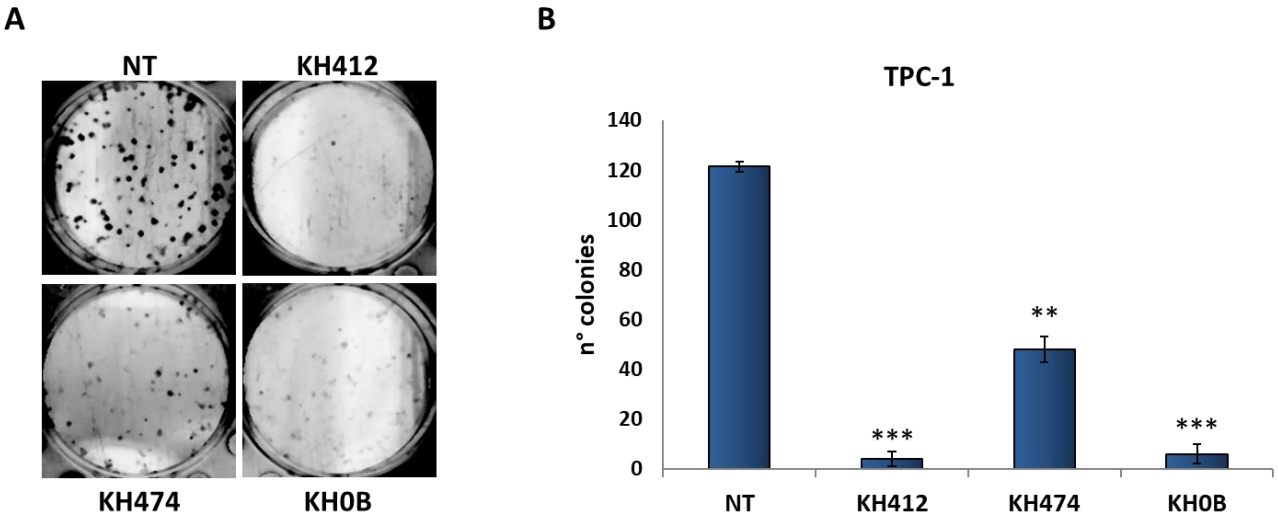


Figure 12: The treatment with KH412, KH474 and KH407 decreases the clonogenic capacity of TPC-1 cells. **(A)** Colony formation assay performed in six-well plates. TPC-1 cells were treated for one week with 300µM of KH412 and KH474 and with 10µM of KH0B. NT= Not-Treated cells. **(B)** Number of colonies were counted using ImageJ software. Data (mean ± SEM) n=3. Asterisks represented ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

To clarify whether these molecules specifically act on respiratory CI, its enzymatic function was determined by hrCNE-PAGE followed by Complex I in-gel activity (CI-IGA) and the expression levels of different CI subunits were evaluated by SDS-PAGE in isolated mitochondria. NTHY 3.1 and TPC-1 cells were incubated for 72 hours with KH412, KH474 and KH0B compounds showing that none of these was able to decrease CI activity compared to not-treated cells in both cancer and non-cancer models (Figure 13). However, though CI assembly and function did not appear to be altered, KH412, KH474 and KH0B treatment reduced the protein levels of several CI subunits (NDUFA9, NDUFS3 and NDUFB8) in TPC-1 cells. Further, it was also observed a decrease of VDAC protein levels, normally expressed at steady-state levels and considered as mitochondrial

loading control (Figure 13). In this context, the most effective compound was KH474, although it promoted a lower effect to decrease the tumorigenic potential *in vitro* compared to KH412 and KH0B. Moreover, in contrast with the latter compounds, TPC-1 treatment with KH474 induced also a lower expression of SDHA protein, the major catalytic subunit of succinate-ubiquinone oxidoreductase (Figure 13). This overall decrease in mitochondrial protein levels may indicate that this compound induced an extensive damage on mitochondria, probably affecting their biogenesis or inducing massive mitophagy.

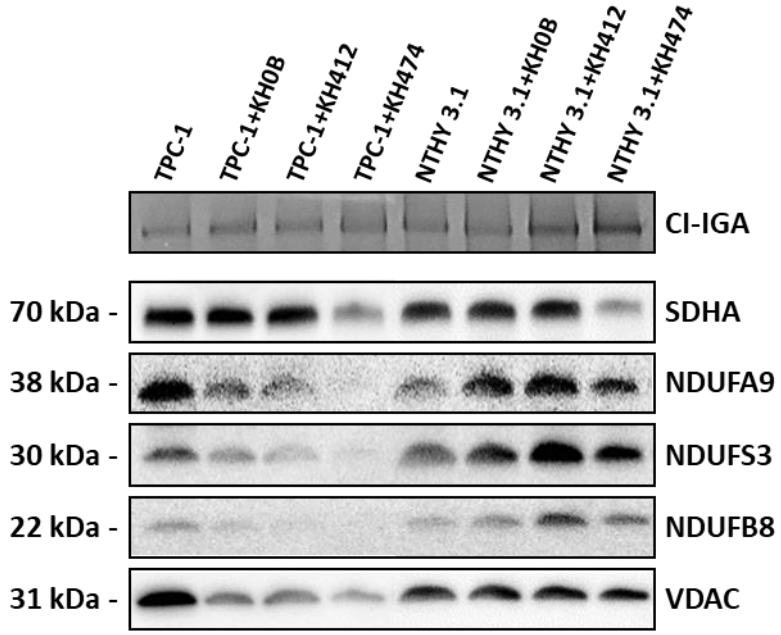


Figure 13: KH-compounds trigger a lower mitochondrial protein expression, included several CI subunits, in TPC-1 cells without affecting CI activity. CI-IGA and western blot analysis of different mitochondrial protein in NTHY 3.1 and TPC-1 isolated mitochondria after treatment for 72 hours with 300µM of KH412 and KH474 and with 10µM of KH0B. VDAC was used as loading control.

In NTHY 3.1 cells, none of the analyzed compounds was able to decrease respiratory CI activity (Figure 13). On the other hand, CI subunits appeared to be more express after treatment with KH-compounds compared to untreated cells and, in contrast to what observed in TPC-1 cells, VDAC protein levels were not altered (Figure 13). It seems that the capacity of KH412, KH474 and KH0B to potentially affect mitochondrial biogenesis or inducing massive mitophagy, as supposed before, is a cell specific response of TPC-1 cells. It may be also possible that the effect promoted by these compounds on mitochondria is cancer specific, but to support this hypothesis further studies are necessary using different cell models. Lastly, KH474 showed the same effect observed in TPC-1 cells reducing SDHA protein levels also in NTHY 3.1 cells (Figure 13).

Overall, these data supported the observation that KH412, KH474 and KH0B may be considered as interesting molecules displaying a specific anti-tumorigenic potential in thyroid cancer models. The reduction of several mitochondrial protein levels promoted by these molecules in TPC-1 cells indicate the existence of a link between their anti-tumorigenic activity and the importance of mitochondria in cancer. Indeed, mitochondria play a central and multi-functional role in malignant tumor progression and targeting mitochondria may provide a therapeutic opportunity. Despite these intriguing observations, none of these compounds was able to inhibit or reduce respiratory CI activity. For that reason, we decided to move away from this research line in order to focus our attention on other compounds capable to induce a pseudonormoxic state in HIF-1-dependent tumors by specifically inhibiting respiratory CI activity.

The anti-tumorigenic potential of metformin mainly derives from CI-independent mechanisms.

Metformin has been widely used for many years for treatment of type II diabetes and several epidemiological studies highlighted its beneficial effect in oncologic patients, in terms of reduced risk to develop disease and better prognosis [129, 130, 131]. Recently, the anti-tumorigenic activity of metformin was associated with its potential activity as respiratory CI inhibitor, although the mechanisms through which it prevents tumor progression are not fully understood and comprises CI-independent activities. To elucidate this point, we exploited a unique cell model generated in our laboratory in which we specifically ablated NDUFS3 gene in osteosarcoma 143B cells via gene editing [156]. NDUFS3 is core nDNA-encoded subunits located in the site of CI responsible for the electron transfer to ubiquinone (Q-module). The knock-out of this subunit abolished CI assembly and activity almost completely [156] and isogenic NDUFS3$^{+/+}$ cells were used as controls. Hence, cells lacking fully assembled respiratory CI missed the major molecular target of metformin and consequently should be less susceptible to the treatment.

First, we evaluated the effect of 10mM and 20mM metformin on cell proliferation, treating 143B NDUFS3$^{+/+}$ (used as controls) and 143B NDUFS3$^{-/-}$ cells for multiple time points. A clear dose-dependent effect of metformin in reducing cell growth after 48 and 72 hours was observed in 143B NDUFS3$^{+/+}$ cells (Figure 14A, 14C). Conversely, 143B NDUFS3$^{-/-}$ viability was significantly decreased after 48 and 72 hours of treatment with 20mM of metformin compared to corresponding not-treated cells, while treatment with 10mM did not affect cell viability (Figure 14B, 14D).

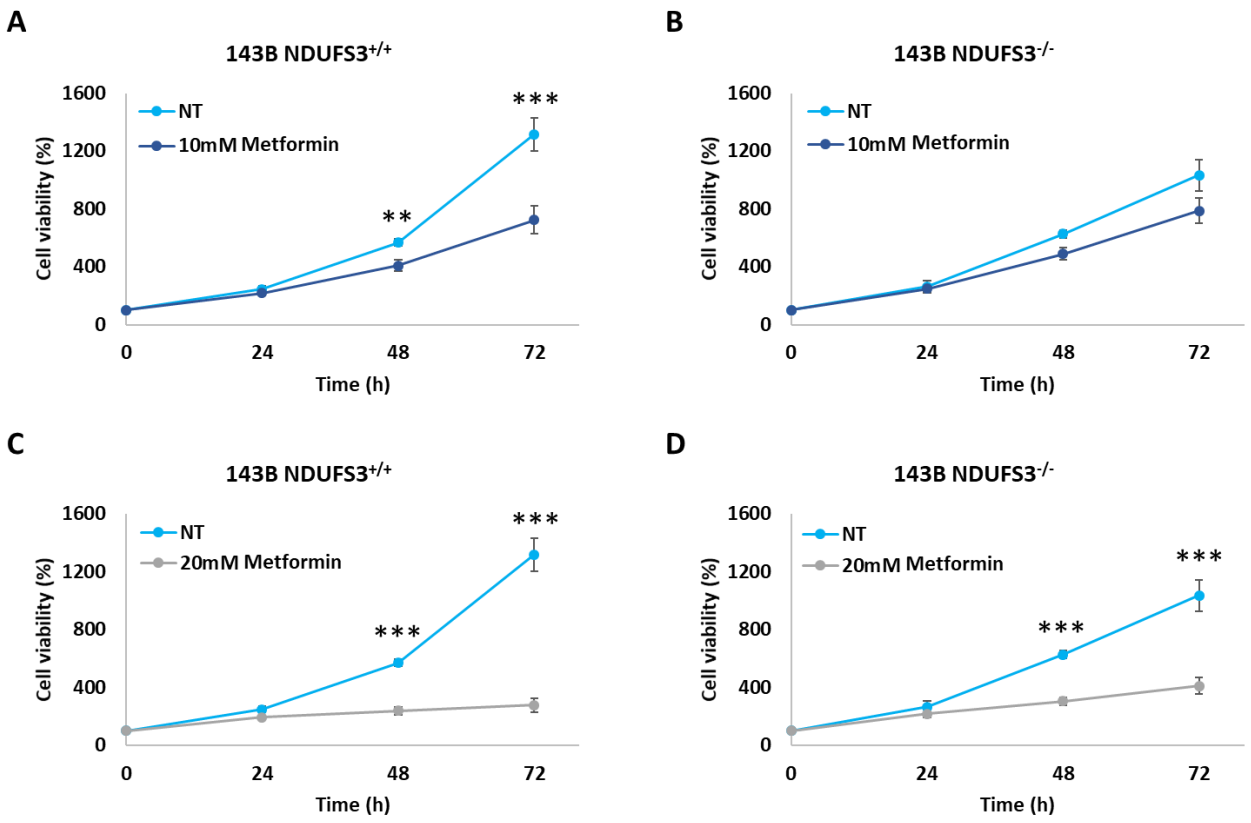


Figure 14: Metformin reduces osteosarcoma cells viability in a specific CI-dependent manner using 10mM of concentration. (A, B) 143B NDUFS3$^{+/+}$ and 143B NDUFS3$^{-/-}$ cells were incubated for 24, 48 and 72 hours with 10mM of metformin. **(C, D)** 143B NDUFS3$^{+/+}$ and 143B NDUFS3$^{-/-}$ cells were incubated for 24, 48 and 72 hours with 20mM of metformin. Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=5). NT= Not-Treated cells. Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

These data suggested that it was possible to observe a decrease of cell proliferation mainly dependent on metformin CI inhibitory effect using 10mM of concentration, whereas not specific effects prevailed when cells were treated with higher doses of drug. Normalizing data on not-treated cells at every time point, it was possible to compare the effect of metformin in 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cells. The cell viability of 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cells was reduced about 40% and 20% respectively compared to not-treated cells after treatment with 10mM of metformin for 72 hours (Figure 15A). Cell growth was reduced by 80% in 143B NDUFS3$^{+/+}$ cells and almost 60% in 143B NDUFS3$^{-/-}$ cells after 72 hours of treatment with 20mM metformin (Figure 15B). These data suggested that at high concentration, metformin exerted a greater effect in reducing cell proliferation *in vitro* independently from the presence of functional respiratory CI and that this drug may impact other unidentified molecular targets at least in osteosarcoma background. Furthermore, the specificity towards 143B NDUFS3$^{+/+}$ cells, rather than NDUFS3$^{-/-}$ cells, was about 20% with

both metformin concentrations tested suggesting that the anti-proliferative effect was not mostly dependent on the inhibition of CI activity.

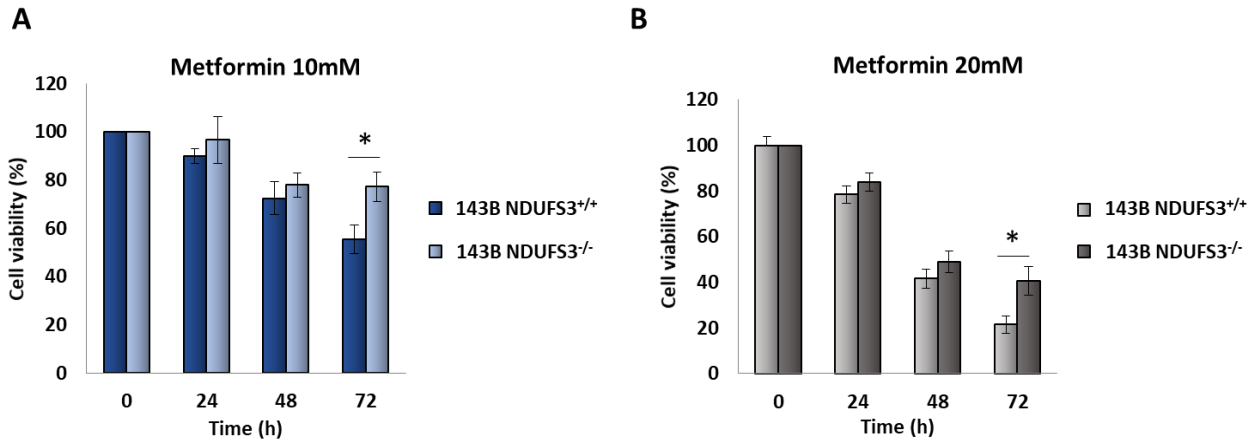


Figure 15: Non-specific effect of metformin mainly determines the decrease of osteosarcoma cell proliferation. (A) 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cells were incubated for 24, 48 and 72 hours with 10mM of metformin. **(B)** 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cells were incubated for 24, 48 and 72 hours with 20mM of metformin. Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the Not-Treated (NT) cells (n=5). Asterisks represented * p-value ≤ 0.05 using Student's T-test.

To investigate the anti-tumorigenic effect of metformin *in vitro* a colony formation assay was performed. 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cells were incubated for 10 days with 10mM of metformin in order to evaluate their clonogenic capacity under inhibition of CI activity. A reduction of colonies size was observed after treatment with metformin, but without significant changes in colonies number compared to non-treated cells in both 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cell models (Figure 16A, 16B, 16C), confirming that metformin did not induce cells death, but prevented cell growth in a manner that was only partially dependent on respiratory CI inhibition.

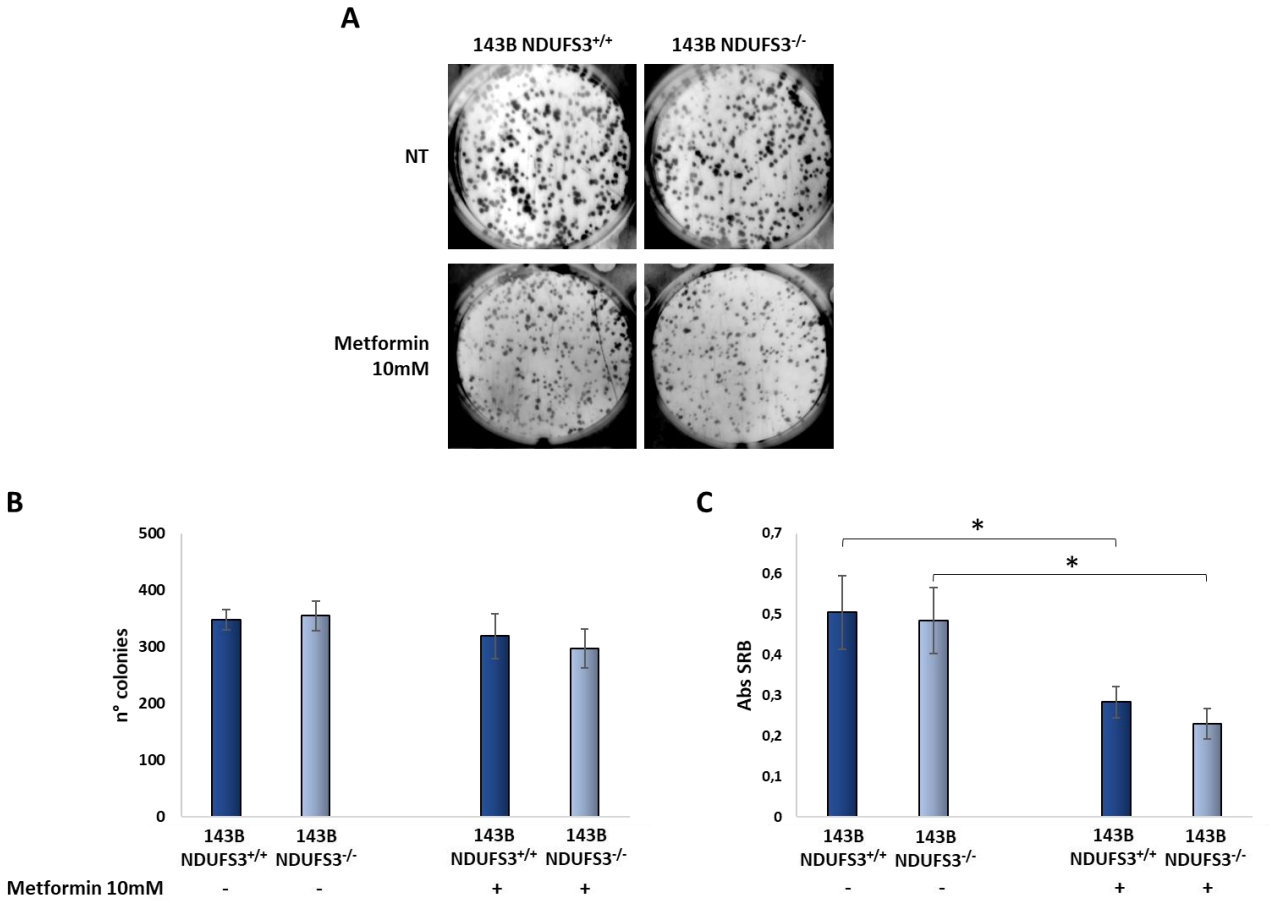


Figure 16: Metformin treatment induces cell growth arrest independently from CI-activity in osteosarcoma cells. (A) Colony formation assay performed in six-well plates. 143B NDUFS3$^{+/+}$ and NDUFS3$^{-/-}$ cells were treated for ten days with 10mM of metformin. NT=Not-Treated cells. **(B)** Number of colonies were counted using ImageJ software. **(C)** The absorbance (abs) of SRB is a measure of the area occupied by cell colonies representing an indirect index of colonies size. Data (mean ± SEM), n=3. Asterisks represented * p-value ≤ 0.05 using Student's T-test comparing not-treated cells to metformin treated cells.

There are evidences in literature regarding the role of metformin to reduce the hypoxic stabilization of HIF-1α protein and HIF-dependent target genes through the inhibition of respiratory CI [133]. This is consistent with the inhibitory effect exerted on HIF-1α by a large amount of canonical and novel CI inhibitors [93]. In line with these findings, although the inhibitory effect on CI did not seem to be the main molecular mechanism through which metformin promoted its anti-tumorigenic potential in our osteosarcoma models, we investigated the impact of metformin treatment on HIF-1α protein levels under hypoxic condition. 143B NDUFS3$^{+/+}$ cells were pre-incubated in hypoxia for 30 minutes (T0) in order to induce HIF-1α stabilization and then cells were treated with metformin for 2, 6, 16 and 24 hours. Western blot analysis showed that drug treatment decreased HIF-1α protein levels compared to non-treated at every time points tested (Figure 17A, 17B). These data allowed us

to speculate that metformin might induce a pseudonormoxic state in hypoxic cells through its respiratory CI-inhibitor effect.

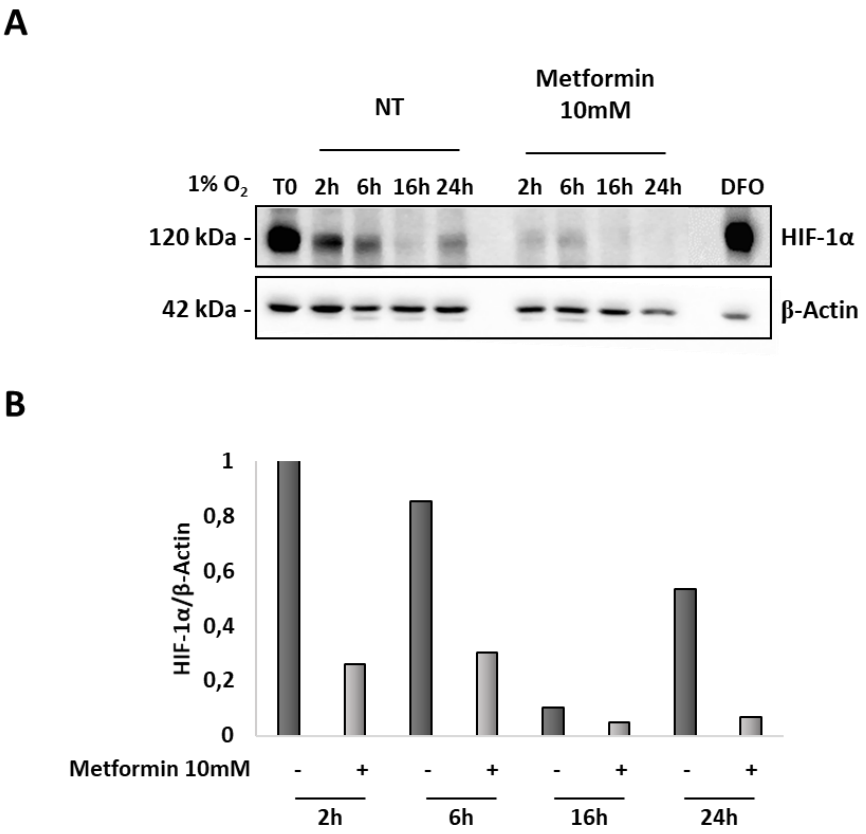


Figure 17: Metformin reduces HIF-1α protein levels in 143B NDUFS3$^{+/+}$ cells under hypoxic condition. (A) Western blot analysis of HIF-1α protein levels after treatment with 10mM of metformin in 143B NDUFS3$^{+/+}$ cells for 2, 6, 16 and 24 hours under hypoxic condition (1% O_2). β-Actin was used as loading control. **(B)** Densitometric evaluation of HIF-1α protein levels normalized on β-Actin. DFO was used as positive control for normoxic HIF-1α induction.

In order to generalize our observations and to determine whether metformin could exert a specific anti-proliferative effect in cancer cells while being not toxic, cancer and non-cancer CI-competent cell models were treated with metformin and cell proliferation, colony formation and HIF-1α stability determined. Cell viability assay was performed in both normoxic (21% O_2) and hypoxic (1% O_2) conditions in thyroid and renal cell models in absence and presence of 10mM of metformin for 24, 48 and 72 hours. Under normoxic condition, metformin treatment induced a slight reduction of NTHY 3.1 and TPC-1 cell proliferation respectively after 72 and 48 hours of treatment compared to corresponding not-treated cells (Figure 18A, 18B). On the contrary, a specific anti-cancer cell proliferation effect was observed under hypoxic condition. In fact, at low oxygen levels, metformin induced only a mild effect on NTHY 3.1 cell proliferation after 72 hours of treatment, whereas

TPC-1 cell growth was largely inhibited already after 48 hours and was reduced 3-fold compared to not-treated cells after 72 hours (Figure 18C, 18D). Hence, metformin differently affected TPC-1 cell proliferation depending on intracellular oxygen levels. The specific anti-cancer effect observed in hypoxia could be explained by a higher capacity of TPC-1 cells to stabilize HIF-1α compared to NTHY 3.1 cells (Figure 25A, 25B), which in turn might explain the greater impact of metformin to reduce TPC-1 cell viability at low oxygen levels.

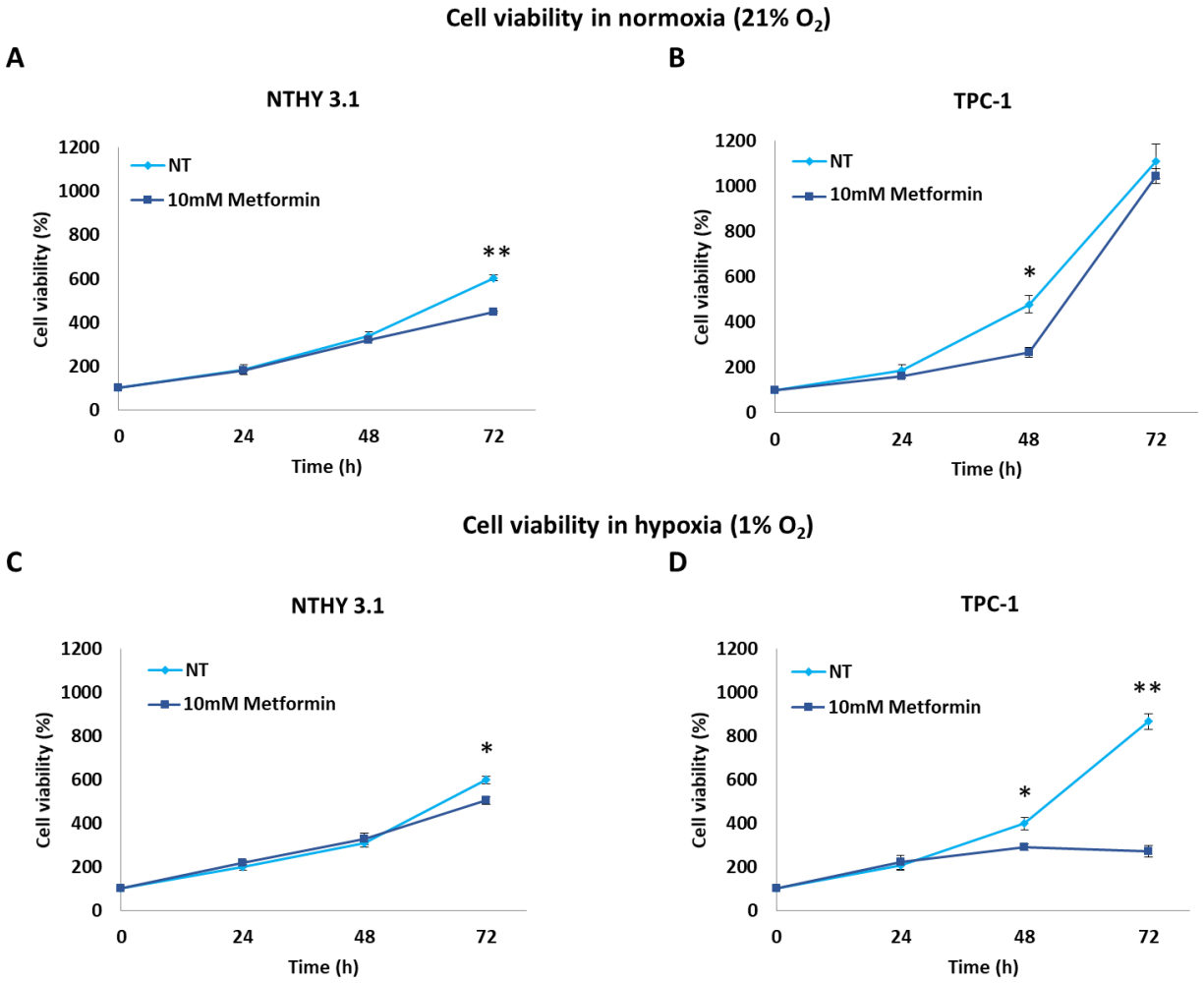


Figure 18: Metformin triggers a specific anti-proliferative effect depending on cellular O_2 levels in thyroid cells. (A, B) NTHY 3.1 and TPC-1 cells were respectively incubated for 24, 48 and 72 hours with 10mM of metformin under normoxic condition (21% O_2). **(C, D)** NTHY 3.1 and TPC-1 cells were incubated for 24, 48 and 72 hours with 10mM of metformin under hypoxic condition (1% O_2). Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). NT= not-treated cells. Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to non-treated cells using Student's T-test.

Furthermore, the TPC-1 clonogenic capacity was completely abolished after metformin treatment for one week (Figure 19A, 19B). In parallel, the western blot showed that also HIF-1α protein levels were reduced compared to corresponding not-treating cells after metformin administration under hypoxic condition for 2, 6, 16 and 24 hours (Figure 19C). Overall, these data suggested that the anti-proliferative effect induced *in vitro* by this drug may be related to its ability to reduce HIF-1α stability in hypoxia. Supported by previous data obtained using osteosarcoma CI-competent cells, it is possible to assume that metformin is able to hamper thyroid tumor growth by promoting a pseudonormoxic state trough CI-inhibition.

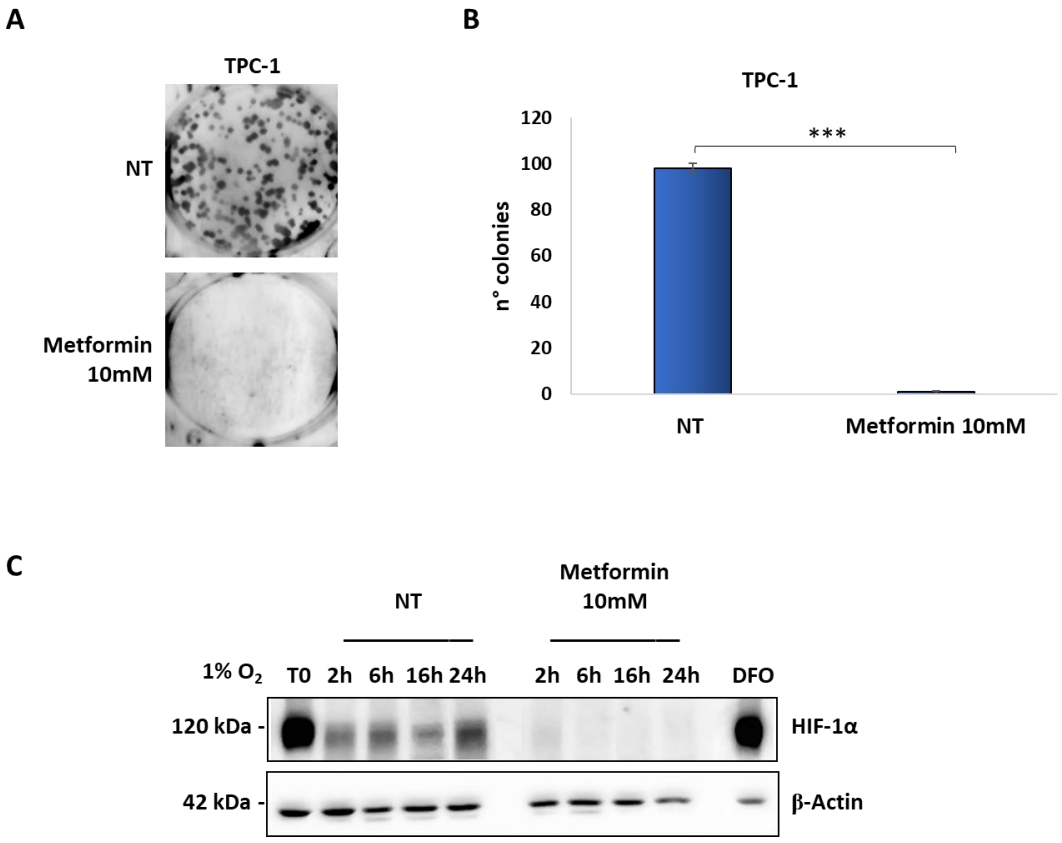


Figure 19: Metformin completely abolishes TPC-1 clonogenic capacity and also reduces HIF-1α protein levels under hypoxic condition. (A) Colony formation assay performed in six-well plates. TPC-1 cells were treated for seven days with 10mM of metformin. NT=Not-Treated cells. **(B)** Number of colonies were counted using ImageJ software. Data (mean ± SEM), n=3. Asterisks represented *** p-value ≤ 0.001 using Student's T-test comparing not-treated cells to metformin treated cells. **(C)** Western blot analysis of HIF-1α protein levels after treatment with 10mM of metformin in TPC-1 cells for 2, 6, 16 and 24 hours under hypoxic condition (1% O_2). β-Actin was used as loading control. DFO was used as positive control for normoxic HIF-1α induction.

In both renal cell models, metformin did not show any effect on cell proliferation compared to not-treated cells under normoxic or hypoxic conditions (Figure 20A, 20B, 20C, 20D), suggesting that the molecular mechanisms through which metformin induces its anti-tumorigenic potential might depend on cell type. However, it is important to consider that UOK-257 cells are characterized by the presence of mutant copy of the folliculin (FLCN) gene [157]. The occurrence of these genomic alteration is related to a higher dependency of UOK-257 on glucose metabolism caused by increased HIF-1α activity and by altered metabolism. This dependence on a single fuel for metabolic production of ATP is known as a loss of "metabolic flexibility", which is a common feature of insulin resistance [158]. These findings may explain the lack of metformin efficacy to decrease renal cancer cell proliferation.

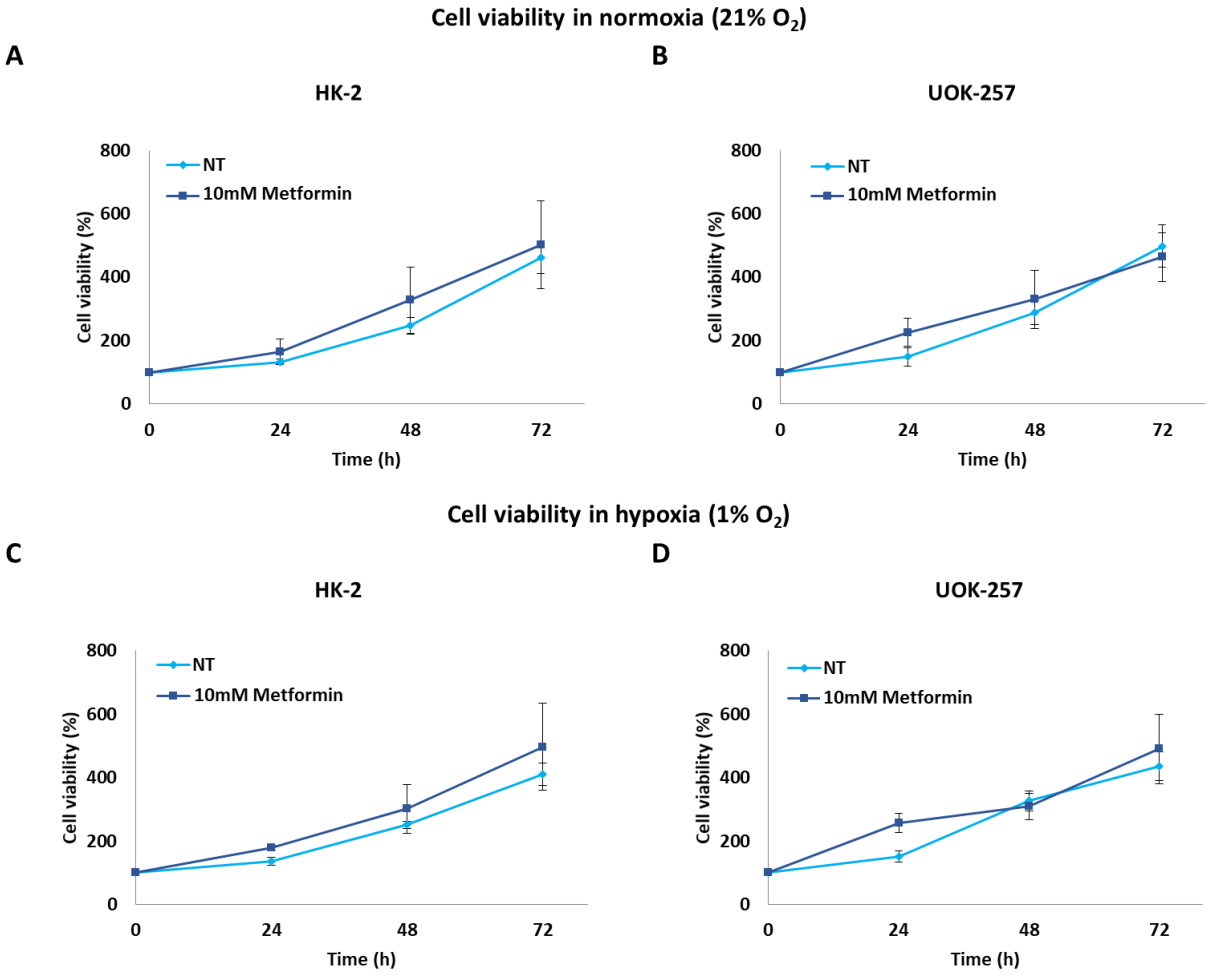


Figure 20: Metformin does not affect cancer cell proliferation in renal cells models. (A, B) HK-2 and UOK-257 cells were respectively incubated for 24, 48 and 72 hours with 10mM of metformin under normoxic condition (21% O_2). **(C, D)** HK-2 and UOK-257 cells were respectively incubated for 24, 48 and 72 hours with 10mM of metformin under hypoxic condition (1% O_2). Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). NT= non-treated cells. Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

Interestingly, more long-term exposure to metformin significantly reduced UOK-257 clonogenic capacity *in vitro* suggesting that this drug might require a specific time to be effective in preventing renal cancer cell growth (Figure 21A, 21B). Indeed, cells were treated for ten days to perform the colony assay formation. In line, metformin treatment reduced HIF-1α protein levels under hypoxic condition 3-fold compared not-treated cells after 24 hours, while just a slight decreased was observed after 2 and 16 hours (Figure 21C, 21D).

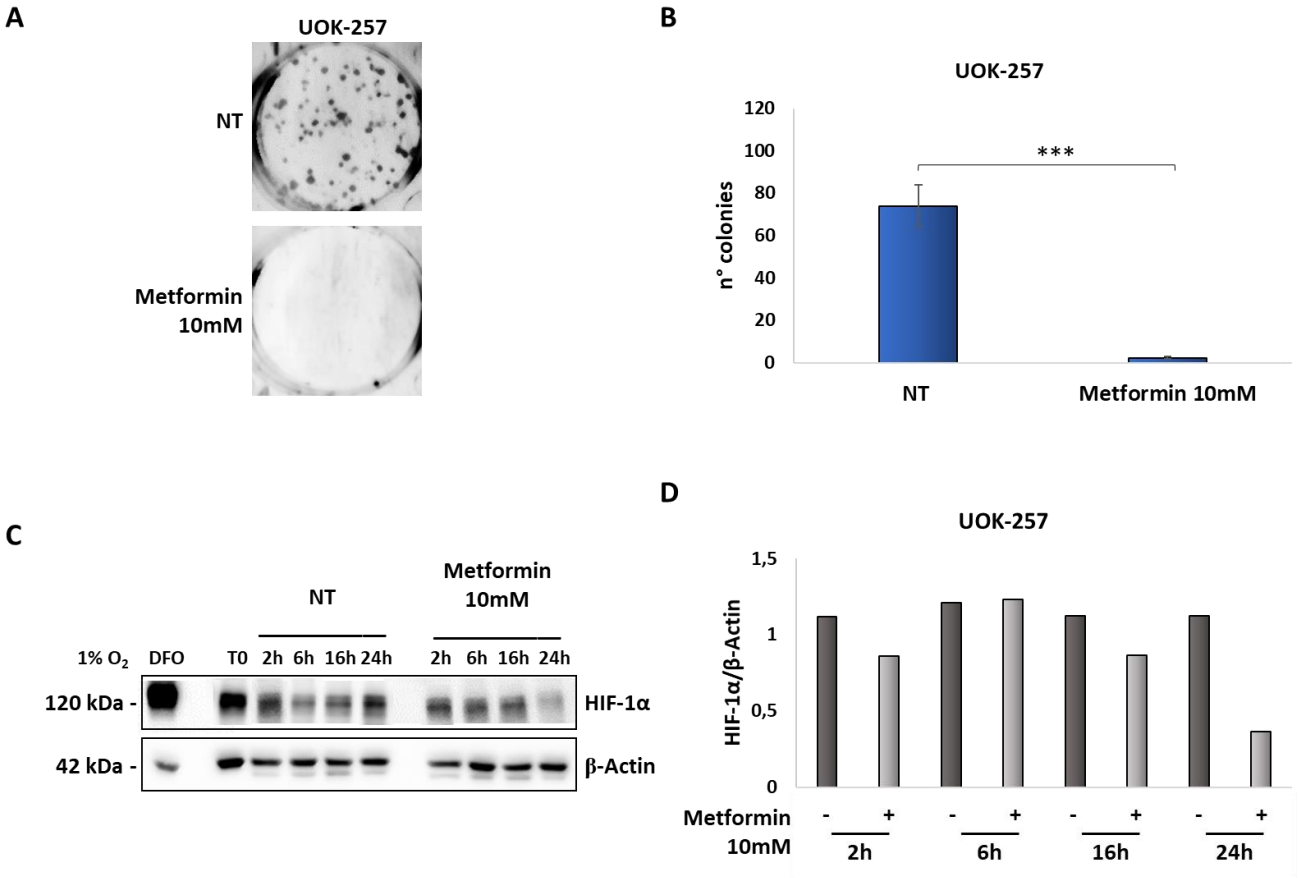


Figure 21: The anti-tumorigenic potential of metformin is dependent on exposure time and on cancer cells type. (A) Colony formation assay performed in six-well plates. UOK-257 cells were treated for seven days with 10mM of metformin. NT=Not-Treated cells. **(B)** Number of colonies were counted using ImageJ software. Data (mean ± SEM), n=3. Asterisks represented *** p-value ≤ 0.001 using Student's T-test comparing not-treated cells to metformin treated cells. **(C)** Western blot analysis of HIF-1α protein levels after treatment with 10mM of metformin in UOK-257 cells for 2, 6, 16 and 24 hours under hypoxic condition (1% O_2). β-Actin was used as loading control. DFO was used as positive control for normoxic HIF-1α induction. **(D)** Densitometric evaluation of HIF-1α protein levels normalized on β-Actin.

In conclusion, our data suggest that metformin reduced tumor growth *in vitro*, although its anti-proliferative effect seemed to be mainly due to systemic effect rather than the specific inhibition of mitochondrial CI activity, at least in our osteosarcoma CI-deficient and CI-competent cells. The anti-tumorigenic activity of metformin also depended on the tissue origin of cancer cells and on the time exposure to the treatment. Interestingly, the treatment did not alter non-cancer cell viability in both thyroid and renal cell models. Furthermore, we demonstrated that this antidiabetic drug reduced HIF-1α protein levels under hypoxic condition in different cancer cell lines. This result is consistent with the possibility that metformin reduces HIF-1α stability through the inhibition of CI since several inhibitors of this mitochondrial enzyme share the ability to target the same pathways, including cancer cell adaptation to hypoxia [93]. In this context, metformin may therefore be a potential therapeutic agent for the treatment of hypoxic tumors.

αKGlogues as a metabolic tool to induce a pseudonormoxic state in hypoxic cancer cells.

The second approach to induce a condition of pseudonormoxia was the direct increase of intracellular αKG levels. However, αKG is a hydrophilic metabolite and does not permeate the plasma membrane. Hence, in collaboration with Prof. Locatelli's group from Department of Pharmacy and Biotechnology (FaBiT) of University of Bologna, seven αKGlogues were designed and synthetized with the aim to boost αKG membrane permeability, to increase its intracellular levels and to promote PHDs activity under hypoxic condition. The experimental plan was to bound carriers characterized by different hydrophobic indices to αKG, in order to allow it to cross the plasma membrane. Once αKGlogues entered into the cell, the ester bound that conjugated the αKG to the hydrophobic carrier should be recognized from cytosolic non-specific diesterases leading to the αKG intracellular accumulation.

Isoprenoid carriers contribute to reduce cancer cells proliferation *per se*.

The treatment with αKGlogues, besides αKG, may also induce the raised intracellular amount of hydrophobic carriers. Most of the carriers selected were phenyl, alkyl or methyl substituents, which are not supposed to show any biological function when released into the cytosol. However, we also tested three hydrophobic carriers (carriers A, B and C) that are natural isoprenoid molecules, some of which are already known to have an anti-cancer activity. For this reason, these three carriers were screened for their possible synergic/antagonist effect with αKG and for their cytotoxicity using the already described thyroid and renal cell lines. Cell viability assay was performed at multiple time points testing different concentrations of these compounds. The cell viability of NTHY 3.1 was reduced already after 24 hours of treatment with all concentration tested of carrier A (Figure 22A). Furthermore, it was observed a cytostatic effect after 72 hours incubating cells with 150µM and 200µM of this molecule. Under these conditions, NTHY 3.1 cell growth decreased ~4-fold compared to corresponding untreated cells, but without going under the number of cells seeded before the treatment with carrier A (time zero, T0), considered as the 100%, indicating an arrest of cell proliferation (Figure 22B). Similarly, viability of TPC-1 was reduced in a dose-dependent manner, except with 2µM of carrier A that did not alter thyroid cancer cell viability at any time points (Figure 22C). In particular, carrier A exerted a cytostatic effect also in TPC-1 using 100µM, 150µM and 200µM of concentration after 72 hours (Figure 22D). These data suggest that this compound could significantly dampen thyroid cell growth, especially at high concentrations (above 100µM) without showing a cytotoxic effect.

The capacity of carrier A to alter renal cells proliferation was weaker compared the effect shown on thyroid cells. In fact, HK-2 cell viability was significantly reduced after 24, 48 and 72 hours of treatment with 200µM of carrier A and after 72 hours with 100µM, 150µM (Figure 22E). Conversely, UOK-257 cell viability was not affected after the incubation with this compound at any concentration tested (Figure 22F).

The second hydrophobic carrier screened for its possible synergistic/antagonist effect with αKG and its cytotoxicity was carrier B. All concentration tested were able to reduce significantly NTHY 3.1 cell proliferation after 24, 48 and 72 hours of treatment (Figure 23A). As observed with compound A, also the carrier B exerted a cytostatic effect inducing the arrest of NTHY 3.1 proliferation after 72 hours of incubation with 150µM and 200µM of concentration (Figure 23B). Conversely, a dose-dependent decrease of TPC-1 cell growth was observed after treatment with carrier B, although without inducing a cytostatic effect as in thyroid non-cancer cells (Figure 23C, 23D). In renal cell models, the treatment with carrier B induced a decrease of HK-2 cell growth that was significant using 50µM, 100µM, 150µM and 200µM of concentration for 72 hours (Figure 23E). In UOK-257, there were not observed alteration on cell viability incubating cells with different concentrations of this compound for multiple time points (Figure 23F). Thus, the effect showed by both carrier A and B was almost the same in renal cells.

The last compound screened was carrier C, which induced a dose-dependent decrease of NTHY 3.1 cell proliferation using 100µM, 150µM and 200µM of concentration (Figure 24A). Differently from other two isoprenoid compounds, the treatment with 150µM and 200µM of carrier C caused a cytotoxic effect on NTHY 3.1 cell growth after 72 hours (Figure 24B). In this case it was not induce a block of cell proliferation since the NTHY 3.1 cell visibility was reduced by 30% and 85% compared the amount of cells seeded before the incubation for 72 hours (T0) with 150µM and 200µM of carrier C, respectively (Figure 24B). In TPC-1 cells, a significant decrease of cell proliferation was observed after treatment with all concentration tested, whereas a cytostatic effect was trigged incubating cells with 200µM of carrier C for 72 hours (Figure 24C, 24D). On the other hand, carrier C treatment did not alter renal cells proliferation compared untreated cells (Figure 24E, 24F).

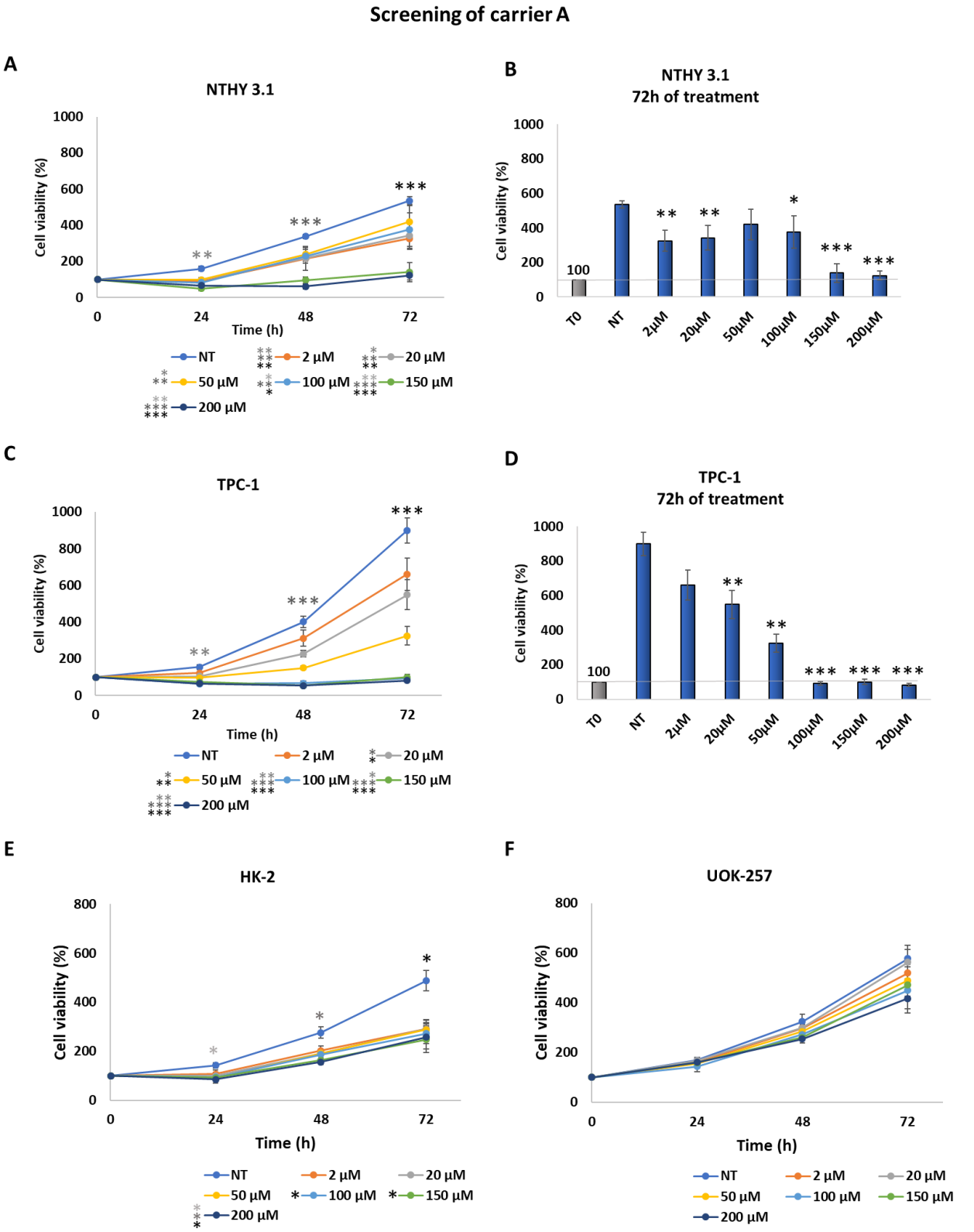


Figure 22: Carrier A induces a cytostatic effect on cell proliferation in both thyroid cancer and non-cancer cells at higher concentrations tested, while it does not alter the proliferative capacity of renal cancer cells.
(A, C) NTHY 3.1 and TPC-1 cells were grown in increasing dose of carrier A (2µM, 20µM, 50µM, 100µM, 150µM and 200µM) for 24, 48 and 72 hours. **(B, D)** Carrier A effect on NTHY 3.1 and TPC-1 cell viability after 72 hours of treatment at different concentrations. **(E, F)** HK-2 and UOK-257 cells were grown in increasing dose of Carrier A (2µM, 20µM, 50µM, 100µM, 150µM and 200µM) for 24, 48 and 72 hours. Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the T0 (time zero) (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

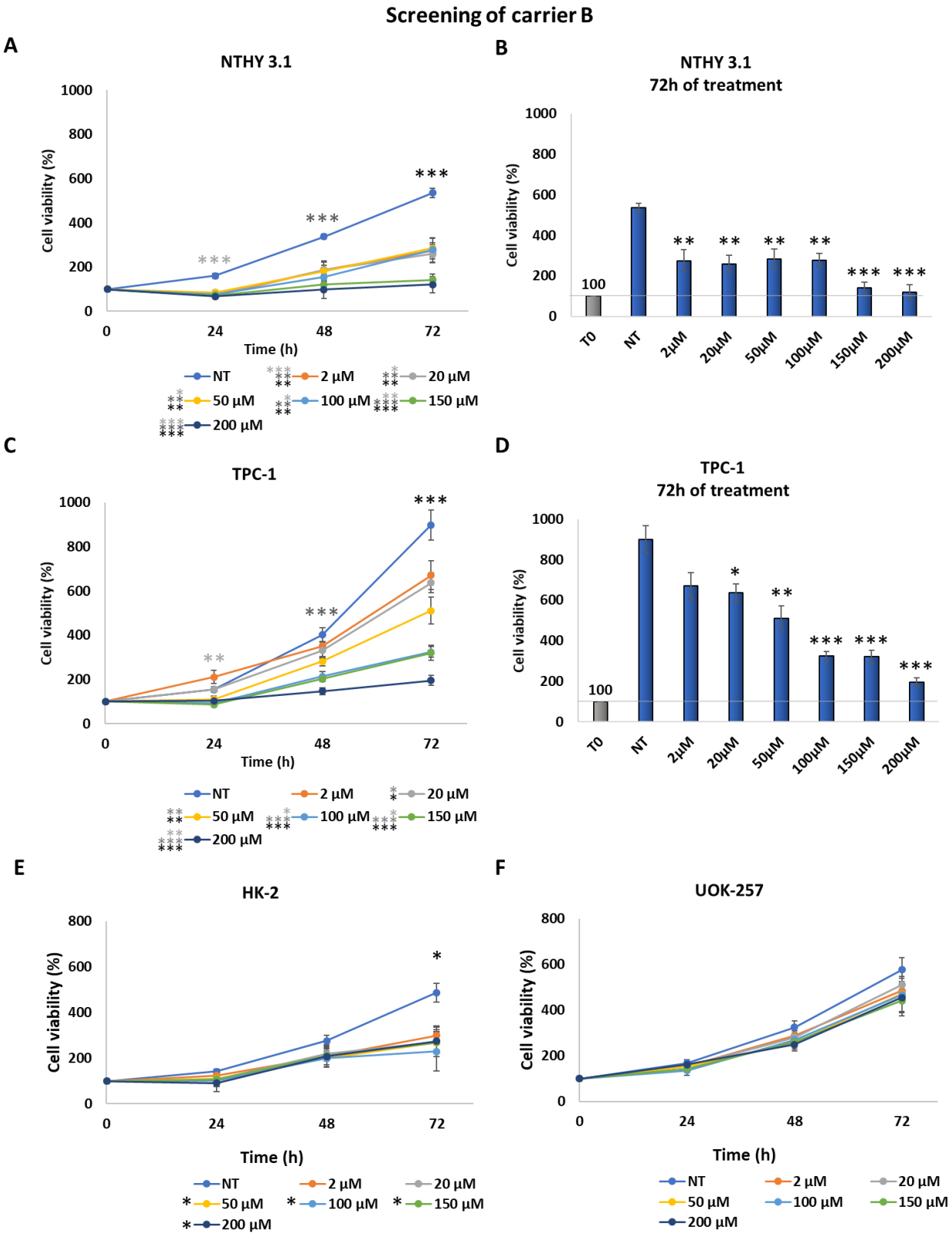


Figure 23: Carrier B significantly decreases thyroid cell growth, but it does not display an anti-proliferative effect on renal cancer cell growth *in vitro*. (A, C) NTHY 3.1 and TPC-1 cells were grown in increasing dose of Carrier B (2µM, 20µM, 50µM, 100µM, 150µM and 200µM) for 24, 48 and 72 hours. **(B, D)** Carrier B effect on NTHY 3.1 and TPC-1 cell viability after 72 hours of treatment at different concentrations. **(E, F)** HK-2 and UOK-257 cells were grown in increasing dose of Carrier B (2µM, 20µM, 50µM, 100µM, 150µM and 200µM) for 24, 48 and 72 hours. Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

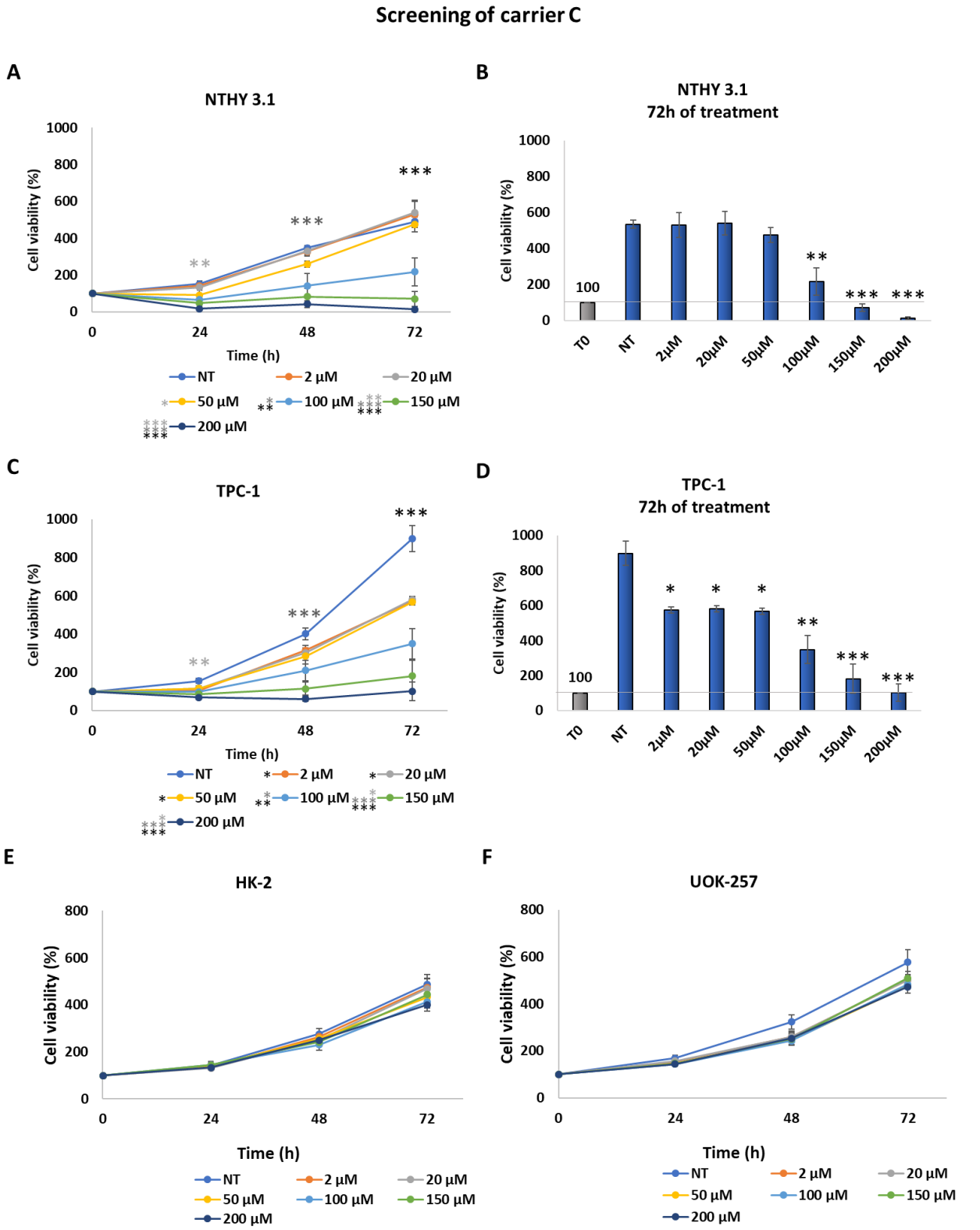


Figure 24: A cytotoxic effect is exerted by carrier C treatment in thyroid non-cancer cells at higher concentration tested. The same treatment does not induce an alteration of renal cell proliferation. (A, C) NTHY 3.1 and TPC-1 cells were grown in increasing dose of carrier C (2µM, 20µM, 50µM, 100µM, 150µM and 200µM) for 24, 48 and 72 hours. **(B, D)** Carrier C effect on NTHY 3.1 and TPC-1 cell viability after 72 hours of treatment at different concentrations **(E, F)** HK-2 and UOK-257 cells were grown in increasing dose of Carrier C (2µM, 20µM, 50µM, 100µM, 150µM and 200µM) for 24, 48 and 72 hours. Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

Overall, these data suggested that carrier A and B may have a synergistic effect with αKG, when conjugated to this metabolite to form the corresponding αKGlogue, to reduce thyroid cancer cells proliferation *in vitro*, specially using 100μM, 150μM and 200μM of concentration. However, it is important to consider that the same compounds induced a block of NTHY 3.1 cell proliferation treating these cells with 150μM and 200μM of concentration for 72 hours. Concerning the carrier C, we decided to exclude this hydrophobic carrier since high concentrations (150μM and 200μM) were able to kill normal thyroid cells.

The effect showed by all isoprenoid carriers tested was completely different in renal cells compared to thyroid models. None of them altered UOK-257 cell proliferation indicating that potential effect of corresponding αKGlogues to reduce renal cancer cell viability *in vitro* could be ascribed exclusively to αKG. Furthermore, carrier A and B promoted a slight but significant decrease of HK-2 proliferation after 72 of treatment with 100μM, 150μM and 200μM of concentration. Therefore, αKG may potentially exerted a synergic effect with these carriers to humper renal normal cells growth that we will take into account using the corresponding αKGlogues. Instead, carrier C did not alter neither renal cancer nor non-cancer cell proliferation suggesting that, when conjugated to αKG, should not have a synergic effect to reduce the tumorigenic potential of cancer cells.

αKGlogues destabilize HIF-1α protein levels under hypoxic condition.

In order to determine the effect of αKGlogues on HIF-1α stabilization under hypoxic conditions (1% O_2), we treated thyroid and renal cells with 200μM of αKGlogues for 90 and 120 minutes and evaluated HIF-1α levels compared to corresponding not-treated cells using western blot analysis. As positive control of HIF-1α stabilization, we incubated the same cell lines under normoxic condition (21% O_2) with 150μM of desferoxamine (DFO), an iron chelator that prevents PHD activity leading to HIF-1α accumulation. Further, dimethyl-2-oxoglutarate was used as a further example of αKG derivative, commercially available, since it has been shown to successfully permeate across the plasma membrane and to increase αKG intracellular levels [68].

Western blot analysis showed that not-treated TPC-1 cells were characterized by a higher capacity to stabilize HIF-1α protein levels after 120 minutes under hypoxic condition compared to NTHY 3.1 cells, highlighting the crucial role of HIF-1α in adaptive responses of the tumor cells to changes in oxygen homeostasis (Figure 25A, 25B). As previously shown, high concentration of carrier C promoted a cytotoxic effect on NTHY 3.1 independently from αKG, thus the corresponding αKGlogue has been excluded this study. NTHY 3.1 and TPC-1 cells were incubated under hypoxic condition for 30 minutes, in order to stabilize HIF-1α. After that, cells were incubated with αKGlogues for 90 and 120 minutes. No reduction of HIF-1α protein levels was observed in NTHY 3.1 cells after αKGlogues treatment; rather the hypoxic transcriptional factor seemed to increase its

stability over time (Figure 25A, 25C). Conversely, αKGlogues B, D and E were selected for their capacity to destabilize HIF-1α specifically in TPC-1 cells already after 90 and 120 minutes of incubation (Figure 25B, 25D).

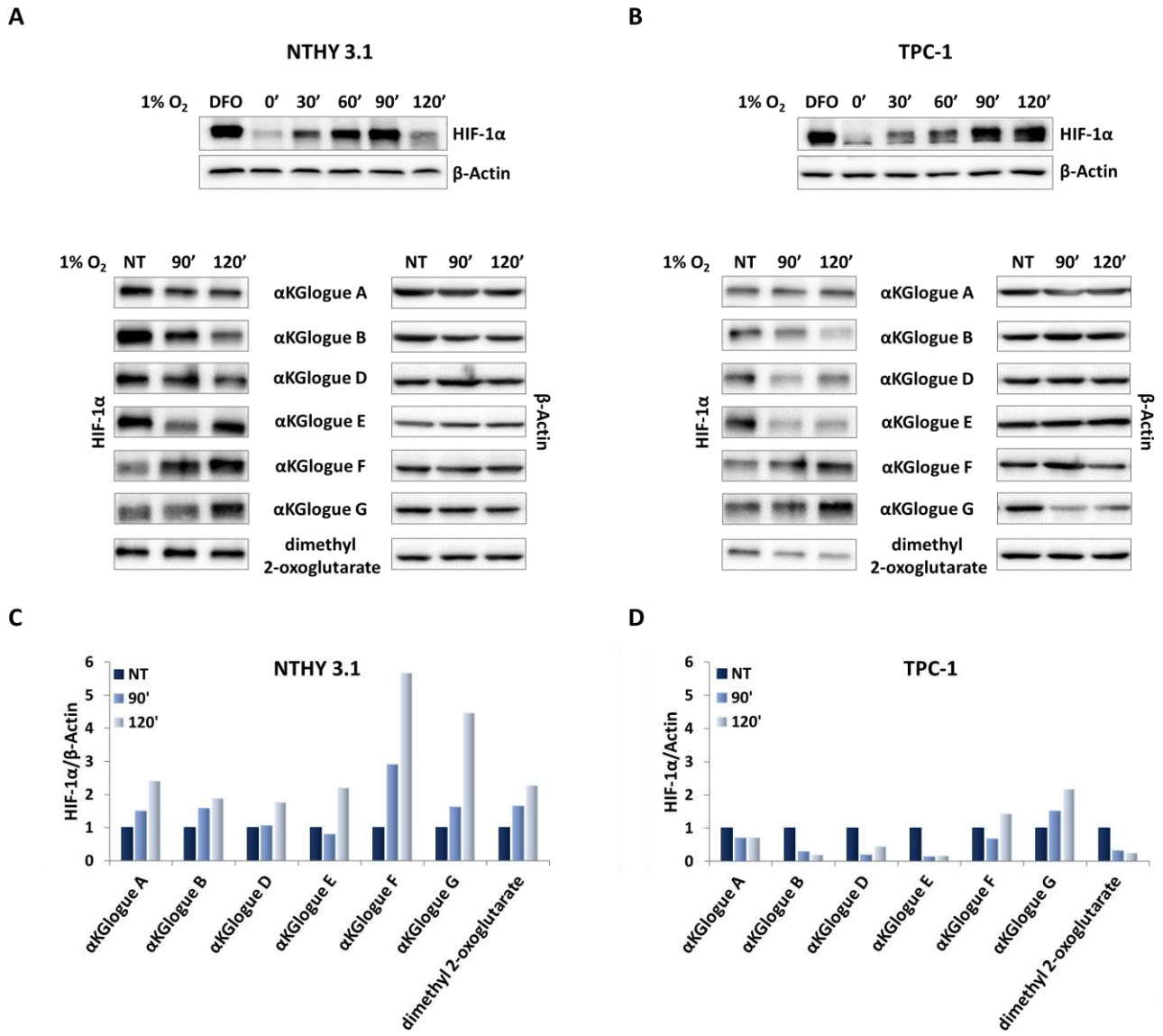


Figure 25: αKGlogue B, D and E reduce HIF-1α protein levels specifically in thyroid cancer cells under hypoxic condition. (A, B) Western blot analysis of HIF-1α levels after treatment with αKGlogues for 90 and 120 minutes in NTHY 3.1 and TPC-1 cells under hypoxic condition (1% O_2). β-Actin was used as loading control. **(C, D)** Densitometric evaluation of HIF-1α. Data were normalized on the corresponding not-treated cells for each time points and on β-Actin. DFO was used as positive control for HIF-1α stabilization.

In renal models, UOK-257 cancer cells showed an increased capacity to stabilize HIF-1α in hypoxia compared to HK-2 normal cells (Figure 26A, 26B), similarly to what observed in thyroid cells. Furthermore, αKGlogues E and F showed a specific effect on cancer cells to reduce HIF-1α protein levels respectively after 120 and 90 minutes of treatment under hypoxic condition. In addition,

αKGlogue G promoted a decrease of HIF-1α stability in both HK-2 and UOK-257 cells after 90 minutes, but only in UOK-257 cells after 120 minutes (Figure 26A, 26B, 26C, 26D). Thus, based on this screening we selected αKGlogues E, F and G for their ability to reduce HIF-1α levels under hypoxic condition in renal cancer cells.

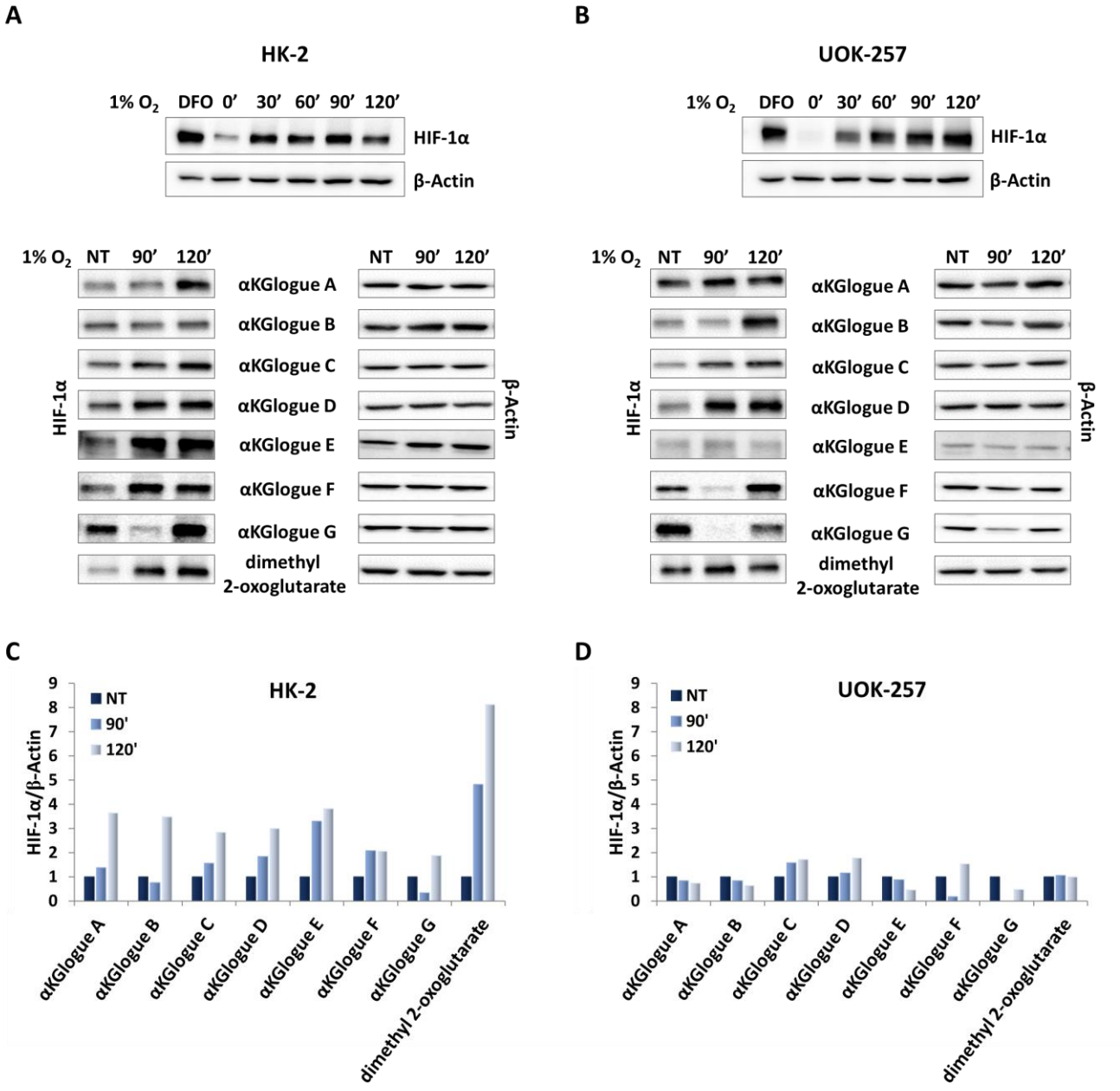


Figure 26: αKGlogue E, F and G are able to destabilize HIF-1α under hypoxic condition in renal cancer cells. **(A, B)** Western blot analysis of HIF-1α levels after treatment with αKGlogues for 90 and 120 minutes in HK-2 and UOK-257 cells under hypoxic condition (1% O_2). β-Actin was used as loading control. **(C, D)** Densitometric evaluation of HIF-1α. Data were normalized on the corresponding not-treated cells for each time points and on β-Actin. DFO was used as positive control for HIF-1α stabilization.

This first phase of the screening had as its main objective to select αKGlogues able to reduce HIF-1α stability under hypoxic condition in each cell models after short period of intense hypoxia. Once the best compounds were identified, we attempted to evaluate how long was the effect on HIF-1α levels. Therefore, we treated thyroid and renal cells for 6, 12 and 24 hours with the previously selected αKGlogues. The treatment with αKGlogues for 24 hours was performed without changing the culture medium or replacing it after 12 hours with fresh medium supplemented with the corresponding αKGlogue. For thyroid cells we selected αKGlogue B, D and E that were able to reduce HIF-1α protein levels in TPC-1 cells in hypoxia, although did not exert any effects in NTHY 3.1. Further evaluations of αKGlogue B ability to destabilize HIF-1α protein levels after more long-term exposure in hypoxia are still ongoing. When NTHY 3.1 cells were exposed to prolonged hypoxia, both αKGlogues D and E slightly reduced HIF-1α stabilization compared to corresponding untreated cells after 6 hours. Conversely, the treatment for 12 and 24 hours did not alter HIF-1α levels (Figure 27A, 27B). The same experiment was performed in TPC-1 cells showing a striking reduction of HIF-1α levels when cells were exposed to αKGlogue D for at least 12 hours (Figure 28A, 28B). In particular, an approximately 3-fold decrease of HIF-1α was observed at incubation times 12 hours and 12+12 hours. Hence, the maximum effect to destabilize HIF-1α under hypoxic condition in TPC-1 cells was obtained after 12 hours of treatment with αKGlogue D, indicating that the timing of drug administration is consistent with a hypothetical therapeutic approach *in vivo*.
Instead, αKGlogue E was able to reduce HIF-1α protein levels only after 24 hours, but refreshing media after 12 hours of treatment (Figure 28A, 28B). Given this prolonged effect in the time, the use of both αKGlogues is promising for a hypothetical pharmacological administration.

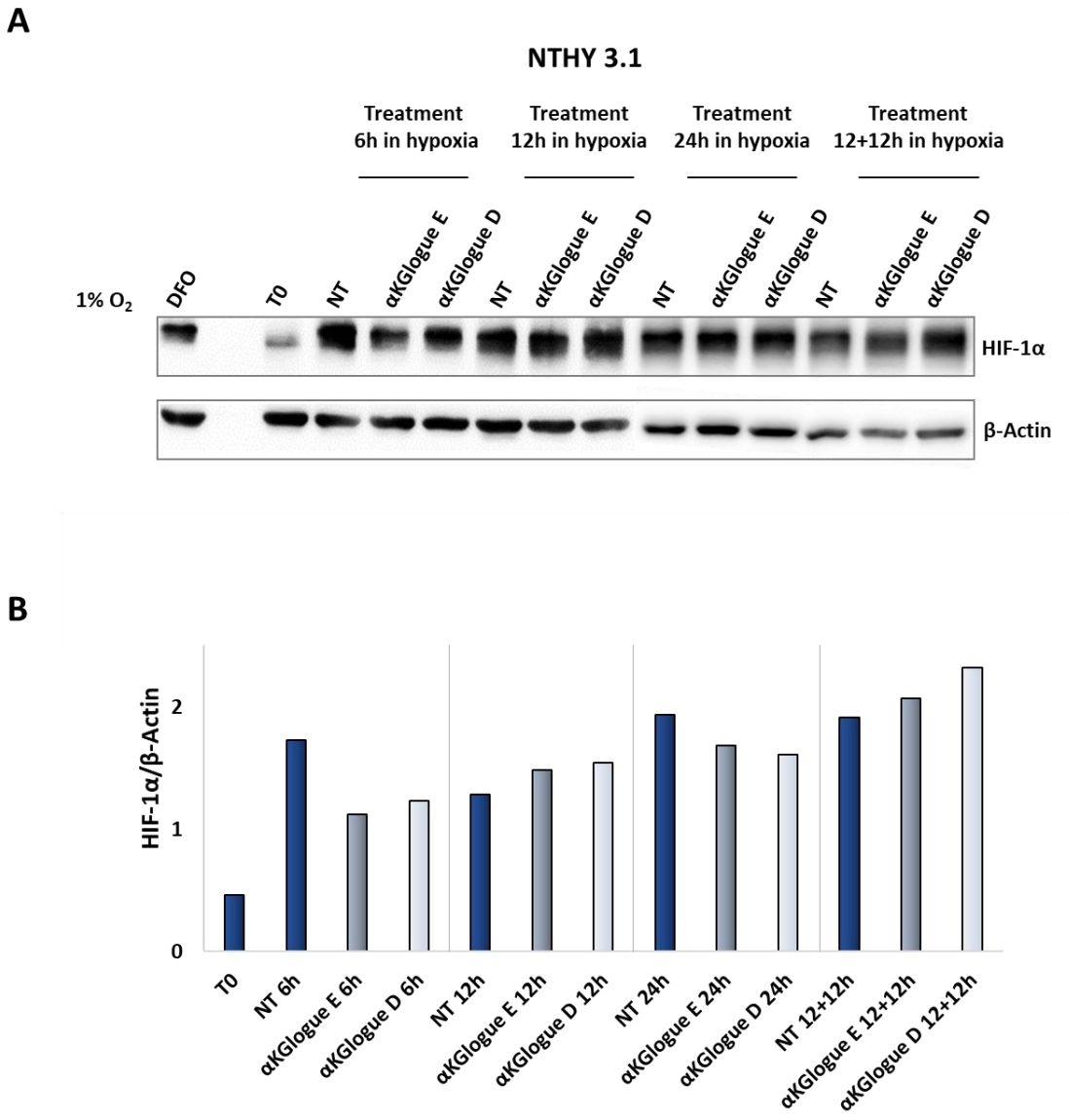


Figure 27: Treatment with αKGlogue D and E slightly reduces HIF-1α protein levels after 6 hours in NTHY 3.1 cells in hypoxia. (A) Western blot analysis of HIF-1α levels after treatment with αKGlogues D and E (200µM) for 6, 12, 24 hours in NTHY 3.1 cells under hypoxic condition (1% O_2). 12+12h seems that cells were treated for 24 hours but refreshing media after 12 hours with new corresponding αKGlogue. β-Actin was used as loading control. **(B)** Densitometric evaluation of HIF-1α. Data were normalized on β-Actin. DFO was used as positive control for HIF-1α stabilization.

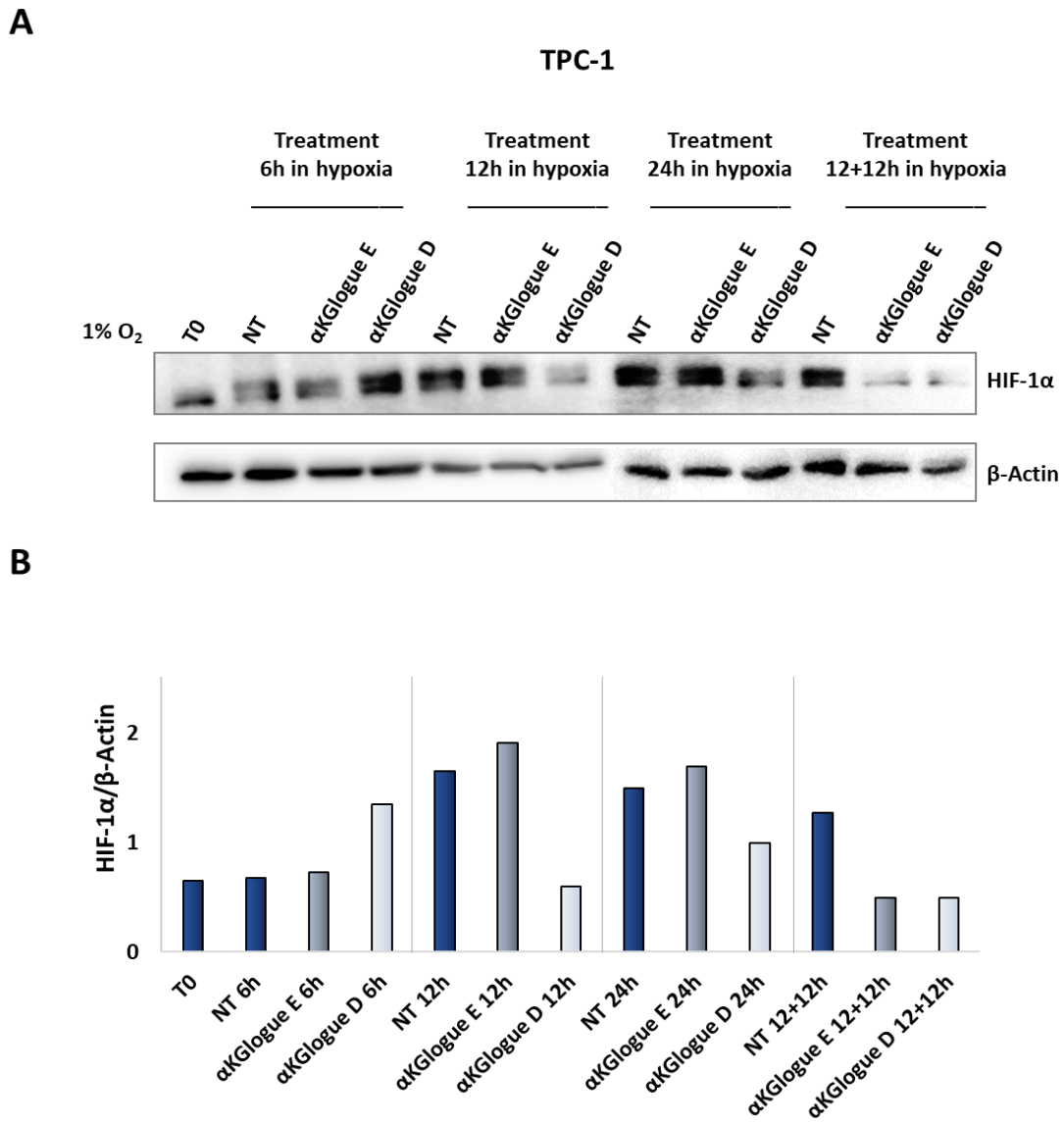


Figure 28: αKGlogue D and E show a more-long term effect to destabilize HIF-1α in TPC-1 cells. (A) Western blot analysis of HIF-1α levels after treatment with αKGlogues D and E (200μM) for 6, 12, 24 hours in TPC-1 cells under hypoxic condition (1% O_2). 12+12h seems that cells were treated for 24 hours but refreshing media after 12 hours with new corresponding αKGlogue. β-Actin was used as loading control. **(B)** Densitometric evaluation of HIF-1α. Data were normalized on β-Actin.

Then we performed this experiment in renal cell models, incubating cancer and non-cancer cells for 6, 12 and 24 hours with αKGlogue E, F and G, which were that compounds able to destabilize HIF-1α in UOK-257 cells after 90 and 120 minutes under hypoxic conditions. As already observed, after 2 hours of treatment these three αKGlogues did not reduce HIF-1α levels in HK-2 cells neither after more long-term exposure (Figure 29A, 29B). It was observed a slight reduction of HIF-1α protein after treatment with αKGlogue G for 6 hours in hypoxia (Figure 29A, 29B).

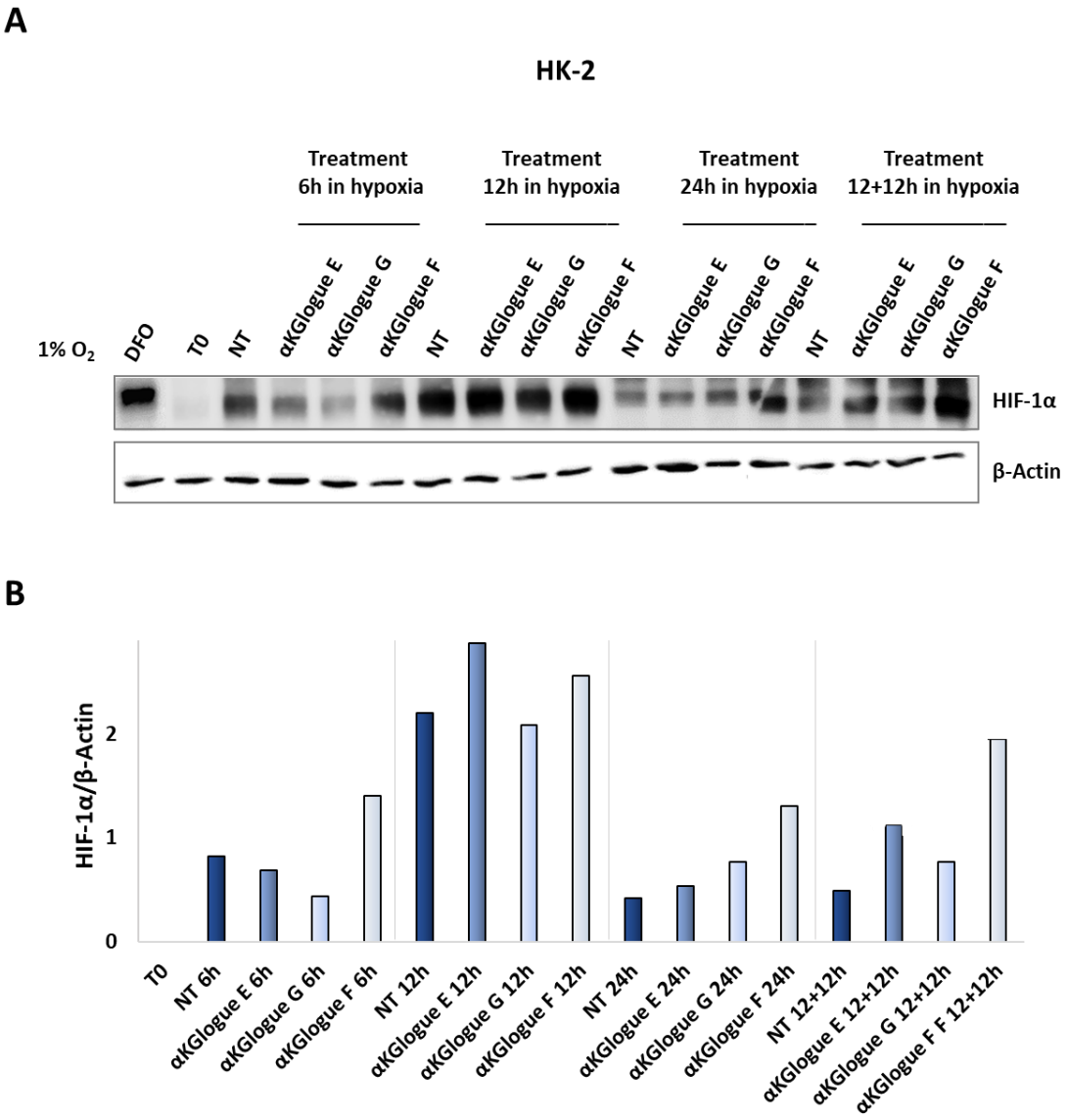


Figure 29: No alterations of HIF-1α levels are observed in HK-2 cells after longer treatments with αKGlogue E, F and G. (A) Western blot analysis of HIF-1α levels after treatment with αKGlogues E, F and G (200µM) for 6, 12, 24 hours in HK-2 cells under hypoxic condition (1% O_2). 12+12h seems that cells were treated for 24 hours but refreshing media after 12 hours with new corresponding αKGlogue. β-Actin was used as loading control. **(B, C)** Densitometric evaluation of HIF-1α. Data were normalized on β-Actin. DFO was used as positive control for HIF-1α stabilization.

Conversely, αKGlogues E, F and G reduced HIF-1α protein levels after 6 and 12 hours of treatment under hypoxic condition in UOK-257 cells (Figure 30A, 30B), indicating that the effect on HIF-1α of these compounds was not limited to very short incubation times but can persist up to 12 hours.

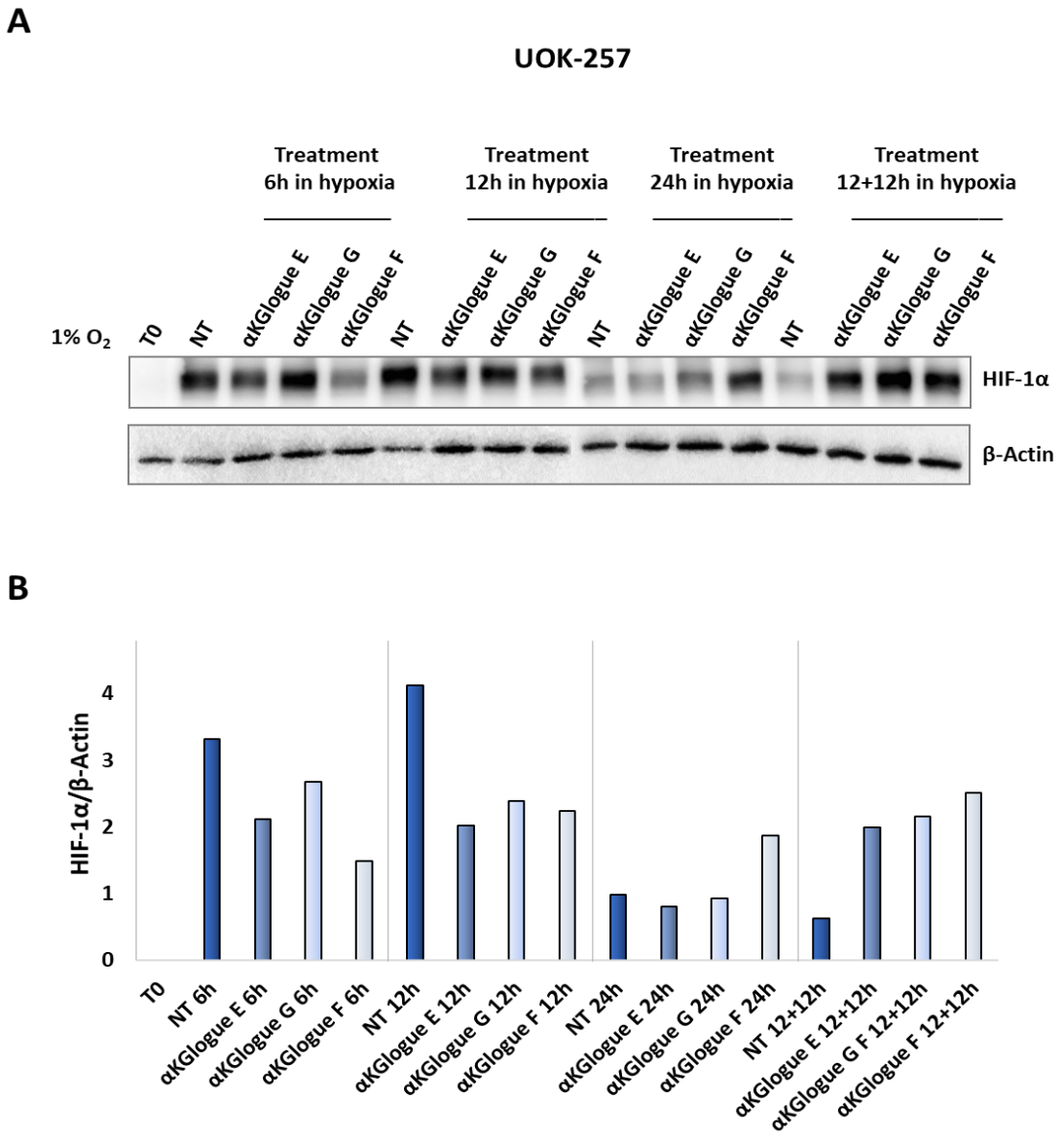


Figure 30: αKGlogues E, F and G reduce HIF-1α protein levels under hypoxic condition after 6 and 12 hours in UOK-257 cells. (A) Western blot analysis of HIF-1α levels after treatment with αKGlogues E, F and G (200µM) for 6, 12, 24 hours in UOK-257 cells under hypoxic condition (1% O_2). 12+12h seems that cells were treated for 24 hours but refreshing media after 12 hours with new corresponding αKGlogue. β-Actin was used as loading control. **(B)** Densitometric evaluation of HIF-1α. Data were normalized on β-Actin.

It should be noted that in both renal cells, HIF-1α stability was reduced after 24 hours under hypoxic condition compared to 6 and 12 hours, independently from αKGlogues treatments. There are evidences in literature supporting a temporal regulation of the HIF-1α and HIF-2α-dependent responses. Under conditions of acute hypoxia (i.e. <24 hours stimulation), transactivation of target genes occurs primarily through the activation of HIF-1α, whereas following longer periods HIF-2α begins to exert more of an influence [159, 160]. It is possible that in renal cells HIF-1α activity reaches its highest levels up to 12 hours to mediate acute responses to hypoxia regulating the activation of its target genes. This may also explain the reason why αKGlogues E, F and G were able to destabilize HIF-1α protein levels only after 6 and 12 hours of treatment supporting the idea that increased αKG intracellular levels induce a psudonormoxic state in HIF-1α dependent tumors.
In conclusion, all αKGlogues previously selected in both thyroid and renal cancer cells for their capacity to reduce HIF-1α levels after short time of incubation (90 and 120 minutes) had shown to maintain their effectiveness for longer periods under hypoxia exposure, thus being eligible to be considered as potential therapeutic agents.

αKGlogues reduce the tumorigenic potential of thyroid and renal cancer cells *in vitro*.

Once established that αKGlogues were able to exert a long-term effect on HIF-1α under hypoxic condition, the next step was to analyze whether the same compounds may display an anti-tumorigenic potential on thyroid and renal cancer cell *in vitro*. To this purpose, we performed a colony formation assay to test whether treatment with different concentrations of selected αKGlogues can reduce the clonogenic properties of tumor cells under hypoxic condition. TPC-1 cells were incubated with three different concentrations (2μM, 20μM and 200μM) of αKGlogue E and D for one week. Despite the long exposure time, treatment with 2μM and 20μM of compounds did not affect the ability of TPC-1 to form colonies. Conversely, the TPC-1 clonogenic capacity was completely abolished treating cells with 200μM of αKGlogue E and D (Figure 31A, 31B, 31C).

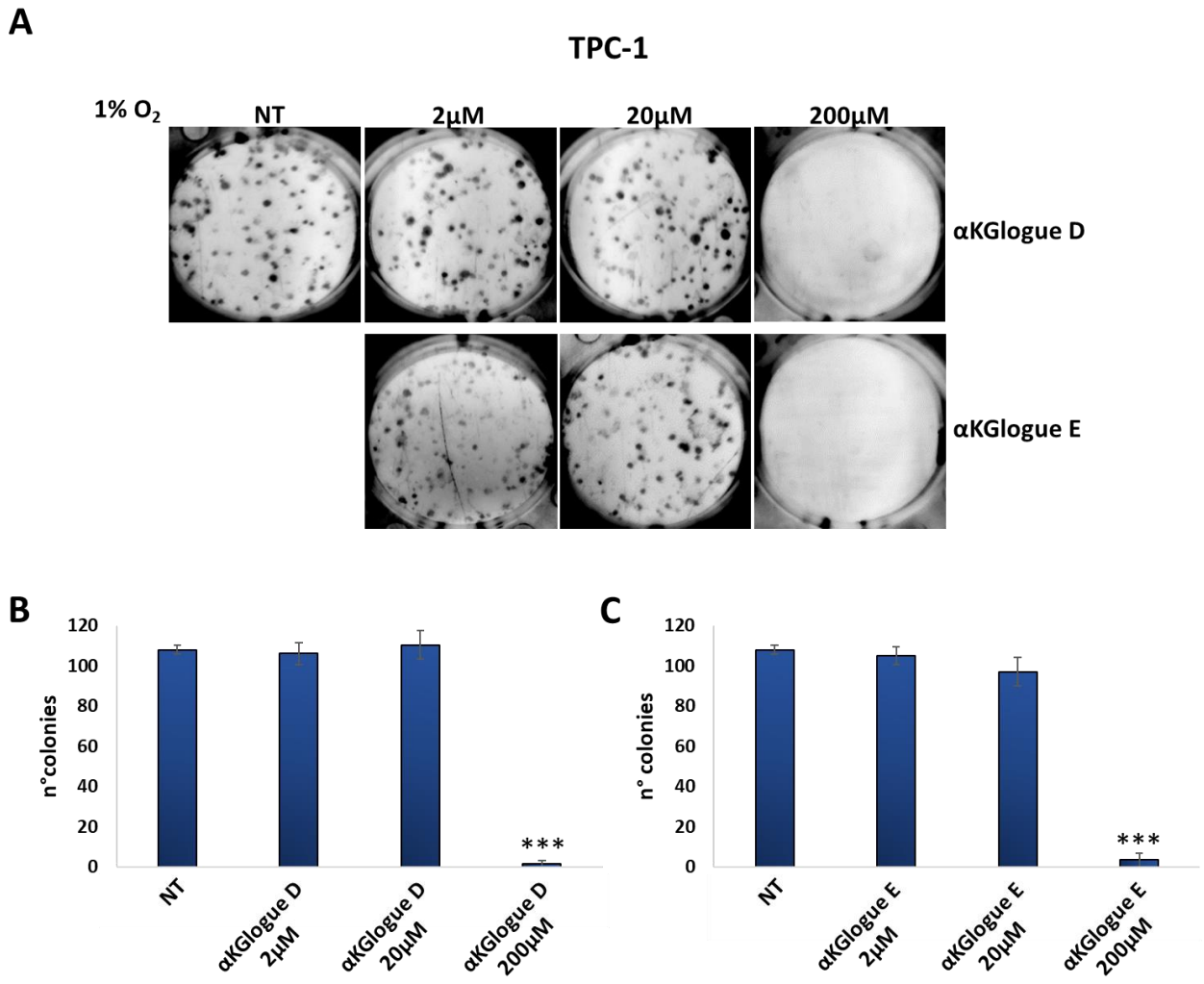


Figure 31: αKGlogues D and E abolish clonogenic survival of TPC-1 cells. (A) TPC-1 cells were treated for one week with 2µM, 20µM and 200µM of αKGlogues D and E under hypoxic condition (1% O_2). **(B, C)** Number of colonies were counted using ImageJ software. Data are media ± SEM, n=3. Asterisks represented *** p-value ≤ 0.001 relative to non-treated cells using Student's T-test.

Then we performed a viability assay treating TPC-1 and NTHY 3.1 cells with 2µM, 20µM and 200µM of αKGlogue D and E for multiple time points in normoxic and hypoxic conditions. As observed in colony formation assay, treatment with 2µM and 20µM of both αKGlogues did not alter neither NTHY 3.1 nor TPC-1 cell viability in normoxia, while a significant reduction of cell growth was observed treating cells with 200µM of compounds (Figures 32 and 33, panels A and B). Similar results were obtained when O_2 availability is limited (Figures 32 and 33, panels C and D).

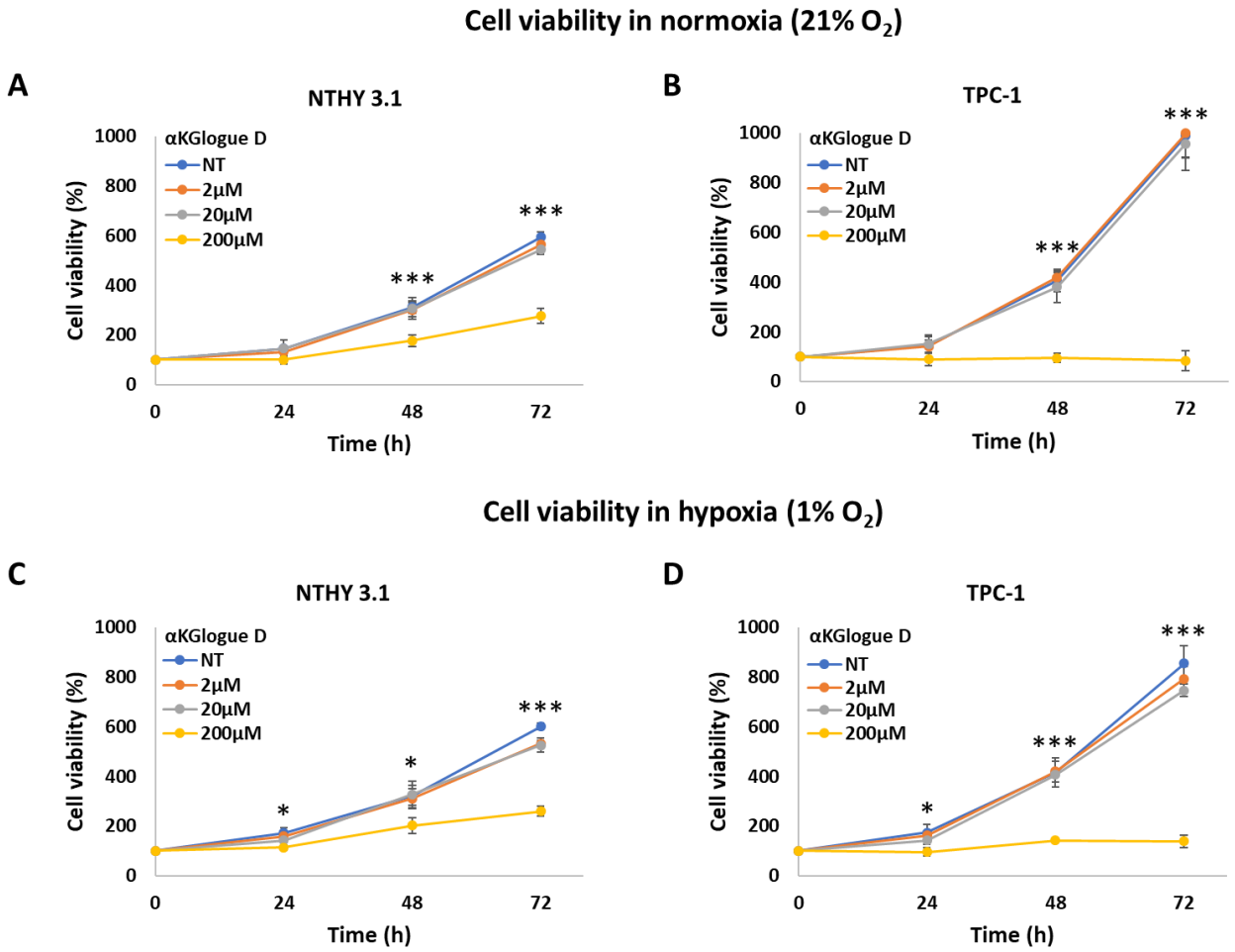


Figure 32: αKGlogue D induces the arrest of TPC-1 cell growth, without having a cytotoxic effect in NTHY 3.1 cells, independently from O_2 levels. (A, B) NTHY 3.1 and TPC-1 cells were grown in increasing dose of αKGlogue D (2µM, 20µM and 200µM) for 24, 48 and 72 hours in normoxic condition (21% O_2). **(C, D)** NTHY 3.1 and TPC-1 cells were grown in increasing dose of αKGlogue D (2µM, 20µM and 200µM) for 24, 48 and 72 hours in hypoxic condition (1% O_2). Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

In particular, a reduction of NTHY 3.1 cell proliferation was induced using 200µM of αKGlogue D under normoxic and hypoxic condition (Figure 32A, 32C, 34A, 34C). Instead, the same treatment promoted the arrest of TPC-1 cell growth, independently from intracellular O_2 levels (Figure 32B, 32D, 34B, 34D). Therefore, the efficacy of αKGlogue D to reduce cell proliferation was greater in thyroid cancer cells rather than in normal cells encouraging future investigations regarding its role to reduce the tumorigenic potential *in vitro* through the inhibition of HIF-1α stabilization and activity.

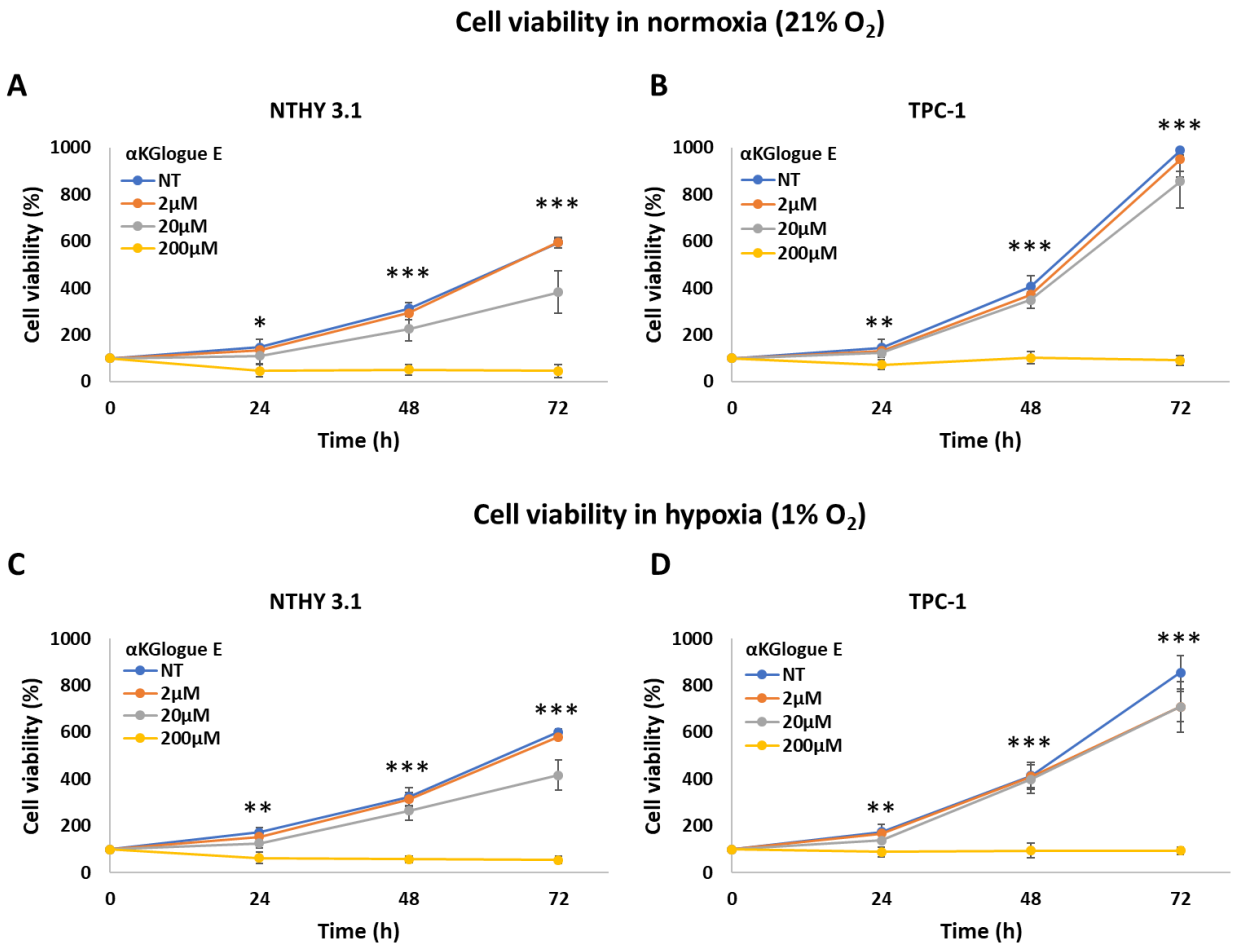


Figure 33: αKGlogue E induces a non-cancer specific cytotoxic effect at higher concentration in thyroid cells. (A, B) NTHY 3.1 and TPC-1 cells were grown in increasing dose of αKGlogue E (2µM, 20µM and 200µM) for 24, 48 and 72 hours in normoxic condition (21% O_2). **(C, D)** NTHY 3.1 and TPC-1 cells were grown in increasing dose of αKGlogue E (2µM, 20µM and 200µM) for 24, 48 and 72 hours in hypoxic condition (1% O_2). Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

The αKGlogue E promoted NTHY 3.1 cell death using 200µM for 24, 48 and 72 hours both in normoxia and hypoxia (Figure 33A, 33C, 34A, 34C). Conversely, a cytostatic effect was observed after treatment of TPC-1 cells after 24, 48 and 72 hours (Figure 33B, 33D, 34B, 34D). However, the cytotoxicity shown in thyroid normal cells lead us to exclude αKGlogue E from subsequent analyses in term of pre-clinical applications.

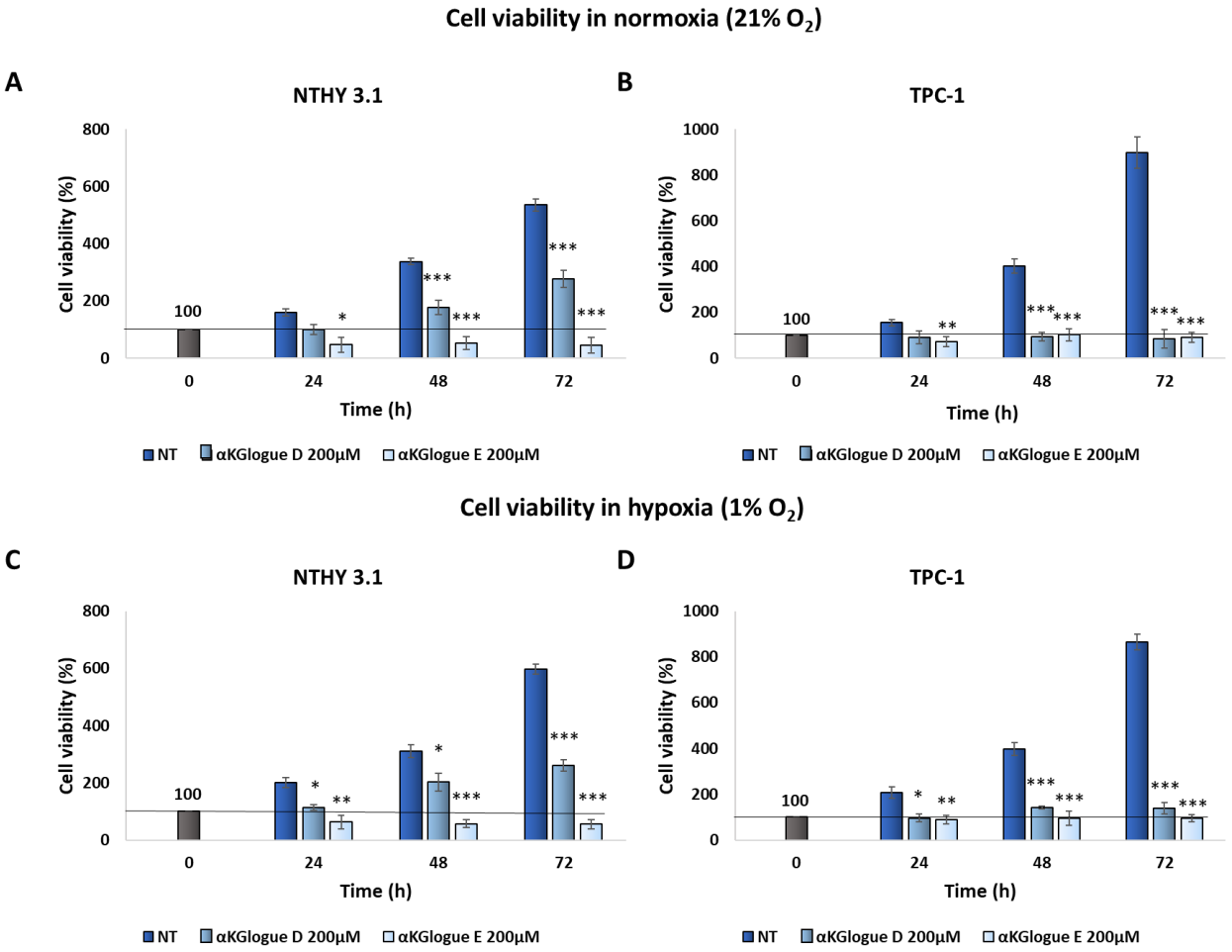


Figure 34: αKGlogues D and E arrest TPC-1 cell proliferation. (A, B) Comparison of the effect of αKGlogue D and E to alter NTHY 3.1 and TPC.1 cell growth under normoxic condition (21% O_2). Cell were treated with 200µM of these αKGlogues for 24, 48 and 72 hours. **(C, D)** Comparison of the effect of αKGlogue D and E to alter NTHY 3.1 and TPC.1 cell growth under hypoxic condition (1% O_2). Cells were treated with 200µM of these αKGlogues for 24, 48 and 72 hours. Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

The same set of experiments was performed in renal cell models using αKGlogues previously selected to reduce HIF-1α protein levels in hypoxic condition in UOK-257 cells (αKGlogues E, F and G) at different concentrations (2µM, 20µM and 200µM). The ability of UOK-257 cells to form colonies under hypoxic condition was not altered by the treatment with 2µM and 20µM of concentrations. On the contrary, the clonogenic capacity was completely abolished treating UOK-257 cells with 200µM of αKGlogues E and G, whereas treatment with αKGlogue F was able to induce a significant reduction of colony numbers at same concentration (Figure 35A, 35B, 35C, 35D). αKGlogues E and G showed a greater ability to reduce the tumorigenic potential of UOK-257 cells in hypoxia compared to αKGlogue F. Therefore, we decided to investigate the effect only of the first two αKGlogues on viability assay in renal cancer and non-cancer cells.

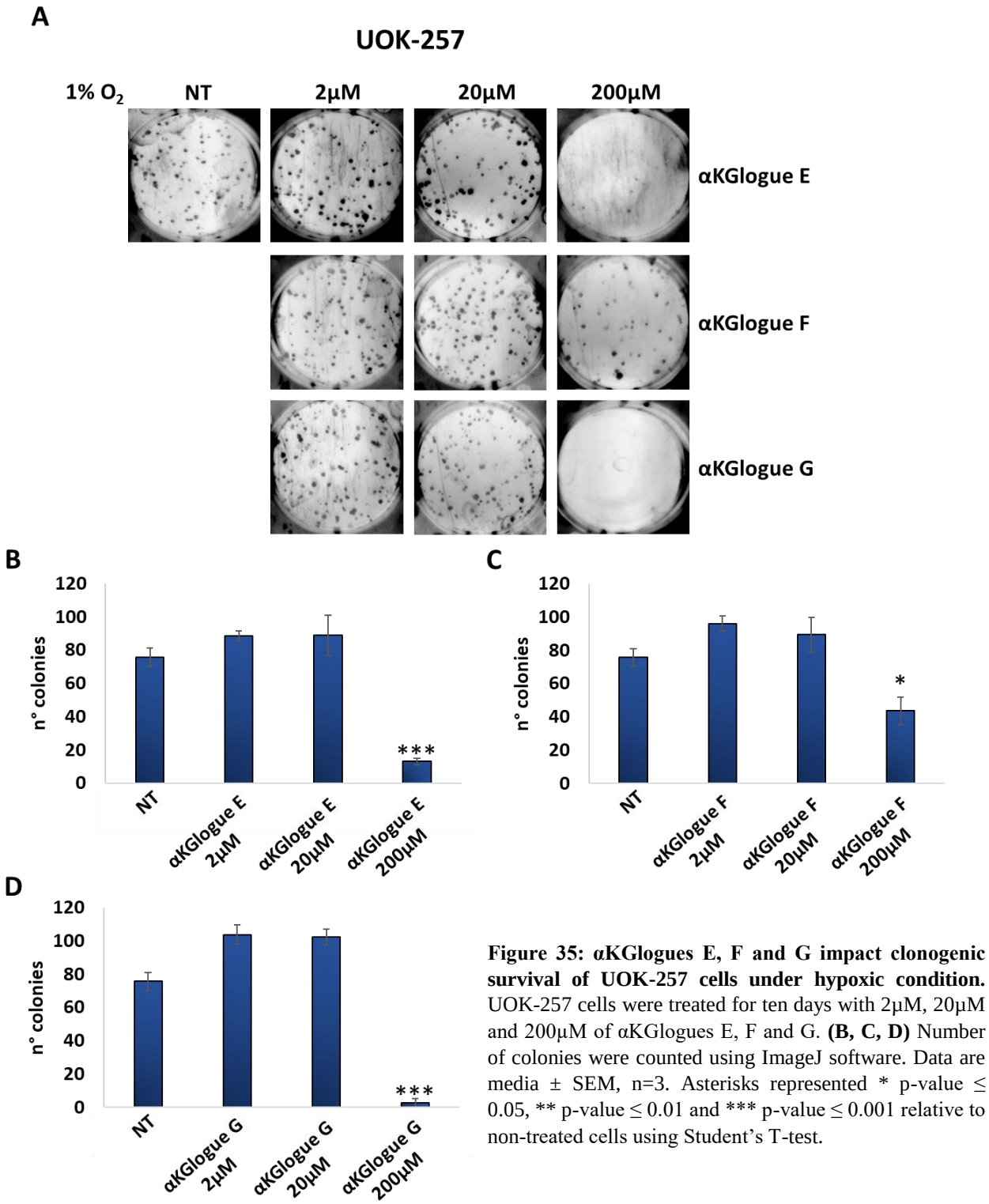


Figure 35: αKGlogues E, F and G impact clonogenic survival of UOK-257 cells under hypoxic condition. UOK-257 cells were treated for ten days with 2µM, 20µM and 200µM of αKGlogues E, F and G. **(B, C, D)** Number of colonies were counted using ImageJ software. Data are media ± SEM, n=3. Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to non-treated cells using Student's T-test.

αKGlogues E and G reduced significantly HK-2 and UOK-257 cell proliferation using 200µM of concentration, while no alteration of cell growth was observed treating the same cells with 2µM and 20µM confirming the anti-proliferative effect seen in colony assay experiments at high concentration (Figure 36 and 37). HK-2 cell viability was significantly reduced after 24, 48 and 72

hours under physiological O_2 levels (21% O_2) and after 72 hours of treatment with 200µM of αKGlogue E under hypoxic condition (Figure 36A, 36C). The incubation with the same compound and amount induced a cytostatic effect on UOK-257 cell proliferation in both normoxia and hypoxia (Figure 36B, 36D). The αKGlogue G showed the same effect of αKGlogue E to decrease HK-2 cell proliferation in different conditions of intracellular O_2 tension (Figure 37A, 37C). Conversely, a cytotoxic effect was observed already after 24 hours treating UOK-257 cells with 200µM of αKGlogue E in normoxia, while a cytostatic effect after 24 and 48 hours followed by cytotoxic effect after 72 hours was observed at low O_2 levels (1% O_2) (Figure 37B, 37D).

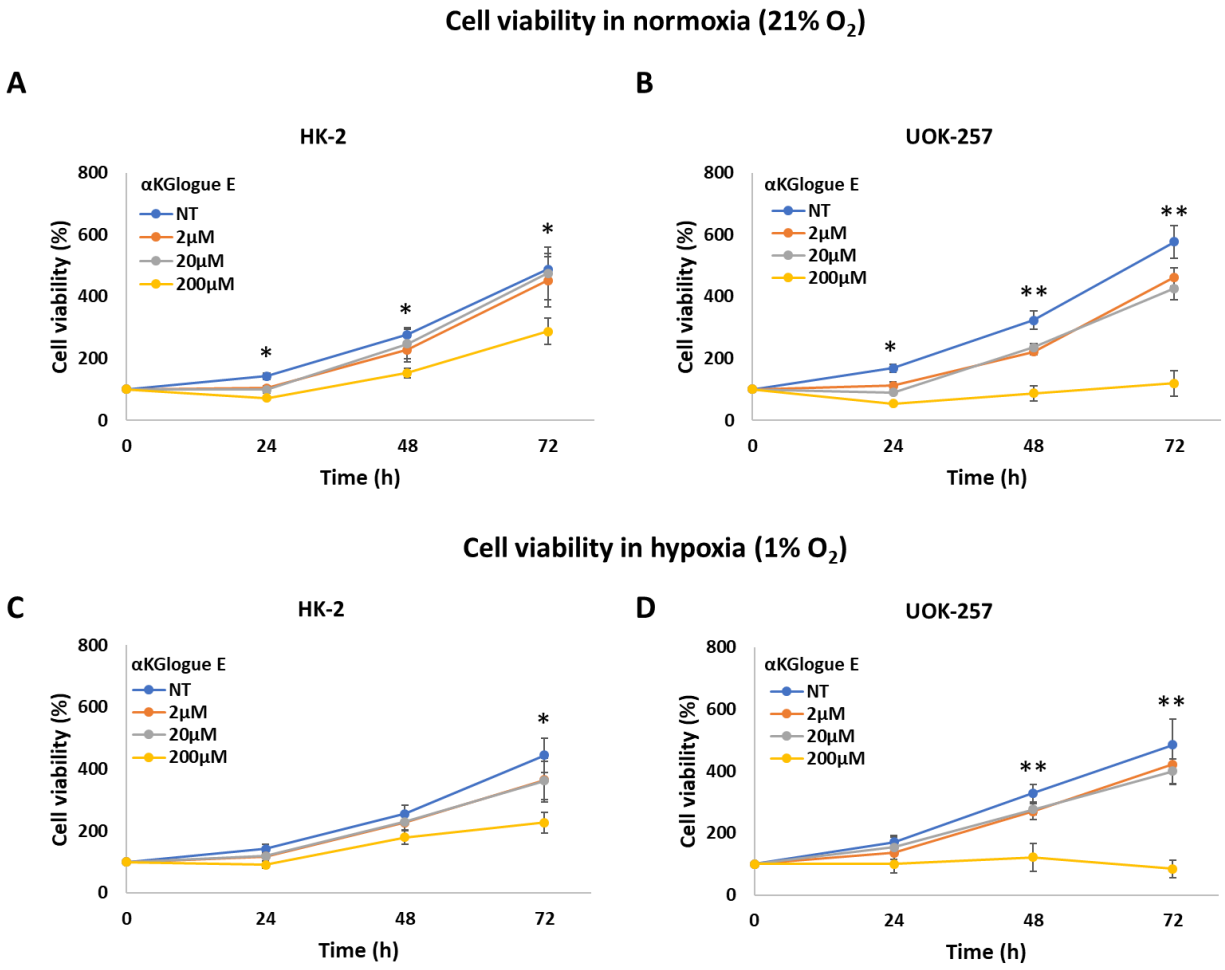


Figure 36: αKGlogue E significantly reduces UOK-257 cell growth, without having a cytotoxic effect on HK-2 cells. (A, B) HK-2 and UOK-257 cells were grown in increasing dose of αKGlogue E (2µM, 20µM and 200µM) for 24, 48 and 72 hours in normoxic condition (21% O_2). **(C, D)** HK-2 and UOK-257 cells were grown in increasing dose of αKGlogue E (2µM, 20µM and 200µM) for 24, 48 and 72 hours in hypoxic condition (1% O_2). Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

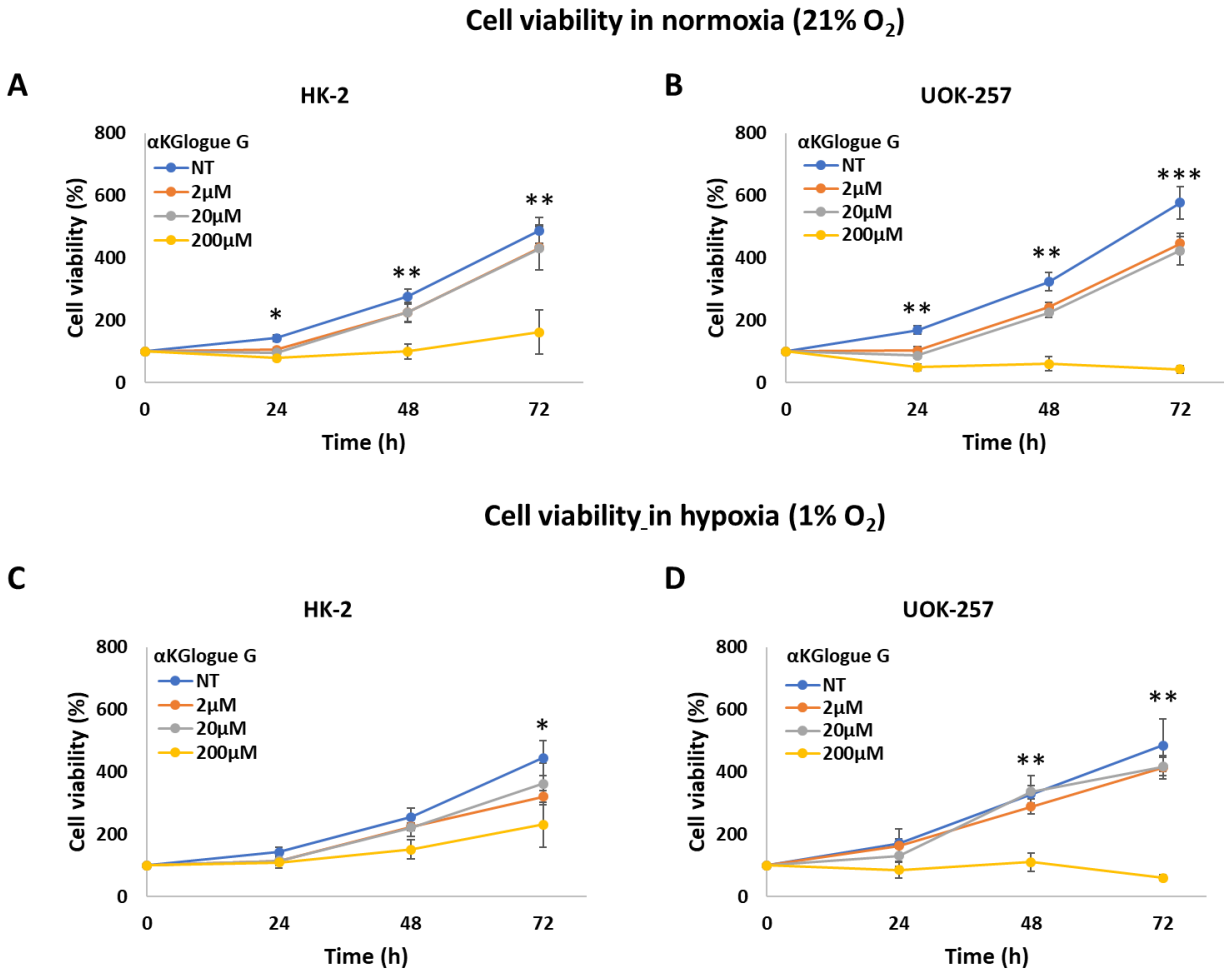


Figure 37: αKGlogue G treatment promotes UOK-257 cell death. (A, B) HK-2 and UOK-257 cells were grown in increasing dose of αKGlogue G (2µM, 20µM and 200µM) for 24, 48 and 72 hours in normoxic condition (21% O_2). **(C, D)** HK-2 and UOK-257 cells were grown in increasing dose of αKGlogue G (2µM, 20µM and 200µM) for 24, 48 and 72 hours in hypoxic condition (1% O_2). Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

Therefore, both αKGlogues were able to induce a growth slowdown in HK-2 cells compared to untreated cells under normoxia and hypoxia, though without showing an arrest of cell proliferation (Figure 38A, 38C). In renal cancer cells, the cell viability was reduced ~7-fold compared to not-treated cells after incubation with αKGlogues E and G for 72 hours in hypoxia inducing an arrest of UOK-257 cell growth (Figure 36D, 37D, 38D). Under normoxic condition, αKGlogues E promoted the same effect shown in hypoxia; conversely, αKGlogues G induced cell death already after 24 hours of incubation. In fact, in this latter case, the number of cells still proliferating went down the 100%, considered as the amount of cancer renal cells seeded before the incubation with αKGlogue G (Figure 38B). In conclusion, αKGlogues E and G had demonstrated to be both good candidates to

be used as therapeutic approach to decrease the renal cancer cells tumorigenic potential, without inducing cell death in normal cells.

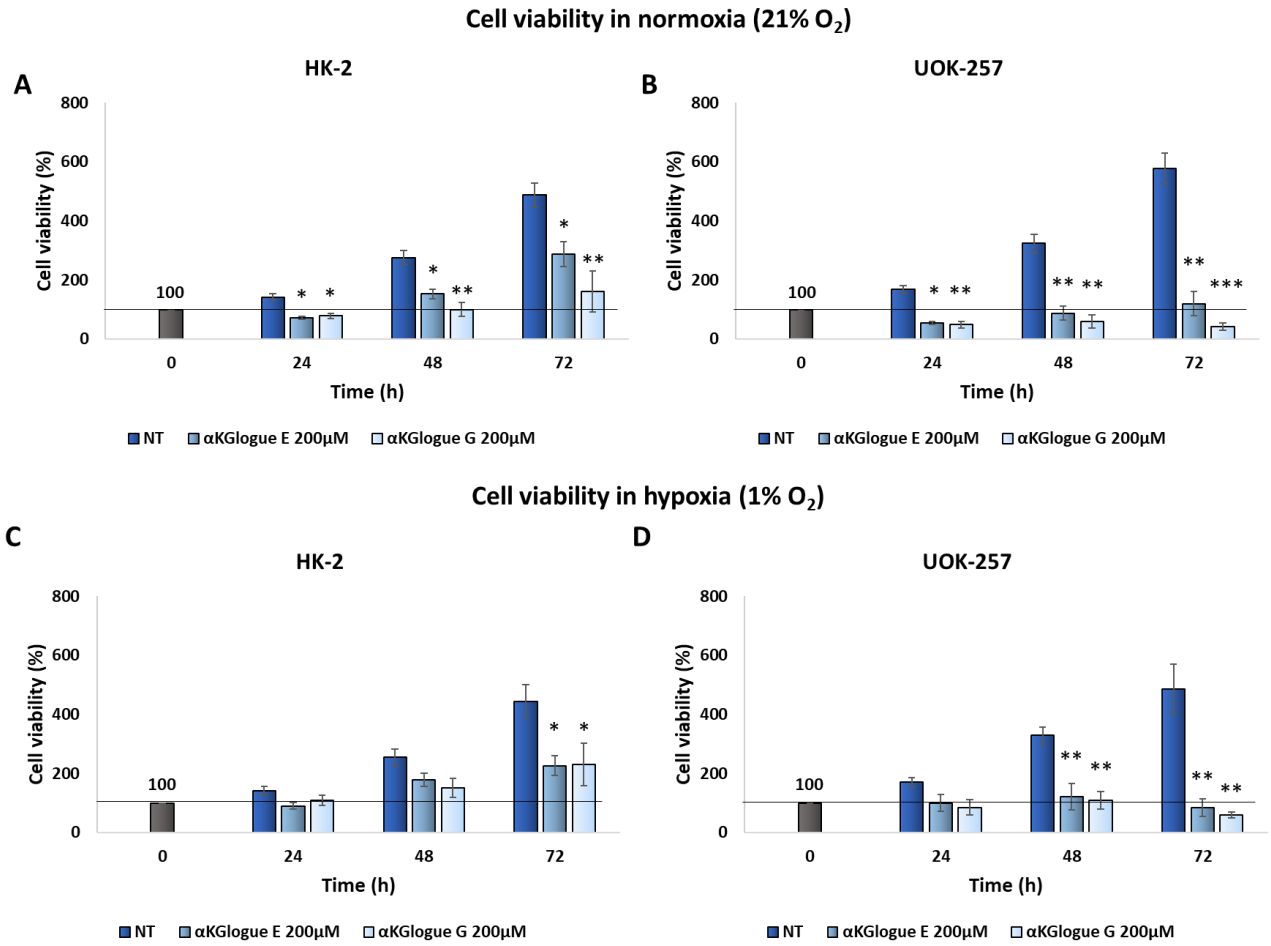


Figure 38: αKGlogues E and G reduce UOK-257 tumorigenic potential *in vitro*. (A, B) Comparison of the effect of αKGlogue E and G to alter HK-2 and UOK-257 cell growth under normoxic condition (21% O_2). Cell were treated with 200µM of these αKGlogues for 24, 48 and 72 hours. **(C, D)** Comparison of the effect of αKGlogue E and G to alter HK-2 and UOK-257 cell growth under hypoxic condition (1% O_2). Cells were treated with 200µM of these αKGlogues for 24, 48 and 72 hours. Cell viability was determined using SRB assay. Data (mean ± SEM) are expressed in percentage considering as 100% the cell viability at T0 (n=3). Asterisks represented * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 relative to not-treated cells using Student's T-test.

Overall, the screening performed in this study led to select αKGlogue D in thyroid cells and αKGlogues E and G in renal cells (Table 4). Further investigations will be required to identify the molecular mechanism through which these ester derivatives of αKG exerted their anti-tumorigenic potential by promoting a pseudonormoxic condition in HIF-1-dependent cancer cells.

Cell model	αKGlogue selected	Effect observed
Thyroid cells	αKGlogue D	- Not cytotoxic for N-THY 3.1 - Inhibition of TPC-1 cell proliferation and clonogenic capacity *in vitro* - Decreased HIF-1α stability after 12h in hypoxic condition in TPC-1 cells
Renal cells	αKGlogues E and G	- Not cytotoxic for HK-2 - Inhibition of UOK-257 cell proliferation and clonogenic capacity *in vitro* - Decreased HIF-1α stability after 6-12h in hypoxic condition

Table 4: αKGlogues selected for their ability to reduce HIF-1α protein levels under hypoxic condition and the tumorigenic potential of thyroid and renal cancer cells *in vitro*.

DISCUSSION

The hypoxic tumor microenvironment is subjected to HIF-driven transcriptional response that induces the activation of genes that are involved in crucial aspects of cancer biology, including angiogenesis, cell survival, glucose metabolism and invasion. This leads cancer cells to acquire properties that allow them to overcome the lack of energy and nutrients supply within hypoxic niches. In this scenario, the transcriptional factor HIF-1α is the main molecular player involved in the early stage of hypoxic response orchestrating these metabolic and genetic changes in cancer cells which are correlated with poor prognosis in oncological patients. Thus, the idea to target HIF-1α is an attractive therapeutic strategy to downregulate tumor aggressiveness interfering with cancer cells metabolism and tumor growth.

HIF-1α stability and activity are finely regulated by intracellular O_2 levels, but, at the same time, several studies revealed the existence of alternative cellular players and molecular pathways able to affect HIF-1α stability by modulating PHDs activity independently from O_2 levels [34]. In line with this, tumors harboring SDH, FH and IDH1/2 mutations are able to stabilize HIF-1α in normoxia as consequence of SA, fumarate and 2-HG accumulation that play as allosterically inhibitors of PHDs enzymatic reaction [48, 49, 50]. Furthermore, 2-HG is also a competitive inhibitor of TETs and KDMs, others αKG-dependent dioxygenase as well as PHDs, and high levels of this oncometabolite may alter the epigenetic landscape of cancer cells [53, 161, 162]. Similarly, recent studies have revealed that together with 2-HG, SA and fumarate can also induce alterations in DNA and histones methylation, thus enhancing cancer formation [53, 163, 58, 60]. Conversely, increased cytosolic concentrations of αKG have a role to reverse the methylation status of both histones and DNA [164]. Moreover, as co-substrate of PHDs, high αKG intracellular levels enhance the PHDs affinity for O_2 and feed their hydroxylation reaction preventing HIF-1α stabilization also in hypoxia [165, 51, 52]. In fact, the estimated K_m of PHDs for αKG is $\sim 50\ \mu M$ indicating that these enzymes are greatly sensitive to αKG oscillations [35, 36]. Accordingly, our group has demonstrated that the imbalance of αKG/SA ratio, towards αKG, was related to the NADH accumulation, due to the inhibition of αKG dehydrogenase, in CI-deficient cells. This alteration of TCA cycle metabolites levels, as consequence of severe mutations that impair respiratory CI activity and assembly, hampered tumor growth promoting the chronic activation of PHD, even al low O_2 levels, and consequent HIF-1α destabilization. These metabolic changes led to a condition that was termed *pseudonormoxia* [38, 39, 40]. Furthermore, these data indicated that respiratory CI is essential for the hypoxic adaptation of cancer cells and may be considered as an interesting target for development of new anti-cancer

therapies. Indeed, in the last few years several mitochondrial CI inhibitors have been shown to share the ability to destabilize HIF-1α and prevent hypoxic adaptation in cancer cells [93].

Supported by these findings, the main aim of this project was to identify novel and safe adjuvant therapeutic agents for aggressive cancers for which adaptation to hypoxia is a necessary step to progress toward malignancy. To this aim, we attended to prove that HIF-1α destabilization and *pseudonormoxia* can be pharmacologically induced by increasing αKG levels indirectly using CI inhibitors or directly through cell permeable αKG ester derivatives.

To identify novel molecules that specifically impair mitochondrial CI activity and destabilize HIF-1α under hypoxic condition, we took advantage by the collaboration with Khondrion, a Dutch company located in Nijmegen primarily focused on the development of innovative therapies for inherited mitochondrial diseases. We screened a panel of six KH-compounds to identify novel molecules able to hamper tumor growth through the specific inhibition of respiratory CI activity. In this regard, KH412, KH474 and KH0-B were firstly selected for their ability to reduce the tumorigenic potential of thyroid cancer cells *in vitro*, and for their safety toward non-cancer cells consistent with a hypothetical therapeutic approach. However, although these molecules reduced the protein levels of several CI subunits (NDUFA9, NDUFS3 and NDUFB8) in thyroid cancer cells, none of them interfered with the physiologic activity of mitochondrial CI. Furthermore, treatment with KH412, KH474 and KH0B induced also a lower protein expression of VDAC and a reduction of SDHA protein levels was observed after treatment with KH474 in isolated mitochondria from thyroid cancer cells. Therefore, it is plausible to hypothesize that the anti-tumorigenic activity of these three KH-compounds may affect mitochondrial biogenesis, respiratory complexes stability or stimulate mitophagy, without exerting a specific effect on CI activity. Despite Warburg effect, cancer cells also need mitochondrial respiration to provide the building blocks for macromolecule (nucleotides, lipids and amino acids) biosynthesis as well as ATP and NADPH, which are essential to acquire a proliferative advantage [166]. In fact, it was demonstrated that enhanced mitochondrial biogenesis is often correlated with cancer cells capacity to grow anchorage independently, invasiveness and metastasis migration [167, 168]. In this regard, the development of new molecules that show the ability to inhibit mitochondrial biogenesis in cancer cells could represent a new therapeutic approach. On the other hand, the anti-tumorigenic potential of KH412, KH474 and KH0B may be also due to their ability to stimulate mitophagy. Mitophagy can have the beneficial effect by eliminating old and/or damaged mitochondria, thus maintaining the integrity of the mitochondrial pool. However, mitophagy is not only limited to the turnover of dysfunctional mitochondria but also promotes reduction of overall mitochondrial mass in response to certain stresses, such as hypoxia and nutrient starvation [169]. This prevents generation of reactive

oxygen species and conserves valuable nutrients from being consumed inefficiently, thereby promoting cellular survival under conditions of energetic stress [169]. In this context, tumor cells are strictly dependent on functional mitophagy to preserve their malignant phenotype and dysregulation of this process may represent a therapeutic strategy to hinder tumor progression. However, further studies are required to better investigate the possible correlation between the anti-tumorigenic activity of KH412, KH474 and KH0B and the impact on mitochondrial biogenesis or altered mitophagy, that was out of the topic of this project.

Hence, we decided to focus our study on others potential CI inhibitors. A large panel of molecules has been studied as anticancer drugs for their capacity to pharmacologically inhibit CI function. It is demonstrated that rotenone, piericidin A and capsaicin induced cancer cells death under metabolic stress condition acting as specific CI inhibitors. Nevertheless, the use of these molecules remains confined into preclinical studies since they shown to stimulate ROS production thus being cytotoxic [125]. Recently, biguanides have been recognized as a new class of CI inhibitors, preventing ubiquinone reduction in a non-competitive manner [132]. In particular, several studies have revealed that metformin administration, already used as clinical approach for type 2 diabetes mellitus, was correlated with lower risk to develop cancer and, at the same time, with a better prognosis in oncologic patients [129, 130, 131]. However, the molecular mechanism trough which metformin might act as anti-cancer agent is still unclear and appears to be related to the combination of intracellular and systemic effects. It is reported that metformin decreased tumor growth by reducing circulating levels of insulin and insulin growth factor 1 (IGF-1), also known as mitogens for cancer cells [170], [171]. Others works ascribed anticancer proprieties of metformin to its ability to activate the cell energy sensor AMPK and consequent inhibition of the mammalian target of rapamycin complex 1 (mTORC1), which plays a pivotal role in metabolism, growth and proliferation [172, 173]. However, this last mechanism cannot exclusively explain the anti-tumorigenic effect of metformin since many cancers unable to activate AMPK are still susceptible to these drugs. Recently, a growing number of studies demonstrated that metformin may exert its anti-proliferative and cytostatic effect in cancer cells through the direct inhibition of mitochondrial CI activity, although the downstream effects remain unclear [132], [133], [134]. Therefore, we investigated the effect of this drug in CI-deficient cells compared to isogenic CI-competent counterparts and we observed that metformin mostly reduced osteosarcoma cell proliferation *in vitro* independently from the presence of functional respiratory CI. Indeed, the difference between CI-competent and CI-null cells in terms of reduced cell proliferation was only about 20%, suggesting that other mechanisms prevail in the cytostatic effect of metformin. Furthermore, it should be noted that metformin-dependent inhibition of CI decreased cell proliferation, but not increasing cell death. Our data were consistent with what observed by

Griss and co-workers regarding the dose-dependent cytostatic effects of metformin exerted on cancer cell proliferation, but without showing major alterations in cell viability [142]. Despite the importance of Warburg profile for cancer cells, it is now known that, to sustain and fuel tumorigenesis, oxidative metabolism can be re-established and a significant level of OXPHOS activity is maintained in cancer cells [174, 175]. The arrest off cell growth, rather than an increase of cell death, it was a consequence of mitochondrial CI inhibition and OXPHOS impairment after metformin administration which leading cancer cell to rely more or exclusively on glycolysis to sustain their proliferation [135, 133]. In fact, it was demonstrated that glycolysis inhibition in metformin-treated cells can lead to metabolic crisis enhancing its anti-proliferative effect [176]. In parallel, there are evidences regarding the cytostatic effects of metformin as consequence of a reduced glucose- and glutamine-dependent anaplerotic flux into the TCA cycle which in turn impacts pathways of mitochondrial-dependent biosynthesis including citrate-dependent *de novo* lipogenesis [142]. In this scenario, metformin could hamper tumor progression by decreasing the availability of several TCA intermediate metabolites essential for tumor cells proliferation. The cytostatic effect induced by metformin through CI inhibition is similar to the unconventional case of oncocytic tumors, a subset of epithelial neoplasms, in which the occurrence of high load of CI-destructive mtDNA mutations have been associated with more benign state of quiescence, typical of oncocytomas [177]. The severe OXPHOS deficiency forces oncocytic cells to adopt alternative metabolic pathways such as increased glycolysis to compensate for ATP production. However, the NAD^+/NADH unbalance impacts the shift from respiration to glycolysis in cancer [178]. Indeed, severe CI damage was shown to induce NADH accumulation, which is a known inhibitor of several TCA cycle enzymes, such as pyruvate dehydrogenase complex, citrate synthase, and αKG dehydrogenase complex. Consequently, oncocytic tumors have been related with accumulation of same TCA cycle metabolites, as αKG, promoting HIF-1α destabilization even during normoxia and preventing the Warburg effect and the progression to a malignant state [39]. In this frame, several studies have demonstrated that also metformin administration was related with the inhibition of HIF-1α expression in oral squamous cell carcinoma, colon cancer and hepatocellular carcinoma cells [145], [133], [179]. In contrast to other CI inhibitors, metformin did not stimulate H_2O_2 production preventing in this way HIF-1α stabilization and activity [133, 179].

Since we were interested in finding CI inhibitors that may have a clinical use in order to destabilize HIF-1α in hypoxic cancer cells, we decided to investigate the effect of metformin on HIF-1α protein levels in osteosarcoma CI-competent cell models. Although the specific CI inhibitor effect did not seem to be the main molecular mechanism by which metformin decreased the tumorigenic potential of cancer cells not CI-deficient *in vitro,* a reduction of HIF-1α levels was observed treating these

cells under hypoxic condition. It is known that metformin treatment stimulated an increase of PHDs levels in hypoxia inducing the reduction of HIF-1α stability and nuclear localization [145]. Further, Griss and co-workers demonstrated that metformin treatment significantly lowered all metabolites of the TCA cycle except αKG, whose increased levels allosterically foster PHDs activity independently from intracellular O_2 availability [142, 51, 52]. Thus, it is possible to hypothesize that in osteosarcoma cells treatment with metformin may boost PHDs activity, upon CI inhibition and consequent intracellular αKG accumulation, promoting HIF-1α destabilization under hypoxic conditions. On the other hand, as described by Prior and colleague, CI inhibition reduced the intracellular oxygen consumption leading to a condition of intracellular normoxia even when the extracellular oxygen tension is low [141]. In line with this, it was demonstrated that metformin efficiently reduced mitochondrial respiration by inhibiting respiratory CI in hepatocellular carcinoma and the decrease of mitochondrial oxygen consumption was correlated to HIF-1α protein degradation [179]. In this scenario, the use of metformin could open the doors to alternative therapeutic strategy to contrast hypoxic adaptation of cancer cells through respiratory CI inhibition. Besides the specificity towards mitochondrial CI, we were also interested to evaluate the efficiency of metformin to reduce the tumorigenic potential in cancer cells considering, at the same time, its effect on non-cancer cells. In this regard, we have reported that metformin administration promoted a reduction of thyroid cancer cells proliferation under hypoxic condition, while no effects were observed at physiological O_2 levels (21% O_2). Conversely, changes in O_2 availability and metformin treatment did not alter thyroid non-cancer cells viability. These data indicated that metformin exerted a specific anti-cancer activity in hypoxia. Our findings are supported by recent Safari and colleagues work, in which it is demonstrated that metformin enhanced its anti-tumor effect under hypoxia condition rather than normoxia in breast cancer cells, without showing side effects on non-cancer cells in the same conditions [180]. In this regard, the microenvironment of cancer cells is different from that of non-cancer cells due to their high proliferation rate and lower energy supplements by nutrient circulation [181]. Therefore, cancer cells alter their metabolic pathways to survive and, unlike non-cancer cells, may adapt better to drastic changes in oxygen availability [181]. The different metabolism may be the key to explain the specific anti-cancer effect of metformin induced under hypoxic condition. Concordantly, we also demonstrated that thyroid cancer cells are characterized by a higher capacity to accumulate and stabilize HIF-1α in hypoxia compared to non-cancer cells. This may justify the lower sensitivity of NTHY 3.1 cells to metformin treatment. However, adding complexity, the enhanced anti-tumor effect shown after metformin treatment under hypoxia in thyroid cell models was not confirmed using renal cells models. In this case, metformin treatment did not alter renal cell viability neither depending on cancer or non-cancer

nature nor depending on O_2 levels. These data confirmed the absence of side effect in normal cells after metformin treatment and suggested an anti-tumorigenic effect dependent on tissue origin and type of tumors. Indeed, UOK-257 is a cell line derived from human renal carcinoma of Birt-Hogg-Dubé (BHD) patient and are characterized by higher dependency on glucose metabolism, which is a common feature of insulin resistance [158]. These findings may explain the not efficacy of metformin to decrease renal cancer cell proliferation after 72 hours of treatment. However, long-term exposure to metformin significantly reduced UOK-257 clonogenic capacity *in vitro* suggesting that this drug might require a specific time to be effective in preventing renal cancer cell growth. In line, metformin treatment was related to a reduction of HIF-1α protein levels under hypoxic condition only after 24 hours in renal cancer cells, while in both osteosarcoma and thyroid cancer models HIF-1α was destabilized already after 2 hours of treatment. These data suggested that systemic effect or effects dependent from multiple molecular player may mainly concur to anti-cancer metformin proprieties. To support this hypothesis further studies will be required using a large panel of different cell models.

In the last few years, an increasing number of works has emphasized the role of CI inhibitors to interfere with HIF-1α stability acting as anti-proliferative and anti-cancer agent and opening the door to new possible therapeutic approaches for solid tumors, since mitochondrial function and adaptation to hypoxia are necessary for tumor progression. However, it is important to consider the double-edged role of respiratory CI in cancer. This metabolic enzyme actively controls the cellular energetic and redox balance, providing a crucial checkpoint of cell metabolism, proliferation and survival. For these reasons, it is crucial to characterize the molecular mechanisms through which metformin, as others CI inhibitors, may influence and humper tumor progression. Further investigations are necessary to clarify these points and to identify compounds that are safe enough to act selectively on cancer cells without drastic side effects.

An alternative and more direct approach to destabilize HIF-1α in hypoxic cancer cells by promoting PHDs enzymatic activity was represented by the use of αKGlogues. Recent studies have brought to light the pleiotropic anti-tumor activity of αKG. This TCA metabolite is involved in various cellular mechanisms in which changes of its intracellular levels might exert an impact in the regulation of signaling pathways engaged in promotion of carcinogenesis [182]. As a Krebs cycle metabolite, it regulates anabolic and catabolic TCA products and substrates, thereby regulating amino acid synthesis, ATP production, and reducing equivalent (NAD^+/NADH) generation, which in turn can influence ROS levels. In this regard, it has been shown that the αKG molecule positively modulates antioxidant enzyme activity in rats during induced hepatocarcinogenesis, restoring the oxidation-reduction homeostasis [183, 184]. Additionally, altered αKG levels may promote epigenetic

modifications by altering the activity of enzymes involved demethylation/hydroxylation reactions which belong to the family of αKG-dependent dioxygenases [182].

Concerning the role of this TCA cycle metabolite to prevent hypoxic adaptation of cancer cells, Gottlieb and his group have shown that the increase in αKG levels is sufficient to oppose hypoxia-mediated activation of HIF1α in SDH-deficient and FH-deficient tumors by promoting chronic PHDs enzymatic activity and consequent HIF-1α hydroxylation and destabilization even at low oxygen levels [51, 52]. Treatment with esters of αKG not only decreased HIF-1α protein levels and the expression of its target genes, but also reduced glucose up-take and lactate production suggesting a decrease in glycolytic flux. In these conditions, cells were less able to use oxygen to synthetizes ATP and an increase of cell death was observed in xenograft tumors [185]. In the light of the positive contribution of increasing αKG intracellular levels to reduce tumor survival and progression, the experimental approach to target tumor hypoxia via PHDs may represent a valid tool for cancer therapy. In this research field, our group has generated a library of novel αKGlogues that induced a more long-term effect on HIF-1α compared to others membrane-permeating ester of αKG, without having a cytotoxic effect on non-cancer cells.

To this purpose, αKG was conjugated with different hydrophobic carriers, which allow it to cross the plasma membrane and accumulate into the cytosol. Same of these carriers were characterized by own anti-tumorigenic activity and, accordingly, they have been evaluated for their possible synergic/antagonist effect with αKG to reduce the tumorigenic potential *in vitro* and for their cytotoxicity. However, the cell susceptibility to these treatments was strictly dependent to their tissue origin. This brought out the difficulty to develop an anti-cancer therapy that, besides to be considerate safe for normal cells, has to face with the problematic of the heterogeneity intra- and inter-tumor.

Afterwards, all αKGlogues synthetized were screened for their ability to destabilize HIF-1α protein levels under hypoxic condition. The first step of the screening has provided for a short exposure to different αKGlogues treatment in order to determine which compounds effectively reduced HIF-1α stability in thyroid and renal cell models. The αKGlogues selected were further evaluated for their capacity to induce a pseudonormoxic state in hypoxic cells after more long-term treatment leading to identify in thyroid and renal cells respectively αKGlogue D and αKGlogues E and G as novel and safe adjuvant therapeutic agents able to pharmacologically induce a pseudonormoxic state in hypoxic cancer cells. It is important to underline that these molecules were selected for their capacity to significantly reduce the tumorigenic potential of thyroid and renal cancer cells *in vitro*, without being cytotoxic for non-cancer cells counterpart. In this context, we also demonstrated that αKGlogues concentrations lower than 200μM, i.e. 2μM and 20μM, did not alter thyroid and renal

cell viability probably because the intracellular concentration of αKG was not sufficient to trigger chronic PHDs activity. Accordingly, as already said, the estimated K_m of PHDs is ~50μM indicating that αKGlogues should be administrated using concentrations higher that this value to induce a pseudormoxic state in hypoxic cancer cells [35, 36].

Furthermore, the effect on HIF-1α of these αKGlogues was not limited to very short incubation times but can persist up to 12 hours under hypoxic condition. Therefore, αKGlogues selected in our laboratory were able to exert a more long-term effect on HIF-1α commensurate with a potential clinical treatment in human patients using a concentration 10 times lower compared to the αKG derivatives used by Tennent and colleague. As a future perspective, it would be intriguing to prove the ability of these compounds to chronically destabilize HIF-1α by promoting PHDs activity in cell system that mimics the complex architecture and functional and metabolic heterogeneity of cancer cells in vivo. In this regard, the use of 3D cell culture may be advantageous in providing more physiologically relevant information and more predictive data for further *in vivo* tests. However, this PhD project has allowed us to collect several data which are promising in term of the development of a metabolic approach able to impinge the aggressiveness of hypoxic cancer cells. In this context, our experimental approach to trigger the intracellular increase of αKG levels may represent a valid strategy to allosterically boost PHDs enzymatic function and thus to prevent hypoxic adaptation.

Overall, the indirect respiratory CI inhibition and the direct use of αKGlogues may have a potential role to prevent hypoxic adaptation and metabolic reprogramming of cancer cells miming a normoxic condition. The consequent inability to adapt to adverse environmental conditions leads to a significant delay in tumor progression opening the doors to additional pharmacological intervention to reduce the aggressiveness of hypoxic mass and improve oncological patients' prognosis.

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