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Role of 5'-nucleotidases in the biology of human lung cancer cells and their response to different external stress conditions

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Résumé

Les 5'-nucléotidases sont des enzymes qui sont principalement impliquées dans le métabolisme des nucléotides en déphosphorylant les nucléotides intracellulaires et extracellulaires. Jusqu'à présent, huit 5'-nucléotidases différentes ont été caractérisées et sont nommées en fonction de leur localisation cellulaire et de leur spécificité du substrat. Parmi celles-ci, la 5'-nucléotidase cytosolique (cN-II) et l'ecto 5'-nucléotidase (eN, CD73) ont montré des rôles importants dans le cancer. Par exemple, les cellules exprimant une cN-II mutée ont montré une résistance accrue aux médicaments cytotoxiques dans la leucémie lymphoïde aiguë chez l'enfant en rechute, et il y a une importance accrue du CD73 comme nouveau point de contrôle pour l'immunothérapie du cancer en raison de son rôle dans la production d'adénosine immunosuppressive. Cependant, la compréhension détaillée de la nature biologique de leurs rôles dans la progression du cancer et la résistance au traitement reste à étudier.

Au cours de ma thèse, nous avons étudié les rôles biologiques de ces enzymes dans la prolifération, la migration et la réponse des cellules cancéreuses aux conditions de stress (stress nucléotidique, médicaments cytotoxiques et hypoxie). Pour ce faire, nous avons utilisé les lignées cellulaires d'adénocarcinome pulmonaire humain (NCI-H292), de cancer du sein humain (MDA-MB-231) et de cancer du sein de souris (4T1) et préparé pour chaque lignée cellulaire des phénotypes distincts exprimant ou non cN-II et / ou CD73. Nos résultats ont montré que le déficit en cN-II et CD73 est associé à une migration cellulaire accrue et implique TIMP-2, MMP-2 et MMP-9 via l'activation de la voie COX-2 / PGE2 / Akt. Nous avons également montré que cN-II et CD73 ont un rôle à jouer dans la réponse des cellules à différentes conditions de stress ainsi que dans la sensibilité à certains médicaments cytotoxiques. En effet, une carence en CD73 a été associée à une sensibilité accrue au stress nucléotidique et à la vincristine alors qu'une diminution de la sensibilité à la mitomycine C.

Ce manuscrit décrit ce travail de doctorat. Les premiers chapitres servent de base à l'hypothèse scientifique en expliquant une vision générale des nucléotides et des 5'-nucléotidases ainsi que leurs rôles physiologiques et pathologiques avec un accent particulier sur le cancer. Sur la base de cette

introduction, l'hypothèse de travail sera développée et les résultats seront expliqués dans les chapitres suivants intégrés sous la forme de deux articles scientifiques soumis. De plus, comme cela sera développé plus loin dans le chapitre « *Aim of the project* », un article de synthèse publié sera également inclus à la fin de cette thèse.

Summary

5'-nucleotidases are enzymes that are primarily involved in nucleotide metabolism by dephosphorylating both intracellular and extracellular nucleotides. So far, eight different 5'-nucleotidases have been characterized and are named based on their cellular localization and substrate specificity. Among these, cytosolic 5'-nucleotidase (cN-II) and ecto 5'-nucleotidase (eN, CD73) have shown significant roles in cancer. For example, cN-II mutant cells have shown an increased resistance to cytotoxic drugs in relapsed childhood acute lymphoid leukemia, and there is an increased importance of CD73 as a novel checkpoint for cancer immunotherapy due to its role in the production of immunosuppressive adenosine. However, the detailed understanding of the biological nature of their roles in cancer progression and resistance towards treatment is still a matter to be studied.

During my PhD, we have studied the biological roles of these enzymes in cancer cell proliferation, migration and response towards stress conditions (nucleotide stress, cytotoxic drugs and hypoxia). To do so, we used human lung adenocarcinoma (NCI-H292) and breast cancer (MDA-MB-231) cell lines as well as mouse breast cancer cell line (4T1) and prepared for each cell line four distinct phenotypes expressing or not cN-II and/or CD73. Our results showed that knocking out cN-II and CD73 is associated with an increased cell migration and involves TIMP-2, MMP-2 and MMP-9 via activation of COX-2/PGE2/Akt pathway. We also showed that both cN-II and CD73 have a role to play in cells response to different stress

conditions as well as sensitivity towards certain cytotoxic drugs. Indeed, CD73-deficiency was associated with increased sensitivity towards nucleotide stress and vincristine whereas decreased sensitivity towards mitomycin C.

This manuscript gives a detailed explanation of the PhD project. Initial chapters serve as the foundation for the scientific hypothesis by explaining a general outlook of nucleotides and 5'-nucleotidases as well as their physiological and pathological roles with a particular focus on cancer. Based on this introduction, the working hypothesis will be developed, and results will be explained in the following chapters integrated as two submitted scientific papers. Moreover, as will be elaborated further in the chapter "*Aim of the project*", one published review article is included at the end of this PhD thesis.

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Dedication

*To my beloved **parents** for making me so able as to win the PhD fellowship*

&

*To my pretty and caring wife, **Hira**, for her unconditional love and support through all thick and thin during the whole period of my PhD*

&

*To my son, **Hayyan**, one of the best blessings of my life.*

Glossary

ADA: Adenosine deaminase

ALL: Acute Lymphoblastic Leukemia

AMP: Adenosine Mono Phosphate

CD73: Ecto 5'-nucleotidase

cN-II: cytosolic 5'-nucleotidase II

CoCl₂: Cobalt Chloride

dCK: deoxyCytidine Kinase

GMP: Guanosine Mono Phosphate

HIF-1 α : Hypoxia Inducible Factor 1 alpha

HSP: Hereditary Spastic Paraplegia

IMP: Inosine Mono Phosphate

MDR: Multidrug-Resistant

MMP: Metalloproteinases

NT5C2: Gene coding cN-II

NT5E: Gene coding CD73

PAP: Prostatic Acid Phosphatase

PRPP: Phosphoribosyl Pyrophosphate

RNR: Ribonucleotide Reductase

RRM2: Ribonucleoside-diphosphate Reductase subunit M2

TME: Tumor Microenvironment

General Introduction

Nucleotides are the important role players in the functioning of cellular machinery. They have a strong strategic position in intracellular as well extracellular activities. Intracellularly, they serve as building blocks for nucleic acids, currency notes for cell's powerhouse, regulators for several metabolic enzymes and mediators for hormonal actions. In the extracellular compartment, nucleotides hold an inevitable position in a wide range of both physiological and pathological processes. Examples include, but are not limited to, cell growth and expansion, neurotransmission, platelet aggregation, blood vessels' tone, regulation of immune system, purinergic signaling and homeostasis of nucleotide pool across cell membrane etc. Many cell types release nucleotides under quiescent conditions (Beigi and Dubyak 2000; Schwiebert et al. 2002) as well as in response to various stress signals *i.e.* mechanical stimulation (Burnstock and Knight 2017; Grierson and Meldolesi 1995; Homolya, Steinberg, and Boucher 2000; G. Yegutkin, Bodin, and Burnstock 2000), treatment with agonists like bradykinin, serotonin and acetylcholine (Q. Yang et al. 1994) and via regulated exocytosis (Leitner et al. 1975; Unsworth and Johnson 1990; Sorensen and Novak 2001). A large number of nucleotide functions is directly or indirectly linked to their metabolism. In eukaryotic cells, there are two functionally distinct nucleotide pools. One pool is present in the nucleus of the cell and is needed for DNA replication and repair whereas other is present in the mitochondria responsible for mitochondrial DNA (mtDNA) production. A third pool also exists in cytoplasm which is not considered as functional rather act as a reservoir to maintain two functional pools mentioned earlier. Studies have shown that an interplay between cytosolic and mitochondrial nucleotide pools is present in the cell (Di Noia et al. 2014). Nucleotides pool sizes move inside the cell during different phases of cell cycle. However, maintenance of intracellular pools of nucleotides is very essential for error free DNA replication and cellular proliferation. This balance is regulated collectively through all enzymes involved either in the biosynthesis or degradation of nucleotides in the cells. Any change or imbalance in their pools might

have drastic impacts on cell fate *e.g.* onset of spontaneous mutations (Mathews 2015). In cancer, for example, it has been shown that abnormal activation of cell proliferation pathways like Rb-E2F as a result of HPV viral protein E7 or cellular oncogene cyclin E leads to genomic and chromosomal instability because of an insufficient pool of nucleotides to support this replication (Bester et al. 2011). Therefore, a strict homeostasis is regulated by 5'-nucleotidases along with nucleoside kinases and other enzymes.

Nucleotides

Nucleotides are the biochemical compounds of fundamental importance in cell biology as they play a substantial role in many cellular processes as energy donors, components of enzyme cofactors, chemical messengers and, above all, the building blocks of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). Undoubtedly, both DNA and RNA serve as the blueprints of all sort of life. Structural details, synthesis and pathophysiological roles of nucleotides are outlined as follows.

Structural insight of nucleotides

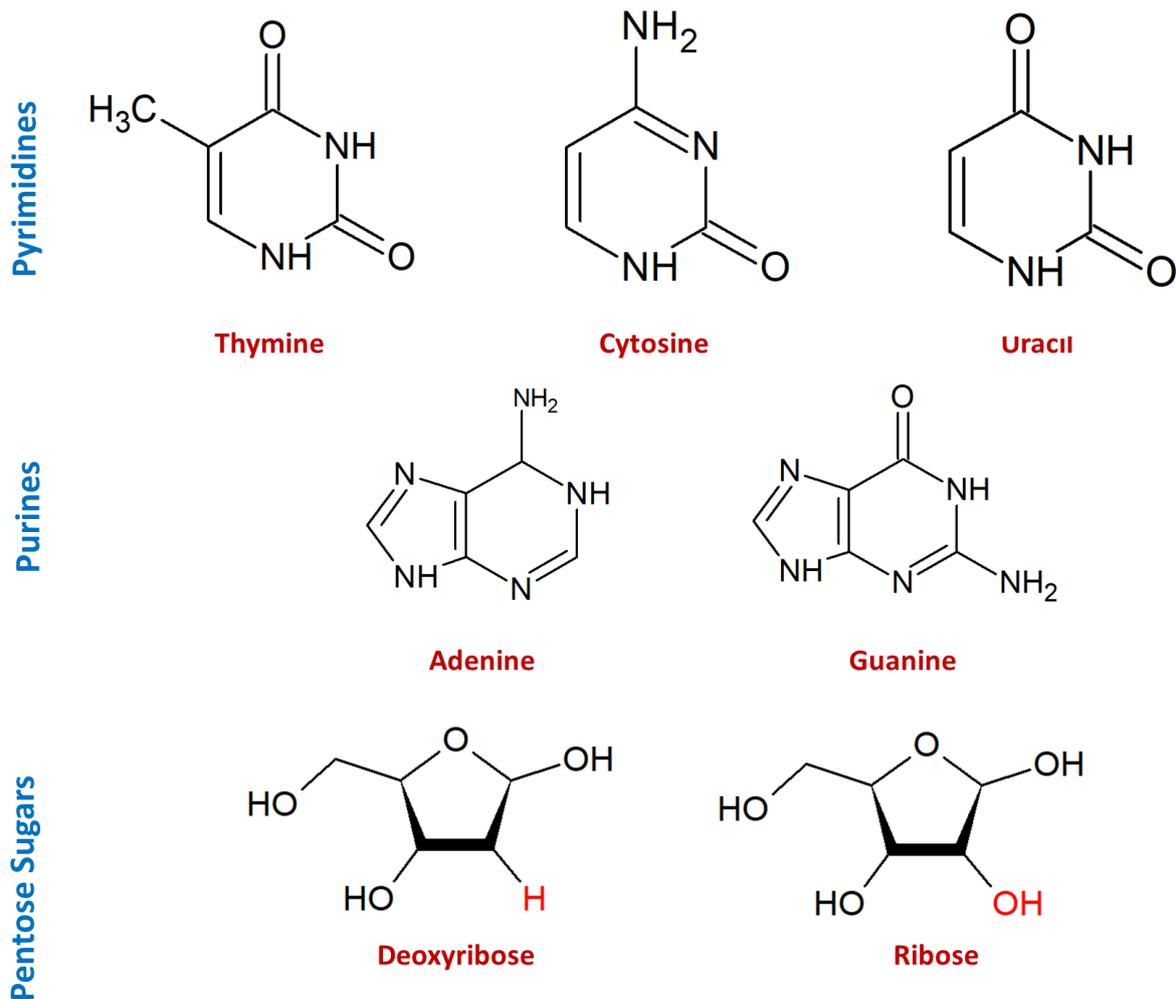
Nucleotides are composed of three distinctive chemical moieties *i.e.* nitrogenous base, pentose sugar and phosphate group(s). Nitrogenous base and pentose sugar covalently link together via glycosidic bonds and constitute nucleosides. Nucleosides, in turn, become nucleotides by attachment of phosphate group(s) with their pentose sugar. Thus, nucleotides are the phosphorylated forms of nucleosides and can be categorized into different types based on the number of phosphate groups attached with the nucleoside, the type of nitrogen base and oxidation status of pentose sugar. *Table 1* shows the nomenclature and classification of main natural nucleosides. However, additional natural variations may exist with methylations, oxidations etc.

Table 2. Nomenclature and classification of main natural nucleotides based on the type of constituent chemical moiety.

Pentose sugar	Nitrogenous bases						Number of phosphate groups
	Purines			Pyrimidines			
	Adenine	Guanine	Hypoxanthine	Thymine	Uracil	Cytosine	
Ribose	AMP	GMP	IMP	TMP	UMP	CMP	One
	ADP	GDP	IDP	TDP	UDP	CDP	Two
	ATP	GTP	ITP	TTP	UTP	CTP	Three
Deoxyribose	dAMP	dGMP	dITP	dTMP	dUMP	dCMP	One
	dADP	dGDP	dIDP	dTDP	dUDP	dCDP	Two
	dATP	dGTP	dITP	dTTP	dUTP	dCTP	Three

Nitrogen base

Nucleotides and nucleosides can be divided into purine and pyrimidine derivatives based on their nitrogen base (Figure 1). The purines consist of a 5-membered imidazole ring fused to a six membered ring structure. Both the rings of purines share two common carbon atoms (at position 4 and 5) and contain 4 nitrogen atoms altogether. Pyrimidines consist of a single ring of six atoms (4 carbons and 2 nitrogen). Purine nitrogenous bases include nine different members but adenine, guanine and hypoxanthine are of highest biological importance as well as for the scope of this thesis. On the other hand, pyrimidine nitrogenous bases include thymine, uracil and cytosine. Among all nitrogenous bases, adenine (A), guanine (G), thymine (T), uracil (U) and cytosine (C) are also known as nucleobases as they are incorporated in the structure of nucleic acids. On the other side, hypoxanthine and its nucleoside inosine (I) are of particular importance thanks to their pivotal position in *de novo* nucleotide synthesis. Additionally, inosine is also occasionally found in nucleic acids *i.e.* in the anticodons of tRNA.



Figures 1: Structures of nitrogenous bases for purine and pyrimidine nucleotides and pentose sugars for ribonucleotides or deoxyribonucleotides.

Pentose sugar

The pentose sugar is a monosaccharide carbohydrate with a five-carbon ring that can be either ribose or deoxyribose, forming ribonucleotide or deoxy-ribonucleotide respectively when attached to nitrogen bases (Figure 1, 2). In the structure of nucleotides, pentose sugar acts as a central unit with its one end connected to nitrogenous base and the other end connected with phosphate group. It is linked to nitrogenous base via an N-glycosidic linkage at carbon 1'. Pentose sugar, along with phosphate group (PO_4^{3-}), serves as the backbone of nucleic acids (DNA/RNA) structure.

Phosphate group

In the structure of a nucleotide, phosphate (PO_4^{3-}) is attached to the pentose sugar (ribose or deoxyribose) of nucleoside at carbon 3' or 5' via ester bonds. Based on the number of phosphate groups attached, there are either mono-, di- or tri-phosphorylated nucleosides. Nucleotides such as ATP and GTP are used not only for RNA or DNA synthesis, but also as energy donors for many cellular reactions. This is because of the energy-rich bonds present in these nucleotides, which release energy on cleavage.

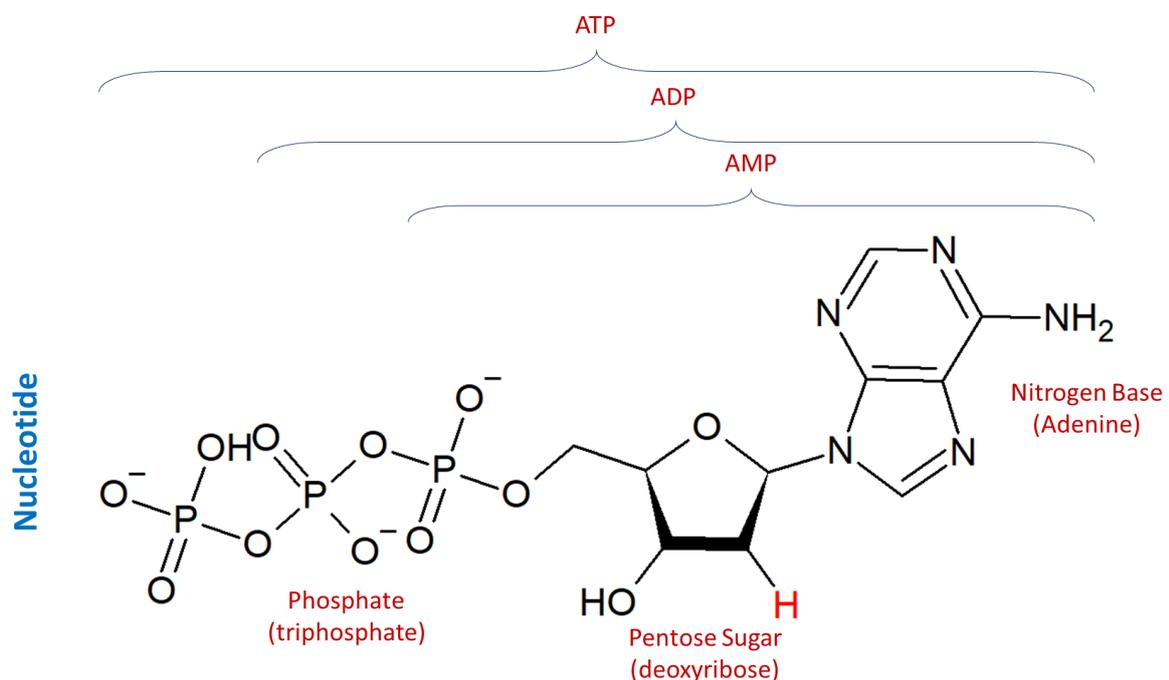


Figure 2: A complete structure of a nucleotide (deoxy Adenosine triphosphate, ATP) showing all three chemical components, Nitrogen base (adenine), pentose sugar (deoxyribose) and three phosphate groups (PO_4).

Nucleotide metabolism

Nucleotides are always in demand in cellular markets, as cells need them not only to proliferate but also to fuel a smooth and uninterrupted functioning of cellular machinery. To maintain adequate

supply-demand chain, nucleotides-manufacturing units are always functional and consuming all the available raw material in the cell cytoplasm.

There are two distinct pathways for the synthesis of nucleotides, salvage and *de novo* pathways (Blakley and Vitols 1968). Both these pathways are active independent of each other. *De novo* pathway can be considered as the one mainly responsible for production of more nucleotides whereas the “salvage pathway” can be considered as a regulatory pathway by maintaining the balance between needed nucleotides and extra nucleotides, adding or taking away phosphates, modifying ribose into deoxyribose etc., based on the demands in cells. However, both these pathways are regulated in order to maintain an equilibrium within different nucleotides’ pools. As this project will focus more on the purine nucleotides and nucleosides, and therefore their synthesis will be explained in more detail in upcoming paragraphs.

De novo synthesis

The *de novo* synthesis, as the name suggests, is the pathway that starts the synthesis of nucleotides from raw material present in the cell *i.e.* amino acids, CO₂, NH₃ and ribose-5-phosphate (Dang 2012; Hartman and Buchanan 1959; Newsholme et al. 2003). It is important to note that purine and pyrimidine synthesis via *de novo* pathway follow different steps (Aird and Zhang 2015; Reichard 1988). Purine *de novo* synthesis is an eleven-step process that takes the 5-membered phosphorylated sugar (ribose-5-phosphate) and start assembling different member-atoms of the nitrogenous base ring to the pentose sugar (Figure 3). Every step is catalyzed by a specific enzyme which gives rise to a specific intermediate. The most important of all the intermediates and the starting point in *de novo* purine synthesis is phosphoribosyl pyrophosphate (PRPP) and is formed from the precursor pentose sugar (Hove-Jensen et al. 2017).

Pyrimidine *de novo* synthesis, on the other hand, is a five-step process that first prepares nitrogenous base and then attaches it with the ribose-5 sugar (Aird and Zhang 2015) (Figure 4).

As this pathway synthesizes purine nucleotides from small precursor molecules, it consumes a lot of cellular energy in the form of ATP and GTP over a cascade of enzymatic reactions. The first purine nucleotide synthesized via the *de novo* pathway is IMP (inosine monophosphate), which then converts into either AMP or GMP. On the other hand, UMP is the first pyrimidine nucleotide which is synthesized from the *de novo* synthesis of pyrimidine nucleotides. UMP is then converted into other pyrimidines nucleotides (Reichard 1988).

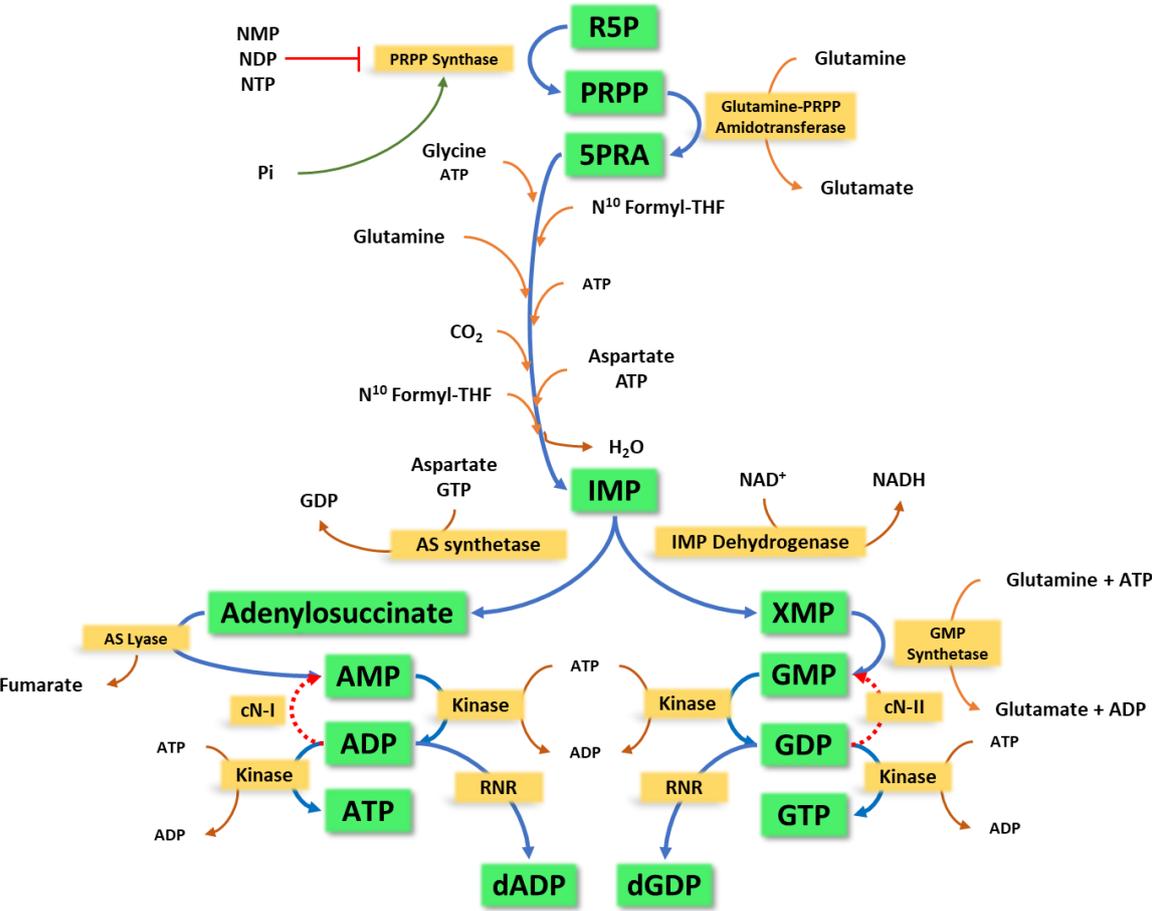


Figure 3: Schematic diagram showing *de novo* synthesis of purine nucleotides.

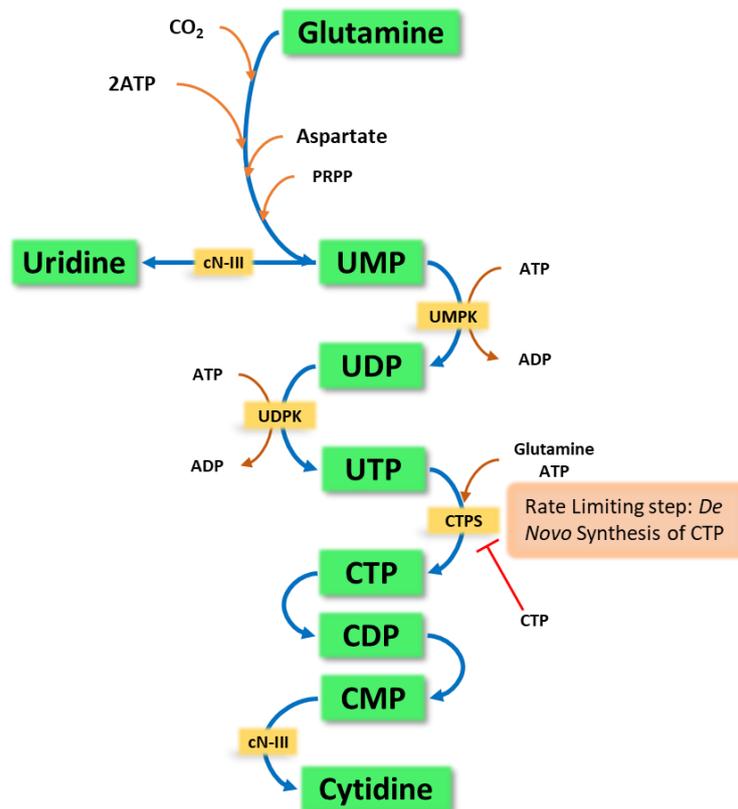


Figure 4: Schematic diagram showing de novo synthesis of pyrimidine nucleotides.

Salvage pathway

The name of the “salvage pathway” of purine/pyrimidines nucleotide synthesis is also self-explaining as it rescues the useful parts of the degraded nucleic material present in the cells (metabolic intermediates of DNA/RNA) and allows the production of nucleotides again. The rescued subunits (nitrogen bases, nucleosides and nucleotides) are reprocessed back to form new nucleotides (Murray 1971). Different enzymes are involved in the salvage pathway depending upon the starting substrate. Both purines and pyrimidines salvage pathway consist of a single phosphorylation step if we only consider the transformation of a nucleoside into the corresponding nucleotide. It is important to note that this process is bypassing the industrious steps to build nucleotides’ subunits from small precursor molecules, as is the case in *de novo* synthesis pathway. Therefore, it consumes lower cellular energy as compared to the *de novo* pathway. For this reason, this pathway can also be termed as energy-saving pathway for nucleotides biosynthesis.

nucleotides and nucleosides in the cells, and the salvage pathway play a major role in this cellular process.

Enzymes of the salvage pathway have been shown to have significant roles in various pathological conditions including cancer (X. Xu and Wu 2014; J. Li et al. 2019; Malami and Abdul 2019).

Enzyme deficiencies and therapeutic targeting

Nucleotides hold an important position in many physiological processes. Uracil nucleotides, for example, stimulate the proliferation and differentiation of dopaminergic neurons (Milosevic et al. 2006). Muscles and plasma level of purines, in particular hypoxanthine, has been shown to be an important predictor of actual sports performance by relating it to the muscles' adaptability to nucleotide metabolism (Zieliński et al. 2019).

On the other hand, nucleotides are also involved in pathophysiology of many diseases as a result of their defective metabolism. As mentioned earlier, nucleotides' pools are tightly regulated by specific enzymes and, therefore, any deficiency of these regulating enzymes may disturb this balance. Examples include but are not limited to different metabolic diseases (Balasubramaniam, Duley, and Christodoulou 2014), Lesch-Nyhan syndrome and adenosine deaminase deficiency (S. Fujimori et al. 1989; Sidi and Mitchell 1985; Shin Fujimori et al. 1990; Whitmore and Gaspar 2016; Harris 2018). Furthermore, inborn defects in purine metabolism have also been associated with pediatric syndromes of neurological origin (Camici et al. 2010). In addition to nervous system and metabolic diseases, nucleotides imbalance is associated with tumor transformation, progression and sensitivity to cytotoxic drugs (Aird and Zhang 2015; Meuth 1989; Weinberg, Ullman, and Martin 1981; Chang et al. 2013), Bloom syndrome (Chabosseau et al. 2011), gout (Boss and Seegmiller 1982), renal diseases (Dobrovolsky et al. 2003; Kimura et al. 2003), aging (Mathews 2006; Burhans and Weinberger 2007), immunodeficiency (Ammann 1985; Boss and Seegmiller 1982) and several mitochondrial disorders (Pitceathly et al. 2012; El-Hattab and Scaglia 2013).

Besides the importance of nucleotide metabolism in different pathological conditions, there is a huge advancement in pharmacological use of nucleotides. Nucleoside analogs, for example, is a group of drugs which have been in use for almost 50 years now and have wide spread use in different diseases (Walker 2012). Currently they are in use as anti-viral drugs against hepatitis B virus, hepatitis C virus, herpes simplex, HIV and coronavirus (Pruijssers and Denison 2019), anti-cancer chemotherapy (C. M. Galmarini et al. 2001; Tuncbilek et al. 2018), anti-platelet drugs (Adamski et al. 2018), first short course disease-modifying drug in multiple sclerosis (Deeks 2018) as well as in rheumatic disorders (Jansen, Scheper, and Dijkmans 2003). Currently, their efficacy as anti-bacterial agents is also under consideration and some of them have shown good potential in preclinical studies (Thomson and Lamont 2019). Addition of several new members in this pharmacological class during past decade represents its potential and interest of researchers in this field (Jordheim, Durantel, et al. 2013). Other than nucleoside analogues, enzymes involved in nucleotide metabolism have gained an exponential importance as potential therapeutic targets in pharmacotherapy of above-mentioned diseases. Examples include but are not limited to SAMHD1 inhibitors and NUDT inhibitors etc. Indeed, equilibrium in nucleotide pools is dependent on the activity of these enzymes and hence, the fine tuning of a particular enzyme can regulate the pools in order to achieve desired therapeutic goals.

Role of nucleotides in cancer pathophysiology

It has been shown that nucleotides are directly or indirectly involved in cancer transformation, progression and its sensitivity towards cytotoxic drugs (Aird and Zhang 2015; Meuth 1989; Weinberg, Ullman, and Martin 1981; Chang et al. 2013). However, the scope of this PhD project demands a detailed explanation of their pathological importance in cancer.

Genomic instability is among the most imperative hallmarks of cancers and is characterized by mutagenesis and results in a rapid and uncontrolled cell growth (L. A. Loeb, Springgate, and Battula 1974; Bielas et al. 2006; Lawrence A. Loeb 2016). Once a normal cell is transformed into a cancer cell, it will always be in a state of proliferative emergency. Nucleotides, being precursors of DNA, hold a

central position in maintaining cellular genetic integrity in normal cells. Any modification in these precursors or disturbance in their respective pools can lead to the incorporation of modified residues into DNA and hence cancer. In this regard, Topal and Baker in 1982 first coined the concept that precursor nucleotides pools are orders of magnitude more susceptible to modification (methylation for example) than the incorporated nucleotides (Topal and Baker 1982). Similarly, in addition to the nucleotides' modification, maintenance of their respective pools has also been considered of critical importance in maintaining the genomic integrity of the cells. Studies have shown that accumulation or depletion of dNTPs pools, both balanced and unbalanced, can stimulate mutagenesis at replication, insertion and/or proofreading steps (Mathews 2006; 2015). For example, an imbalanced dNTP pool can drive substitution mutagenesis either by competition at nucleotide insertion step or by a next-nucleotide effect resulting in point mutation, transversion as well as a frameshift realignment. These mutagenic pathways have already been described in details (Mathews 2015).

From the above, it can be concluded that modification in precursor nucleotides and dysregulation of their respective pools imperatively contribute towards genomic instability. Later studies identified certain enzymes as nucleotide pool sanitizers because of their enzymatic activity to safeguard cells from incorporation of mutagenic nucleotides into their replicating DNAs (Mathews 2016). MTH-1, for example, described by *Mo et al.* (Mo, Maki, and Sekiguchi 1992) is one of the earliest identified enzymes known to be a dNTPs pool sanitizer which functions by hydrolyzing the mutagenic dNTP (8-oxo-dGTP). However, its role as a tumor suppressor or an effective therapeutic target has been a controversy since 2014 (Mathews 2016) and out of the scope of this discussion. Similarly, enzymes involved in nucleotides metabolism and maintenance of their pools (e.g. RRM1, RRM2, dCK, hENT1, thymidylate synthase, 5'-nucleotidases etc.) are considered to be major stakeholders in the broader pathophysiology of cancer because of their role in fine tuning of nucleotides pools in cells. For example, studies have shown significant prognostic value of cN-II in adults with AML (C. M. Galmarini et al. 2001) and thymidylate synthase for patients with Duke's B and C colon carcinoma (Allegra et al. 2003) as well as prognostic and predictive values of hENT1, dCK, RRM1, and RRM2 for gemcitabine sensitivity of

patients with PDAC (Fujita et al. 2010; Sierzega et al. 2017; Bird et al. 2017; Maréchal et al. 2012). Furthermore, a study has shown the prognostic value of RRM2 for colorectal cancer (CRC) patients and its potential as a predictor for CRC chemotherapy (Xiyong Liu et al. 2013).

On the other hand, once a normal cell is converted into a cancer cell, it starts replicating at an accelerated pace. It means a higher replication of the genetic material and hence a higher demand to produce enough dNTPs. Therefore, cancer cell undergoes certain metabolic reprogramming in order to meet the escalating demands of dNTPs biosynthesis. Altered expression of oncogene *MYC* (Satoh et al. 2017), *MTOR* (Guertin and Sabatini 2007), PI3K-AKT pathway (Tong, Zhao, and Thompson 2009), *ERK-MAPK* pathway (Burotto et al. 2014), *G6PD* (Patra and Hay 2014), *RRM1* (Buj and Aird 2018) as well as tumor suppressor gene *TP53* (J. Liu, Zhang, and Feng 2014) causes an accelerated biosynthesis of nucleotides in cancer cells. Different mediators of nucleotides biosynthesis are also reprogrammed in cancer cells in favor of mounted production of dNTPs and other nucleotides (Jo et al. 2016; Berg et al. 2010). All these metabolic changes further favor cancer progression by disturbing the nucleotides pools in the cell. Studies have also shown that decreased dNTP pools as a result of rapid proliferation of cancer cells play an important role in Oncogene-Induced Senescence (OIS) via replication stress and DNA damage response activation (Aird and Zhang 2015). Apart from this, nucleosides can also function as immunomodulators. Adenosine, for example, produced extracellularly as a result of enzymatic activity of CD73, has been shown to favor cancer progression as it may function as an immunosuppressor via adenosine receptors expressed on various immune cells (Kumar 2013). The immunosuppressive effects of adenosine in tumor microenvironment help cancer cells to proliferate under covers, scientifically known as immune evasion (Young et al. 2018).

To date, there is a convincing number of anticancer therapies targeting nucleotide metabolism. One of the potential targets is ribonucleotide reductase (RNR) (C. Zhang et al. 2014; Mannargudi and Deb 2017). Free radical scavengers (hydroxyurea, trimidox) (Szekeres et al. 1994; Madaan, Kaushik, and Verma 2012), iron chelators (3-AP, Triapine) (Yu et al. 2012) and antisense oligonucleotide to RRM2

(GTI-2040) (Malik, Zwiebel, and Cooper 2018; Leighl et al. 2009) are three important classes of anti-cancer drugs which target RNR. More importantly, antimetabolites constitute a class of drug which has truly revolutionized the cancer therapy and have turned cancer into a, somehow, curable disease. These are further subdivided into two different sub classes. *i.e.* antifolates (Bertino 2009) and nucleoside analogues (Tsesmetzis et al. 2018).

Based on the information outlined above, the role of enzymes involved in nucleotides metabolism as potential therapeutic target and as a prognostic and predictive factor can be well justified in the context of cancer. Indeed, targeting these enzymes will clearly help in manipulating the nucleotides pools in cancer cells in the favor of treatment. However, there is an utter need of a better and in-depth understanding of their biological roles in normal and cancer cells to avoid off target effects of nucleotide metabolism-targeting pharmacotherapy. In this project, we studied the biological roles of two 5'-nucleotidases (cN-II and CD73) in cancer. These enzymes hold pivotal position in nucleotide metabolism and maintenance of their pools in the cell. A detailed explanation of different 5'-nucleotidases is outlined as follows.

5'-nucleotidases

5'-nucleotidases (also known as nucleoside monophosphate phosphohydrolases) are a group of enzymes that catalyze the dephosphorylation of non-cyclic nucleotide monophosphates at the 5'-carbon position of its pentose sugar. Both ribo- and deoxyribonucleotide monophosphates serve as substrates for 5'-nucleotidases. These enzymes differ based on the sequence, structure, substrate specificity and subcellular localization. *Table 2* shows the nomenclature, genetic codes, subcellular localization and substrates for all members of 5'-nucleotidase family. Out of eight 5'-nucleotidases, six are present in the cytosol, one in mitochondria whereas one is attached to the extracellular plasma membrane. Some 5'-nucleotidases (CD73, cN-II, cdN and mdN) are ubiquitously expressed in wide range of species and organ systems of the human beings while others (cN-IA, cN-IIIA) show a tissue specific expression. By converting a mono-phosphate in to the corresponding nucleosides, 5'-nucleotidases play a very crucial role in maintaining the nucleotide pools in the cells (Bianchi, Pontis, and Reichard 1986; Hunsucker, Mitchell, and Spychala 2005). It is important to note here that both cytosol and mitochondria express their own kinases and 5'-nucleotidases, and hence maintain their nucleotide pools either alone or in collaboration amongst each other.

5'-nucleotidases are getting higher attention by scientists working on different diseases. In this project, we focused on two of these enzymes *i.e.* cytosolic 5'-nucleotidase-II (cN-II) and ecto 5'-nucleotidase (CD73). The general introduction and other characteristics associated with these two enzymes are outlined in detail here after.

Table 2: A summary of the basic information related to eight different human 5'-nucleotidases. Number of amino acids in each 5'-nucleotidase represents its isoform 1 where applicable. Substrates in bold letters represent the ones for which the enzyme has higher sensitivity.

Name	Gene	Cytogenetic location	Protein size (kDa)	No. of amino acids	Substrates	Localization	Crystal structure (Reference + PDB number)
cN-IA	NT5C1A	1p34.2	47.7	368	AMP , Pyrimidine-dNMPs	Cytoplasm	-
cN-IB	NT5C1B	2p24.2	68.8	610	AMP	Cytoplasm	-
cN-II	NT5C2	10q24.32-q24.33	65	561	IMP/GMP , AMP	Cytoplasm	(2J2C) (Walldén et al. 2007)
cN-IIIA	NT5C3A	7p14.3	37.9	331	CMP , UMP , dUMP, dCMP, dTMP	Cytoplasm	(2CN1) (Walldén et al. 2007)
cN-IIIB	NT5C3B	17q21.2	34.4	300	M⁷GMP	Cytoplasm	-
CD73	NT5E	6q14.3	69	574	AMP , Broad specificity, both pyrimidine and purine (d)NMPs	Extracellular	4H2F, 4H2G (Knapp et al. 2012)
cdN	NT5C	17q25.1	45	201	dUMP , dTMP , dGMP, dIMP	Cytoplasm	(4L57) (Pachl et al. 2014)
mdN	NT5M	17p11.2	25.9	228	dUMP , dTMP , 3'-dTMP, 3'-UMP	Mitochondria	(4L6A) (Rinaldo-Matthis et al. 2002)

Cytosolic 5'-nucleotidase II

General introduction

Cytosolic-5'-nucleotidase-II (cN-II, coded by gene *NT5C2*), also known as high K_m 5'-nucleotidase, IMP-GMP specific 5'-nucleotidase or purine 5'-nucleotidase, is an IMP/GMP-preferring, bifunctional, allosterically regulated and structurally strongly conserved cytosolic 5'-nucleotidase (Banditelli et al. 1996; Oka et al. 1994; Itoh, Mitsui, and Tsushima 1967; Hunsucker, Mitchell, and Spychala 2005; Spychala, Madrid-Marina, and Fox 1988). cN-II is ubiquitously expressed in almost all organs and tissues of vertebrates at a low but constant level. The highest expression of cN-II has been reported to be in skeletal muscle, pancreas and heart muscle (Allegrini et al. 1997). Historically, cN-II was first partially purified from the liver of a chicken in 1967 (Itoh, Mitsui, and Tsushima 1967). Later, human cN-II and bovine cN-II was cloned from a human placenta cDNA library and calf thymus, respectively (Oka et al. 1994; Allegrini et al. 1997). Human cN-II shows highly conserved structure with a lowest homology of 98% with other mammalian cN-II (Oka et al. 1994). The gene location for human cN-II is described as on chromosome 10q24.32. The human cN-II is a 561 amino acids long peptide chain with a calculated molecular mass of 65 kDa, deduced from open reading human cDNA sequence (Oka et al. 1994). However, on SDS-PAGE, cN-II from various sources migrates at a relative mass in the range of 50-70 kDa (Spychala et al. 1999; C. Rampazzo et al. 1999).

Structural insight and allosteric regulation of cN-II

Structurally, cytosolic-5'-nucleotidase II (cN-II) belongs to the haloacid dehalogenase (HAD) superfamily. This family is defined by an α/β -Rossmann-like domain and a smaller 4-helix bundle domain, which, however, is not present in all HAD superfamily enzymes (Burroughs et al. 2006). cN-II is a tetrameric enzyme that consists of four crystallographically related subunits, two interfaces (interface A and interface B), one active site per subunit and two regulatory/effector sites as (Walldén et al. 2007).

Overall, the structure of cN-II has been reported in three different conformations *i.e.* native structure, a transient phosphoenzyme intermediate and active conformation resolved by crystallography at a resolution of 2.2 Å, 2.15 Å and 1.5 Å respectively (Walldén et al. 2007). Each of the four subunits of cN-II is composed of α -helices and β -strands. cN-II structure can be divided into two domains *i.e.* “core domain” and “cap domain”. The binding site for the phosphate of substrate nucleotide is located in the core domain which resembles the α/β -Rossmann-like “core domain” shared by the other members of HAD superfamily (Burroughs et al. 2006; Walldén et al. 2007). On the other hand, cap domain is involved in effector binding and subunit interaction within the tetrameric structure of cN-II (Walldén et al. 2007). The tetramer consists of two identical dimers having two different interfaces, interface A and interface B. Interface A is present at the subunit-subunit contacts within a dimer whereas interface B is responsible for the binding of two dimers. Interface B maintains the tetramer conformational integrity of cN-II by hydrogen bonding (Walldén et al. 2007).

Other than the abovementioned conformations, several other crystal structures for human cN-II have been proposed including several cN-II mutants (single and double mutants), wild-type cN-II complexed with its chemical inhibitors as well as mutant cN-II bound with dATP and free phosphate. (Walldén et al. 2007; Walldén and Nordlund 2011; Jordheim, Marton, et al. 2013; Marton et al. 2015; Hnízda et al. 2016; C. L. Dieck et al. 2018; Hnízda et al. 2018a; Guillon et al. 2019).

Allosteric regulation of cN-II: As mentioned, cN-II is an allosterically regulated 5'-nucleotidase and several effectors can regulate its enzymatic activity. Bivalent metallic cations, preferably Mg^{2+} , are required for the activation of cN-II from native conformation (Worku and Newby 1982). In addition to that, substrate concentration, ATP, ADP, 2,3-bisphosphoglycerate (F. Bontemps, Berghe, and Hers 1988; Françoise Bontemps et al. 1989), dinucleosides polyphosphate or diadenosine tetraphosphate (activators) (Pinto et al. 1986; Marques et al. 1998) and inorganic phosphate (P_i) (inhibitor) (Spychala, Madrid-Marina, and Fox 1988; Itoh 1981; Berghe, Pottelsberghe, and Hers 1977; Sychala et al. 1999) can also allosterically affect the enzymatic activity of the enzyme. However, it is important to note that

the effector molecules are not involved in the reaction stoichiometry but rather play a role in allosteric regulation, combined stabilization and activation (Tozzi et al. 1991; Bretonnet et al. 2005). It has been shown that, like many other HAD family members, cN-II functions by forming a phosphoenzyme intermediate during its enzymatic activity (Worku and Newby 1982; Baiocchi et al. 1996). Finally studies have shown that certain genetic mutations of cN-II are clustered into hotspots driving intersubunit stimulation (Hnízda et al. 2018b) and cause cN-II to become constitutively active without the need of allosteric activators (Hnízda et al. 2016).

Physiological roles of cN-II

As mentioned earlier, cN-II has a highly conserved primary structure from human to nematodes with a great percentage of homology with other mammals. This evolutionarily conserved structure, not limited only to the active site, suggest that cN-II plays an important physiological role in living organisms. Furthermore, it suggests that the physiological importance of cN-II is not only due to its enzymatic activity but a more general mechanism including expression and protein-protein interaction as well. As the physiological roles of cN-II are not very well defined, there is an increasing interest in exploring them. Although cN-II has been considered as a key modulator of intracellular AMP, a couple of studies have shown its contradictory role in promoting AMP-activated protein kinase (AMPK) phosphorylation and metabolism in human and mouse skeletal muscles (Kulkarni et al., 2011; Kviklyte et al., 2017). A recent study on *NT5C2*^{-/-} mice, it is observed that deletion of cN-II in mice protects against high fat diet-induced weight gain, adiposity, insulin resistance and associated hyperglycemia (Johanns et al. 2019). However, these observations were not dependent on AMPK. On the other hand, interesting physiological roles of cN-II in human brain and human neural progenitor cells (hNPCs) were also revealed in a recent study (Duarte et al. 2019). It has shown that cN-II plays an important role in the human nervous system development, regulation of AMPK signaling and protein translation as well as its implications in psychiatric disorders. The same study showed that *NT5C2* gene is also involved in motility behavior of *D. melanogaster*.

As described earlier, cN-II preferably catalyzes the dephosphorylation of 5'-monophosphates of 6-hydroxy purine nucleosides (IMP, GMP and XMP), it still has an unneglectable affinity for other monophosphate nucleotides (Carson et al. 1991). Therefore, it has been shown that cN-II helps maintain the pools of adenosine nucleotides in the tissues where cN-I (for which preferred substrates are adenosine nucleotides) has low expression *e.g.* brain and liver tissues (Itoh 2013). However, it has also been shown that cN-II silencing in skeletal muscle directly or indirectly increases intracellular AMP concentrations (Kulkarni et al. 2011). A possible explanation to this observation was suggested by MG Tozzi et al. in 2013 that cN-II silencing in skeletal muscles may cause an accumulation of IMP in the cell which will then be converted to AMP (purine cycle). An eventual drop in energy charge stimulate AMPK pathway to restore it (Tozzi, Pesi, and Allegrini 2013). It has also been observed that cN-II is particularly active in the livers of birds, crocodiles, lizards and snakes. The study has shown that its activity in chicken liver increases 2-folds by feeding a high-protein diet (Itoh 2013). This shows that cN-II is involved in protein metabolism and helps in the production of uric acid via IMP dephosphorylation. cN-II also helps replenish the increased demands of inosine in other cells by converting surplus IMP into diffusible forms *i.e.* inosine (Itoh 2013). Sufficient evidences have shown that cN-II activity is increased in response to enhanced accumulation of IMP in oxygen-independent sports *e.g.* 400 m and 800 m running race (Ipata and Pesi 2018; Hellsten et al. 1999). cN-II also holds a key position in the oxypurine cycle and its metabolomics interaction with other cycles like the purine cycle (Ipata and Pesi 2018). It is important to note here that among all other 5'-nucleotidases, only cN-II and cN-III possess phosphotransferase activities.

Pharmacological importance of cN-II

Nucleoside analogues: cN-II has been studied as an important modulator of pharmacotherapeutic effects of cytotoxic nucleoside analogues. Nucleoside analogues, as the name suggests, are structural analogues of physiological nucleosides and therefore upon administration, compete with them for transport proteins, metabolic enzymes and integration into nucleic acid synthesis. It is worth

mentioning that these drugs are administered in prodrug form and, therefore, must be converted to active forms by phosphorylation. This major step in their mechanism of action is achieved largely by the action of corresponding nucleoside kinases present in the cells, notably deoxycytidine kinase (dCK). Monophosphates are subsequently converted into di- and tri-phosphates with the help of corresponding enzymes (nucleoside monophosphate kinase and nucleoside diphosphate kinase). These triphosphate forms of nucleoside analogues then interact with various intracellular targets (RNR, DNA polymerase and CTP synthase etc.) and may finally integrate into the newly synthesized nucleic acids (DNA/RNA) of cancer cells to induce their pharmacological action. cN-II silencing in cultured cells, on the other hand, have shown an increased cytotoxic effect of gemcitabine (L. Li et al. 2012). However, this is still an hypothesis as it lacks enough experimental support as if this increased sensitivity is due to the direct effect of cN-II catalytic activity or indirectly because of the disturbance in other purines and pyrimidines pools in the cells (Tozzi, Pesi, and Allegrini 2013; Itoh 2013). Formerly it has been shown that dFdCMP (monophosphorylated gemcitabine) is not a good substrate for cN-II (Mazzon et al. 2003). Therefore, an optimum balance of expression as well as enzymatic activity is needed between cN-II and other enzymes involved in the transportation, activation and metabolism of nucleoside analogues for their required therapeutic efficacy.

From the above discussion, we can now imagine that any change in the expression profile or enzymatic activity of cN-II can have a direct or an indirect impact on the therapeutic effect of nucleoside analogues. There is a plethora of studies showing the association of cN-II expression and/or activity with the sensitivity towards nucleoside analogues. For example, it has been shown that deoxyadenosine-induced resistant human T lymphoblastoid CCRF-CEM cells have a 4-folds increased enzymatic activity of cN-II (Carson et al. 1991). These resistant CCRF-CEM cells have shown decreased sensitivity towards deoxyadenosine, deoxyguanosine, araC and araG (Carson et al. 1991). Furthermore, a 2.2-2.7 folds increase in cN-II activity has observed in cladribine-resistant human promyelocytic leukemia cells (HL-60) (Schirmer et al. 1998). Here, it is worth mentioning that these resistant cells showed only marginal decrease in the expression level or enzymatic activity of dCK,

confirming that increased cN-II activity is largely responsible for disturbing the crucial enzymatic balance explained earlier in this paragraph.

On the other hand, there are certain studies showing that the role of cN-II is not sufficiently evident as the reason for increased resistance towards nucleoside analogues. In this regard, human erythroleukemic cells K562, in addition to augmented cN-II activity, have also shown an increased activity of other enzymes involved in the metabolism of nucleoside analogues, in particular dCK (Dumontet et al. 1999). Similarly, CCRF-CEM cell model, when induced with etoposide (topoisomerase II inhibitor), showed cross-resistance towards both purine and pyrimidine nucleoside analogues (Lotfi et al. 2001) whereas increased cN-II activity is mainly associated with purine nucleoside analogues (Schirmer et al. 1998). Furthermore, this resistant cell model showed a 2-fold increase in cN-II activity. Another study has shown that cladribine- and clofarabine-resistant cells show an increased cN-II activity along with a huge decrease in dCK activity (39-46-folds) (Månsson et al. 2003). Based on all these evidences, it is very obvious that cN-II has a role to play in cells' sensitivity towards nucleoside analogues. However, discussion can be made on whether cN-II is maintaining this balance single handedly or just as a main collaborator with partner enzymes including dCK and others.

Certain studies have used another approach to study the role of cN-II activity in pharmacological effect of nucleoside analogues *i.e.* inhibition of cN-II expression using RNAi approach. Based on opposing results obtained using this approach in different cell models, it has been suggested that role of cN-II in cytotoxic effects of nucleoside analogues is tissue-specific or related to endogenous expression of cN-II *i.e.* low cN-II expressing cells are less sensitive as compared to high cN-II-expressing cells towards changing in the expression of cN-II and sensitivity towards nucleoside analogues (L. Li et al. 2008; Jordheim and Chaloin 2013; Wrabel et al. 2006; L. Li et al. 2008; Jordheim et al. 2015a). In this context, our group has shown that cells with short hairpin RNA (shRNA)-mediated inhibition of cN-II expression are associated with decreased enzymatic activity and increased sensitivity to purine nucleoside analogues and nucleobases (Jordheim et al. 2015b). It was also shown in the same study that increased

enzymatic activity of cN-II as a result of introduction of a hyperactive mutant (R367Q) in the cells, is associated with a decreased sensitivity to the same drugs (Jordheim et al. 2015b).

cN-II has shown an interesting association with nucleoside analogues as several hyperactive cN-II mutants (R238W, R367Q, S445F, R359Q, D407A and others) have been identified which play a role in resistance towards thiopurine nucleoside analogues in relapsed ALL patients. These relapse-specific mutations were first identified in cN-II gene (*NT5C2*) and result in a hyperactive cN-II enzyme (Meyer et al. 2013; Tzoneva et al. 2013). Later, another study demonstrated the rise and fall of clones in pediatric ALL patients from diagnosis to relapse and identified further *NT5C2* mutations and validated that increased mutation rate in *NT5C2* gene is significantly associated with early relapse (Ma et al. 2015). However, these mutations are not clonal in primary human tumors but may be subclonal. Also, a study has suggested that these mutations may not play an important role in the development of human tumor as these are very rare in new leukemia and solid tumors, with only two cases *i.e.* one in adult AML and the other in colon cancer (H. R. Oh et al. 2016). A 1.3 years old B-ALL patient was treated as a high risk ALL and 2.3 years post-complete remission relapsed and showed two *NT5C2* mutations (R367Q and D407V) in bone marrow and testicular relapse samples (Ding et al. 2017). In other studies, these hyperactive *NT5C2* mutations were shown to be associated with the relapse in leukemic patients (Richter-Pechańska et al. 2017) , T-ALL patients (Kunz et al. 2015) and acute promyelocytic leukemic patients (Lehmann-Che et al. 2018). Recent studies have further elaborated the underlying structural mechanisms of cN-II hyperactivity in its relapse-specific mutants. As mentioned earlier it has been shown that these genetic mutations are clustered into hotspots driving intersubunit stimulation (Hnízda et al. 2018b) and cause cN-II to become constitutively active without the need of allosteric activators (Hnízda et al. 2016). Furthermore, R367Q mutant phenotype is associated with increased purines export to the extracellular space resulting in a depletion of intracellular purine-nucleotides pools and is considered to be the key driver in the evolution of relapsed ALL (Tzoneva et al. 2018). The same group explained the in-depth structure-function analysis of constitutively active *NT5C2* mutations (K359Q and L375F). They showed that these mutations reconfigure the catalytic center for substrate

access and catalysis in the absence of allosteric activators (C. L. Dieck et al. 2018; C. L. Dieck and Ferrando 2019). A recent study has further elaborated that these cN-II mutants not only modulate thiopurine metabolism and cellular disposition, but also have an impact on endogenous nucleotide homeostasis (Moriyama et al. 2019). Based on these interesting findings and observations related to the role of relapse-specific cN-II hyperactive mutations in resistance towards 6-MP, efforts are now directed to develop small molecule inhibitors to reverse the effects of these mutations. Initial high throughput screens have identified the HTP_2 as a lead compound against *NT5C2* R367Q mutant recombinant protein (C. Dieck et al. 2019). However, there is an acute need of screening more soluble and potent analogue in order to move forwards into *in vivo* and clinical studies (C. Dieck 2019).

cN-II as a therapeutic target: In addition to the abovementioned roles of cN-II, it can also serve as a potential therapeutic target in cancer. It is based on the results shown by human astrocytoma cells which, when transfected with shRNA to downregulate cN-II expression, underwent cell death by apoptosis even in the absence of any anticancer drug (Careddu et al. 2008). Furthermore, as mentioned earlier, studies have shown that cN-II silencing is associated with increased sensitivity towards nucleoside analogues whereas its overexpression or hyperactive mutant is responsible for resistance towards these drugs. Therefore, these and other observations suggest that targeting cN-II alone or in combination with nucleoside analogues can have improved therapeutic benefits (Chiara Rampazzo et al. 2016). Several teams have done interesting research in developing chemical inhibitors for cN-II. In this regard, our team with the help of collaborators developed and studied cN-II inhibitors using three different approaches. One series of inhibitors came from virtual screening (Jordheim, Marton, et al. 2013), one is composed of substrate analogues that can not be dephosphorylated (Gallier et al. 2011; Meurillon et al. 2014; Nguyen Van et al. 2016), and the last is non-competitive cN-II inhibitors using fragment based drug designing approach (Marton et al. 2015; Guillon et al. 2019).

Biomarker properties of cN-II: Studies have shown that cN-II can serve as a predictive biomarker of response for certain cytotoxic drugs. Formerly, our group had shown that high cN-II expression in acute

myeloid leukaemia patients treated with cytarabine predicts shorter disease free survival (DFS) (C. M. Galmarini et al. 2001; Carlos María Galmarini et al. 2003; Carlos M. Galmarini, Jordheim, and Dumontet 2003). On the contrary, this is not the case in CLL patients treated with fludarabine (Mackey et al. 2005).

Since then, several studies on samples from patients with hematological malignancies have shown that either cN-II alone or both dCK and cN-II determines the intracellular accumulation of active phosphates of nucleoside analogues and ultimately their clinical outcome (Jordheim and Chaloin 2013). Earlier, *H Kawasaki et al.* had shown a relationship between dCK/cN-II ratio and response to cladribine (Kawasaki et al. 1993). They demonstrated that responders have significantly higher ratio of dCK/cN-II than non responders. Years later, another study showed a correlation between fludarabine monophosphate dephosphorylation and cN-II activity in peripheral blood cells of CLL patients (Albertioni et al. 2005). Furthermore, higher ratio of dCK/cN-II has been shown to be associated with a better clinical outcome for CLL patients treated with either fludarabine or cladribine and vice versa (Albertioni et al. 2007). Similar results were observed in another study regarding the association of dCK/cN-II ratio with the production of ara-CTP (phosphorylated and active form of cytarabine) both in blast cells from leukemic patients as well as in HL-60 cultured cell lines (Yamauchi et al. 2009). Another study using HapMap cells has also shown similar association of cN-II with cytarabine sensitivity (Mitra et al. 2011). This study also suggested a genetic variant to be predictive for clinical outcome of araC therapy due to its association with both cN-II expression and araC sensitivity. Moreover, cN-II gene has also shown correlation with the pharmacokinetic parameter of gemcitabine treated solid tumor patients (Mitra et al. 2011). Our group has shown that downregulation of cN-II expression using shRNA causes a decrease in its enzymatic activity and is associated with an increased sensitivity to nucleoside analogues (Jordheim et al. 2015a). On the other hand, stable transfection of cells with hyperactive cN-II mutants (R367Q) caused a decreased sensitivity towards nucleoside analogues (Jordheim et al. 2015a). On the contrary, solid tumor patients treated with gemcitabine showed better survival when cN-II expression

was high (Sève et al. 2005). Recently, a study has also shown cN-II as negative predictive factor for gemcitabine/platinum drugs combination in NSCLC treatment (Toffalorio et al. 2018).

In addition to the above-mentioned observations related to the role of cN-II in predicting the response of certain nucleoside analogues, several studies have also shown that certain mutations in cN-II gene (*NT5C2*) render the enzyme hyperactive and as a result cause resistance to thiopurine chemotherapy (C. L. Dieck and Ferrando 2019; Tzoneva et al. 2013; Meyer et al. 2013; Hnízda et al. 2018b). The detailed discussion on these mutations has been made in the section “nucleoside analogues” under the heading of “pharmacological roles of cN-II”. Finally a study has also demonstrated that circ-NT5C2 RNA expression was upregulated in certain osteosarcomas and that its silencing suppressed the proliferation and invasion of cancer *in vitro* and tumor growth *in vivo* (Xunfa Liu et al. 2017).

All these interesting publications and data suggest very strong basis for the use of cN-II both as diagnostic and predictive biomarker in the progression of disease and treatment with nucleoside analogues respectively. However, large multicenter studies have not been performed in order to validate this in large cohorts. Also, it is still difficult to determine cN-II expression or activity in a reliable manner in routine clinical settings.

Pathological functions of cN-II

Holding a central position in purine metabolism, cN-II has been gaining exponential attention of researchers from all domains of science for its direct or indirect involvement in different pathological conditions as well as its clinical relevance. The enzymatic activities and biochemical perspectives of cN-II have shown to be responsible for its diverse pathological roles which are both biological and genetic in nature (Jordheim 2018).

Hereditary spastic paraplegia (HSP) is considered to be of various genetic origins. The involvement of cN-II in HSP was first demonstrated in 2009 when it was observed that a locus of *NT5C2* gene is associated with HSP in a consanguineous family (Dursun et al. 2009). Since then several studies have

further identified different mutations in *NT5C2* gene which are related to HSP (Novarino et al. 2014; Elsaid et al. 2017; Darvish et al. 2017; Straussberg et al. 2017).

An increasing number of studies have also shown the role of cN-II in schizophrenia since the first linkage between *NT5C2*-harboring locus and this pathological condition was established (Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium 2011). Several gene loci in the genomic region of *NT5C2* have been demonstrated to be associated with different psychiatric disorders including schizophrenia (Aberg et al. 2013; Bergen et al. 2012; Cross-Disorder Group of the Psychiatric Genomics Consortium 2013; Guan et al. 2016).

Interestingly, a SNP in the intergenic region between *NT5C2* and *CNNM2* has been shown to be linked with the regulation of systolic blood pressure in European as well as Asian population (International Consortium for Blood Pressure Genome-Wide Association Studies et al. 2011; Kato et al. 2011; C. Li et al. 2017). It was further demonstrated in a study that hypertensive military pilots have higher expression of cN-II than control pilots (X.-C. Zhao et al. 2018). Finally, certain SNPs in the genomic region of cN-II have also been shown to have a link with body mass index (BMI) and body fat (Hotta et al. 2012; Wen et al. 2014). However, all these studies have demonstrated a mere correlation with blood pressure and BMI, and further evidences are required to establish a functional involvement of cN-II in these disorders.

In addition to the catalytic roles of cN-II in different pathological as well as physiological functions, our team has also initiated the hypothesis of its regulatory role through its physical protein interactors. In this regard, we have shown that cN-II interacts with LRR domain containing Ipaf, a protein which belongs to the family of NBS-LRR (nucleotide-binding site and leucine-rich repeat), and has been shown to be involved in the intracellular pathways associated with inflammatory responses (Federico Cividini, Tozzi, et al. 2015). However, functional impact of this protein-protein interaction needs further investigation.

Importance of cN-II in cancer cell biology

In my PhD project, we are interested in deciphering the role of cN-II in cancer cell biology. In addition to the already mentioned roles as a biomarker for cancer treatments, the mutations described as increasing its activity in relapses patients, the effect of over- and underexpression of cN-II in cell models, and the druggability of the enzyme, several studies have described the roles of cN-II in the biology of various cancer cell models.

In particular, our group and the one of MG Tozzi in Pisa, have developed and studied several cell models in which cN-II expression and activity is modulated by stable shRNA, CRISPR-Cas9 or transfection of wild-type or mutant cN-II. A decreased expression of cN-II in human glioblastoma cells (ADF) causes a modified cell morphology and a decrease in cell viability via activation of apoptotic pathways (F. Cividini et al. 2015). Further, a decreased proliferation rate in cN-II downregulated cells and increased rate in cN-II over-expressing cells was also observed. In the same study, it was also shown that cN-II is involved in the cellular response to fludarabine and cisplatin (F. Cividini et al. 2015). This observation was later confirmed in another cell model of human lung cancer (A549) which expresses higher cN-II than the average value measured in different normal tissues. Here, silencing of cN-II expression was associated with a less proliferative and more oxidative phenotype (Pesi et al. 2018). However, formerly our team has shown that knocking down of cN-II expression using shRNA caused no difference in the cell proliferation in HL-60 and RL cell lines (Jordheim et al. 2015a). The same observations were made in the cell lines of solid tumors *i.e.* MDA-MB-231, MIA PaCa-2, HCT-116 and NCI-H292 (Bricard et al. 2016).

In a separate study, cN-II overexpression has been shown to be linked with a general disturbance in nucleotides pools and an increased resistance towards fludarabine, gemcitabine and cytarabine (Federico Cividini, Filoni, et al. 2015). As monophosphate forms of these drugs are not the substrate of cN-II, therefore, it was suggested that this lower sensitivity might not be due certain regulatory effects which are independent of the enzymatic activity of cN-II (Federico Cividini, Filoni, et al. 2015). This idea

was later strengthened by the identification of Ipaf being a protein interactor of cN-II suggesting roles beyond the catalytic activity, potentially through the regulation of other proteins by direct interaction (Federico Cividini, Tozzi, et al. 2015). Furthermore, our group has recently shown that downregulation of cN-II in breast cancer cells (MDA-MB-231) make them better adapt to glucose deprivation and also show enhanced *in vivo* tumor growth under normoxic conditions (Bricard et al. 2017). However, under hypoxic conditions, cN-II downregulation profile is more sensitive to decreased glucose in culture media (Bricard et al. 2017). These observations suggest that cN-II has a regulatory involvement in cellular biology under certain stress conditions and this role of cN-II is apparently independent of its enzymatic activity.

Despite all these advances in understanding the importance of cN-II in cancer, much is still undone, in particular about the implications of the enzyme on different biological aspects of cancer cell.

Ecto 5'-nucleotidase – CD73

General introduction

Ecto-5'-nucleotidase (eN, eNTDase, EC 3.1.3.5, CD73) is a 574 aa long cell surface enzyme encoded by *NT5E*. It was given cluster of differentiation (CD) 73 in 1987 because it is abundantly expressed on vascular endothelium and some lymphocyte subpopulation and hence used as differentiation marker for lymphocytes (Thompson, Ruedi, and Low 1987). It is a 70-kDa glycosylphosphatidylinositol-anchored glycoprotein which holds a pivotal position in maintaining the extracellular pool of the nucleosides and nucleotides (Zimmermann 1992). CD73 can be cleaved from the cell surface and, therefore, maybe found in soluble forms (Vogel et al. 1992). It shows its physiological functions in close collaboration with other like-minded enzymes present in its vicinity. These include, but are not limited to, CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1; ecto-NTPDase1) and E-NPP (ecto-pyrophosphate-phosphodiesterases). Any change in the extracellular pool of nucleotides not only disturb the regulation of cellular microenvironment but also affect the intracellular pool by resultant

cross-membrane movement of nucleosides as well as signaling through ATP and adenosine receptors. Therefore, a critical balance between nucleotides and nucleosides is needed in order to ensure proper physiological functioning of the cell. It is well documented that ATP is released in the extracellular space by stressed or damaged cells which is converted into ADP and AMP by the enzymatic activity of CD39. However, NPP-1, a dimeric enzyme from alkaline phosphatases (ALPs) family of enzymes, converts extracellular ATP directly into AMP and pyrophosphate. Furthermore, TNAP, is the enzyme which can convert ATP directly into adenosine. AMP is generated extracellularly in three different ways *i.e.* i) by the enzymatic activity of CD39 using ATP as substrate, ii) from extracellular NAD by the enzymes CD38 and CD203a iii) by CD73 using NAD as substrate and generating AMP. However, AMP generated extracellularly via different alternative pathways, in turn, serves as a substrate for CD73 that finally hydrolyzes it into adenosine. Therefore, among different enzymes involved in maintaining nucleotide balance, CD73 is holding the key position as it catalyzes the final step of nucleotide conversion into nucleoside. However, it is noteworthy that CD73 is not the only enzyme responsible for the extracellular hydrolysis of AMP, rather prostatic acid phosphatase (PAP) has also been identified as an ectonucleotidase which, alongwith many other established physiological functions, also generate adenosine by hydrolyzing extracellular CD73 (Zylka et al. 2008). Extracellular adenosine has diverse biological functions, both physiological and pathophysiological. Furthermore, catalytic activity of CD73 also affects extracellular regulation of microenvironment as adenosine can act on all cells and not only on the cell expressing CD73.

Structural insight and enzymatic regulation of CD73

CD73 is believed to be a member of a large superfamily of distantly related metallo-phosphoesterases, which include protein phosphatases, nucleotidases and nucleases. Based on primary sequence similarity, eukaryotic eN is considered to be related to the bacterial counterpart called 5NT. However, there are obvious structural and functional differences between bacterial and eukaryotic 5NT. For example, bacterial 5NT is a monomeric structure whereas eukaryotic is a dimeric one. Also, bacterial

5NT catalyzes AMP, ADP and ATP whereas eukaryotic 5NT specifically hydrolyzes nucleotides monophosphates (NMPs) (Zimmermann 1992). No structure of human CD73 was available until 2012 when *Knapp et al.* proposed the crystal structure of human CD73 and identified important structural and functional domains in it. This helped a lot in terms of structure-based drug design as well as molecular basis of enzyme functioning.

Human CD73 exists as a non-covalent homodimer composed of 60-80 kDa subunits (Zimmermann 1992) in two distinct open conformations, crystal form I (110.6 x 78.5 x 55.2 Å) and crystal form II (127.0 x 70.5 x 57.0 Å) and one elongated closed conformation known as crystal form III (141.1 x 57.9 x 58.5 Å) (Knapp et al. 2012). Each subunit of the dimer is composed of two structural domains: the N-terminal domain (residues 27–317) and the C-terminal domain (residues 337–549). Furthermore, the structure of CD73 harbors several important sites as well *i.e.* metal ion-binding site, substrate-binding site dimerization interface and active site (Knapp et al. 2012). Metal ion-binding site is present in the N-terminal domain whereas substrate binding site and dimerization interface is located on the C-terminal domain of CD73 dimer. Importantly, active site is located at the interface between two domains and comprised of amino acids from both domains. Both N- and C-terminal domains are linked together via single α helix (residues 318–336) in such a way that it forms a hinge region which allows the enzyme to undergo extensive domain movements. (Knapp et al. 2012). Open conformation of CD73 is important in terms of substrate binding and product release whereas closed conformation corresponds to the substrate-enzyme complex.

Several crystal structures of human CD73 in open or closed conformation have been published so far. They include the structures of CD73 in soluble form (Heuts et al. 2012), CD73 complex with antibody IPH53 (Perrot et al. 2019), adenosine (both crystal forms I and II) and chemical inhibitors (PSB11552, baicalin and AMPCP) (Knapp et al. 2012).

It is important to briefly mention here that one of the major differences between human CD73 and other species is that in human, alternative splicing causes the expression of several transcripts variants

(Snider et al. 2014). Five different splice variants in human has been identified so far. Among these, only NT5E-1 and NT5E-201 (commonly known as NT5E2) have been studied in detail. The only difference between the two transcripts is that NT5E-2 lacks exon 7. The protein transcribed by NT5E-1 is termed as canonical CD73 (also CD73L, L for long) and by NT5E-2 is termed as shorter CD73 (CD73S) as the latter is short of 50 amino acids (404-453) at C-terminal domain. Lack of 50 amino acids, including the key functional amino acid Phe417 and dimerization domain, in the C-terminal domain makes CD73S to have compromised enzymatic activity and distinct dimerization properties. Unlike canonical CD73, CD73S is expressed in the cytoplasm complexed with an endoplasmic reticulum chaperon, calnexin.

Physiological roles of CD73

CD73 regulates a wide range of physiological and pathophysiological functions in the cells. These functions are mediated either by the production of adenosine from AMP or via adenosine-independent CD73 signaling. In the latter case, CD73 shows its effects via its glycosylphosphatidylinositol anchor, protein-protein interactions, and other mechanisms. According to *The human protein atlas* CD73 protein and mRNA is ubiquitously expressed in a wide range of human tissues as shown in the figure 6.

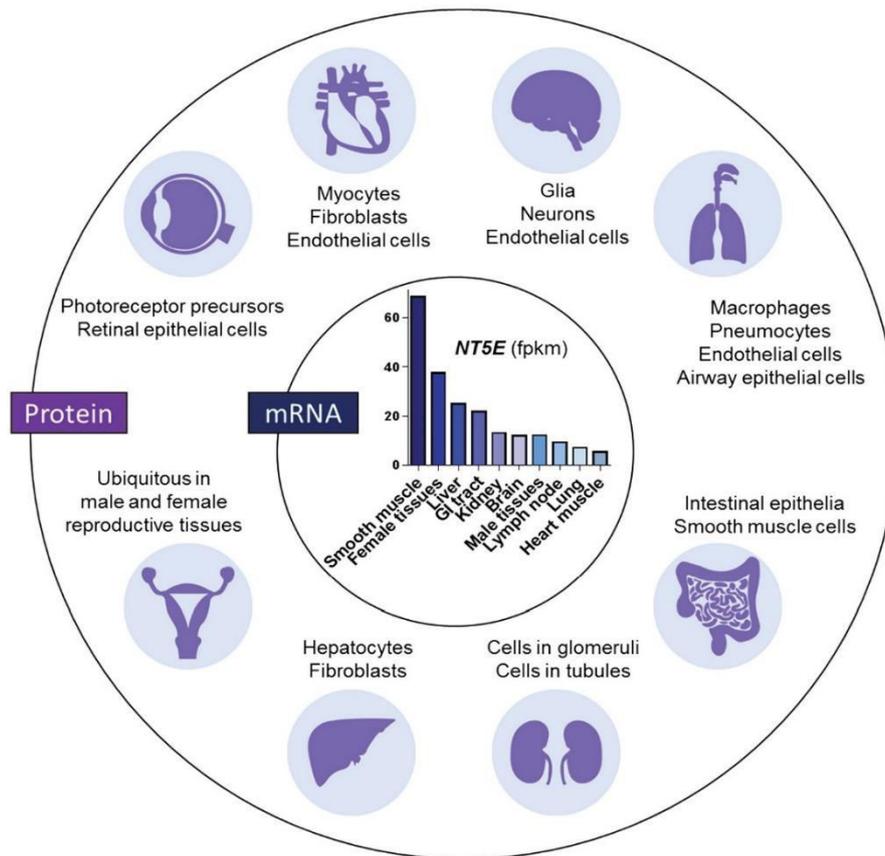


Figure 6: Tissue-specific expression (at mRNA and protein level) of human CD73. Figure adapted from (Minor et al. 2019a).

Molecular functions: There are numerous physiological functions, which are regulated by CD73-generated adenosine, depending on the tissue, cell type and of course the expression of receptors and the corresponding intracellular machinery for signaling. These roles include maintenance of epithelial transport and integrity, ischemia, inflammation and hypoxia (Colgan et al. 2006; Minor et al. 2019b). On the other hand, adenosine-independent functions of CD73 include interaction of CD73 with components of extracellular matrix *e.g.* glycoprotein, tenascin-C. The same study showed that this interaction might have a functional role in the adhesion and migration of MDA-MB-231 breast cancer cells (Rafal Sadej et al. 2008). Furthermore, CD73 serve as a costimulatory molecule in T cell activation (Resta and Thompson 1997), facilitator for binding of lymphocyte to the endothelium (Airas et al. 1995; Airas, Niemelä, and Jalkanen 2000) and protector against TRAIL-induced apoptosis (Mikhailov et al. 2008). Additionally, it plays its role by inducing phosphorylation of certain proteins in lymphocytes and

endothelial cells in response to antibody ligation (Airas et al. 1997; Dianzani et al. 1993), as well as by inhibiting metastasis of human breast cancer cells in response to anti-human CD73 monoclonal antibody (Stagg et al. 2011; Terp et al. 2013). Apart from abovementioned functions, there are other roles of CD73 which still need further investigation in order to be sure if they are mediated through adenosine or independent of its AMPase activity. Interestingly, it was proposed about two decades ago that CD73 might be considered as a receptor for a putative endogenous ligand (Resta and Thompson 1997).

Physiological functions: CD73 has shown to be involved in physiological functioning of different human tissues including CNS, heart, liver and kidney. A brief detail of the major physiological roles of CD73 is outlined below.

Heart and epithelium The role of CD73 in heart and CVS has been well discussed and established in scientific community (Olsson 2004; Minor et al. 2019b). CD73 has been shown to be expressed in different tissues related to cardiac physiology *i.e.* endothelial cells (Pluskota et al. 2013; Ohta et al. 2013), smooth muscle cells (J. Yang et al. 2015) as well as resident cardiac immune cells (Bönner et al. 2012). However, there is a good deal of discrepancy related to the expression of CD73 in different tissues related to cardiovascular system (Minor et al. 2019b). Upregulated CD73 on granulocytes and T-cells infiltrating injured heart, in collaboration with CD39 and other ATP catalyzing enzymes, helps in healing process via adenosine production after an ischemic injury (Bönner et al. 2012) and cardiac arrest (Ryzhov et al. 2019a). On the other hand, CD73-mediated adenosine production and interaction with tenascin-C promote cytokine production through epicardium-derived cells (EPDCs) after myocardial infarction (MI) (Hesse et al. 2017a). Additionally, CD73 expression has been reported to be upregulated in multiple T cell populations in a mouse model of heart failure. This increased expression of CD73 is considered to play a protective role via its anti-inflammatory effects through adenosine production (Quast et al. 2017a). All this data shows that CD73 expression and contradictory roles in

different cardiac physiological processes demand a careful consideration while developing anti-CD73 therapy in diseases like cancer.

Lungs Human Protein Atlas has reported higher expression of CD73 in pneumocytes which makes CD73 as one of the major contributors to extracellular adenosine production (Thul et al. 2017; Picher et al. 2003). This extracellular adenosine plays its part in physiological functioning of human airways epithelial cells like proper cilia beating frequency and ion transport through adenosine receptors expressed on these cells (Morse, Smullen, and Davis 2001; Lazarowski et al. 1992). Additionally, it has shown to have protective roles in the times of crisis like inflammation, bacterial infection and acute lung injury by virtue of its anti-inflammatory and anti-fibrotic effects (Volmer, Thompson, and Blackburn 2006; Ehrentraut et al. 2013), regulation of pulmonary neutrophils recruitment (Bou Ghanem et al. 2015) and by maintaining vascular endothelial integrity, respectively (Eckle et al. 2007). Therefore, adenosine accelerates the healing process in certain chronic pulmonary diseases like asthma and chronic obstructive pulmonary disease (COPD) which are characterized by inflammation. On the contrary, CD73 enhances radiation-induced lung fibrosis associated with increased tissue infiltration of immune cells and a significant increased adenosine concentration (Wirsdörfer et al. 2016a). IFN-beta upregulates the expression of CD73 and is associated with a reduced mortality rate in patients with acute respiratory distress syndrome (Kiss et al. 2007; Bellingan et al. 2014). In hyperoxic lung injury, CD73-generated adenosine promotes vascular barrier function and minimizes abnormal alveolar development in newborn mouse model of hyperoxia (Davies et al. 2014; H. Li et al. 2017).

Liver CD73 is expressed on the apical membrane of hepatocytes as well as the endothelial cells (Matsuura et al. 1984). Recruitment of regulatory B cells in response to activated invariant natural killer T cells (iNKT) suppress hepatic inflammation in a CD73-dependent pathway (Almishri, Deans, and Swain 2015). Furthermore, CD73 has been shown to have protective roles during murine hepatic IP (Hart et al. 2008)

Kidneys CD73 collaborates with tissue nonspecific alkaline phosphatases (TNAP) for the production of adenosine via metabolizing luminally applied AMP (Jackson et al. 2014). CD73 has been shown to mediate adenosine-mediated protective roles in different renal disorders including but not limited to renal ischemia reperfusion (Kim et al. 2013; Sung et al. 2017) and diabetic nephropathy (Tak et al. 2014; Oyarzún et al. 2015; Taşkıran et al. 2016).

CNS Several studies have shown the role of CD73 in brain physiological functions including locomotion and social behavior (Augusto et al. 2013; Kuleskaya et al. 2013), auditory cortex plasticity (Blundon et al. 2017), brain cognitive functions (Zlomuzica et al. 2013), regulation of sleep (Zielinski et al. 2012) and temperature (Muzzi et al. 2013), infections (Bynoe et al. 2012) and protective roles in inflammation (Mills et al. 2008; Petrovic-Djergovic et al. 2012; Shun Xu et al. 2018).

Bone Role of CD73 in osteophysiology is mediated through adenosine which is involved in triggering osteoblast differentiation process (Costa et al. 2011; Takedachi et al. 2012), bone marrow-derived mesenchymal stem cell development as well as protective roles in bone injuries (Katebi, Soleimani, and Cronstein 2009) and bone formation (Mediero et al. 2015). Furthermore, accumulative data has shown the imperative role played by adenosine and its receptors in metabolism of osteoblasts and osteoclasts (Mediero and Cronstein 2013).

Endothelial integrity CD73-generated adenosine protects endothelial barrier functions as CD73 deficient mice suffered from massive vascular leakage and pulmonary edema under hypoxic conditions (Bowser and Broaddus 2016; Colgan et al. 2006). The studies have also shown that mRNA expression of CD73 increases in mucosal inflammations which causes an increased CD73-generated extracellular adenosine. This adenosine, in turn, works as a negative regulator of inflammation and shows protective functions (Bynoe et al. 2012; Louis et al. 2008). Another study has shown the evidence that CD73 and adenosine receptor signaling attenuate the toxin induced damage to epithelial barrier integrity by certain bacterial toxins like *Clostridium difficile* (Schenck et al. 2013).

Pathological functions of CD73

Considering the very vital roles and physiological importance of CD73 in different organ systems, it is obvious that any change in its expression or catalytic activity might have pathological outcomes. In order to explore these unwanted outcomes, Thompson et al generated CD73 knockout mice to show the role of CD73 in vascular leakage during hypoxia (Thompson et al. 2004a). Since then, these mice have been used in several studies related to this subject. Like its physiological roles, CD73 shows its pathological roles in several ways. *i.e.* I) via extracellular production of adenosine and its function in extracellular domain II) via adenosine produced extracellularly and showing its effects intracellularly either through adenosine receptors or adenosine transporters III) via protein expression of CD73. Several important studies suggesting the role of CD73 in different disease conditions are outlined in the *Table 3*. However, this table is just a representative of different roles of CD73 in various disorders related to different organ systems. Therefore, its pathological roles should not be considered limited only to the information in the table. The detailed explanation of CD73 role in all pathological conditions is out of the scope of this thesis.

Table 3: Role of CD73 in different pathological conditions.

Organ	Disease	Role of CD73	Reference
CNS	Hypolocomotion and decreased working memory performance	Mediates the formation of adenosine for striatal adenosine A2AR functions	(Augusto et al. 2013)
	Decreased auditory cortex plasticity	Mediates the formation of adenosine to activate A1R from auditory thalamus in mice	(Blundon et al. 2017)
	Brain inflammation	Regulates the entry of lymphocytes into CNS during experimental autoimmune encephalomyelitis Regulates leukocytes trafficking in Ischemic brain	(Mills et al. 2008; Petrovic-Djergovic et al. 2012; Shun Xu et al. 2018)

		Attenuates inflammation by promoting macrophages/microglia M2 polarization in mice.	
	Nociception	A1R-mediated regulation of pain sensory neurons	(Sowa, Voss, and Zylka 2010; Street et al. 2013)
Liver	Fatty liver	Mediates ethanol-induced fatty liver	(Peng et al. 2009)
	Hepatic fibrosis	Mediates adenosine production Involved in CCl ₄ - and bile duct ligation-induced liver fibrosis in mice Transcriptional upregulation of CD73 in activated hepatic stellate cells	(Peng et al. 2008; P. Yang et al. 2010)
	Mallory-Denk bodies (MDBs)	Involves in liver enlargement, absence of p62 induction and aggregation of K8/K18 Decreased mRNA expression of CD73 in human NAFLD biopsy specimen	(Snider et al. 2013)
	Hepatic cirrhosis	Mediates effect through adenosine via A _{2A} Receptor	(Chan et al. 2006)
	Hepatic Steatosis	NT5E gene deletion reduced the development of hepatic steatosis in mice	(Peng et al. 2009)
Lungs	Vascular leakage	NT5E ^{-/-} mice showed vascular leakage in response to hypoxia CD73 is upregulated in response to IFN-β ₁	(Thompson et al. 2004a; Bellingan et al. 2014)
	Hyperoxia	Upregulates in hyperoxia NT5E ^{-/-} mice develop more severe pulmonary edema	(Davies et al. 2014; H. Li et al. 2017)
	Inflammation and fibrosis	Enhances radiation-induced lung fibrosis via adenosine production	(Wirsdörfer et al. 2016b)
Heart	Myocardial Infarction	Increased expression of CD73 in human monocytes <i>in vitro</i> by MSCs Mediates cytokine production by epicardium-derived cells formed after myocardial infarction	(Monguió-Tortajada et al. 2017; Hesse et al. 2017b)
	Cardiac arrest	Increased number of CD73 ⁺ lymphocytes is associated with improved survival	(Ryzhov et al. 2019b)
	Heart Failure	Inhibits cardiac inflammation and fibrosis via adenosine production by CD73 on T cells	(Quast et al. 2017b)
Kidney	Chronic Kidney Disease	Overexpression and leads to a more aggressive disease via adenosine and HIF-1α dependent pathway	(W. Zhang et al. 2013)

In addition to the outlined pathological roles of CD73, missense loss-of-function mutations in CD73 gene (NT5E) were identified to be a cause of a rare disease known as “arterial calcification due to deficiency of CD73 (ACDC)” characterized by symptomatic arterial and joint calcification (St Hilaire et al. 2011; H. Jin et al. 2016). Furthermore, it has been shown that NT5E-2 is expressed at low levels in

most human tissues under normal conditions, however, in liver cirrhosis and cancer the expression level of both NT5E-2 and its protein CD73S are upregulated (Snider et al. 2014). CD73 expressed on vascular smooth muscles facilitate the formation of atherosclerotic plaque (J. Yang et al. 2015). Additionally, it is gaining importance as a key therapeutic target for antifibrotic treatments in liver diseases as it is upregulated in the activated liver myofibroblasts (Fausther et al. 2012). CD73 expression on effector T cells sustained by TGF- β facilitates tumor resistance to anti-4-1BB/CD137 therapy (S. Chen, Fan, et al. 2019). In livers, CD73-mediated extracellular adenosine production has been shown to play a critical role in the pathogenesis of hepatic fibrosis (Peng et al. 2008). It has also been suggested that CD73 may serve as a novel cellular marker of activated liver fibroblasts as it is upregulated in hepatic stellate cells after myofibroblast differentiation (Fausther et al. 2012). Furthermore, there are evidences of the role played by CD73 and consequent adenosine production in the development of ethanol-induced fatty liver in mice (Peng et al. 2009). CD73 also plays an important role in severe hepatocyte injury as knockout mice have shown no signs for Mallory-Denk bodies (MDBs) aggregates, characteristic of hepatocytic injury (Snider et al. 2013).

CD73 expression is increased in hypertensive chronic kidney disease patients, and this in turn induces the expression of ADORA2B receptors via adenosine production. This increased adenosinergic signaling profile is responsible to aggravate the hypertensive CKD via HIF-1 α dependent endothelin-1 induction (W. Zhang et al. 2013).

Role of CD73 in cancer

CD73 has been shown to play an important role in tumor progression in different ways. A cancer cell harbors growth and survival mechanisms at the same time as uncontrolled growth is its hallmark and this in turn creates a challenging microenvironment lacking all the important growth factors like oxygen, glucose and nutrients (Weber and Kuo 2012). This challenging environment is further spiced up by the invasion of host army of immune cells once the cancer cell is identified. So, cancer cell introduces several game changing modulations in order not only to survive but also to grow under such

harsh environment (Hanahan and Weinberg 2011). These reprogramming mechanisms are characterized by altered expression of proteins, catalytic activities of certain enzymes, production and release of protumoral factors etc. Among many other proteins, a plethora of studies has shown that CD73 is overexpressed in cancer cells (Antonioli, Pacher, et al. 2013; Antonioli, Blandizzi, et al. 2013). Different studies demonstrating the role of CD73 in tumor development or progression has been summarized in *Table 4*.

Table 4: Roles of CD73 in different cancer types identified so far with the references to the respective study

Cancer Type	Experimental models	Role of CD73	Reference
Brain	C6, U138MG	Expression of CD73 is associated with increased cell proliferation	(Bavaresco et al. 2008)
	CD4+ T lymphocytes and malignant glioma cells of patients	Glioma-derived CD73 collaborates with CD39 of infiltrating CD4+ T lymphocytes and results in immunosuppression	(Shuo Xu et al. 2013)
	T98G, SVGp12	High expression is associated with multi-drug resistance in glioblastoma	(Quezada et al. 2013)
	Daoy, ONS76, D283	Absence of CD73 expression in D283 (metastatic medulloblastoma cell line)	(Cappellari et al. 2012)
Melanoma	SKMel2, SKMel23, WM35, Mel501, Mel505, C81-61	Methylation-dependent transcriptional silencing of CD73 mRNA in primary and metastatic melanomas. Lack of CD73 methylation is associated with relapse	(H. Wang et al. 2012)
	A375	Decreased expression is associated with decreased cell migration and invasion	(Rafal Sadej and Skladanowski 2012; R. Sadej, Spychala, and Skladanowski 2006)
Breast Cancer	Normal mammary gland and breast carcinoma cells	CD73 is differentially expressed in different types of normal and cancerous breast cells	(Krüger et al. 1991)
	Breast cancer cells and tissues	CD73 regulates the expression and phosphorylation of EGFR	(Zhi et al. 2012)
	MDA-MB-231	Interaction with Tenascin C and generates adenosine CD73 may promote tumor growth	(Rafal Sadej et al. 2008; Xuerui Zhou et al. 2007)
	T-47D, MDA-MB-231	Overexpression increases invasion, migration and adhesion	(Li Wang et al. 2008; P. Zhou et al. 2007)

	4T1.2, E0771	Anti-CD73 mAb therapy delayed <i>In vivo</i> tumor growth and metastasis	(Stagg et al. 2010)
	Breast cancer Patients (stage I-III)	Increased expression is associated with a better prognosis	(Supernat et al. 2012)
	Patients (TNBC, luminal BC, HER2+ BC)	Overexpression of CD73 confers resistance to doxorubicin, <i>NT5E</i> gene expression is significantly associated with poor prognosis in TNBC patients but not in luminal or HER2+ patients	(Loi et al. 2013)
Pancreas	Panc-1, AsPC-1, SW1990, BxPC-3, MIAPaCa-2, and CFPAC-1, HPDE6-C7	CD73 is overexpressed in PDAC Poor prognosis	(L. Zhou et al. 2019; Haun et al. 2015)
Cervical cancer	Hela, SiHa	Overexpression promotes cell proliferation and migration independent of its enzymatic activity	(Z.-W. Gao et al. 2017)
	CRC patients	High expression as a poor prognostic biomarker	(Wu et al. 2012; N. Liu, Fang, and Vadis 2012)
Bladder	RT4, T24	Higher enzymatic activity in T24 grade 3 bladder cancer cell line than in RT4 grade 1 cells	(Stella et al. 2010)
Gallbladder cancer	Cancer patients	NT5E expression was associated with decreased survival time	(Xiong et al. 2014)
Colon	CRC Tissues	Higher CD73 expression as a poor prognostic biomarker	(Wu et al. 2012)
	Peritumoral tissues	Increased CD73 expression in both tumoral and stromal compartments Combined CD73 expression in malignant epithelial cells and tumor stroma have a better prognostic value	(B. Zhang et al. 2015)
Ovarian cancer	Ovarian cancer patients	Overexpression is associated with better prognosis	(H. K. Oh et al. 2012)
	SKOV3	High expression of functional CD73	(D. Jin et al. 2010)
Gastric Carcinoma	Cancer patients	CD73 poor prognostic indicator	(Lu et al. 2013)
Leukemia	Patients (leukemia, myelodysplastic syndrome, non-leukemic, healthy adults)	CD73 expression is associated with leukemia subtype, differentiation and development. In B-lymphoid leukemia, there is an inverse relationship with CD73 expression but no such relation in T-ALL	(S.-X. Zhao et al. 2011)
	Patients (chronic lymphoblastic leukemia)	Overexpression is associated with more aggressive and proliferative disease	(Serra et al. 2011)
	Patients children (ALL)	No prognostic value of CD73 expression	(Wieten et al. 2011)
Prostate cancer	Prostate cancer patients	Overexpression is associated with lymph node metastasis	(Qing Yang, Du, and Zu 2013)

Head and Neck	Head and Neck squamous cell carcinoma (HNSCC) patients with active disease and no evident disease after therapy	Increased expression of CD73 and CD39 in T reg cells of HNSCC patients	(Mandapathil et al. 2009)
Thyroid	Fresh thyroid tissue samples normal thyroid (3), nodular goitre (3), follicular adenoma (3), papillary thyroid carcinoma (PTC) (3)	Increased expression and enzymatic activity of CD73 in PTC	(Kondo et al. 2006)

Role of CD73 in *in vivo* tumor growth: Role of CD73 in tumor progression and initiation has also been shown in several *in vivo* studies. Indeed, CD73 deficient mice have shown decreased growth of primary tumors as well as metastasis in a melanoma model (G. G. Yegutkin et al. 2011). Similarly, CD73 deficient mice has shown resistance to the development of 3-methylcholanthrene (MCA)-induced fibrosarcomas (Stagg et al. 2012), subcutaneous tumors and lung metastasis (Stagg et al. 2011). However it is important to mention that *in vivo* tumor growth is also dependent upon the immune-status of the mice as well as CD73 expression on both cancer cells and immune cells of the mice.

Drug resistance and CD73: CD73 has been shown to have a role in resistance towards certain cytotoxic drugs (Ujházy et al. 1994; Quezada et al. 2013; Holohan et al. 2013). Initially, a study has shown that, AMP-CP (alpha,beta-methyleneadenosine 5'-diphosphate), an inhibitor of CD73 enzymatic activity, reverses the resistance of CD73 positive MDR cells towards doxorubicin (Ujházy et al. 1996). Further study using breast cancer mouse models and TNBC patients has shown that CD73 promotes anthracycline resistance and poor prognosis (Loi et al. 2013). Gene expression of CD73 and HER3 has been identified as a potential predictive marker for the efficacy and resistance of cetuximab in metastatic colorectal cancer (Cushman et al. 2015). A recent study has shown that CD73 expression on infiltrating CD8⁺ T cells sustained by TGF- β , richly present in tumor microenvironment, confers resistance to agonistic anti-4-1BB/CD137 Ab therapy (S. Chen, Fan, et al. 2019).

CD73 and onco-immunology: An accelerated effort is ongoing in the field of cancer immunotherapy to develop antibodies against CD73 as it is considered to be an effective and emerging checkpoint in cancer immunotherapy (Hammami et al. 2019; S. Chen, Wainwright, et al. 2019). Apart from the biological roles of CD73 in cancer, increasing evidences have shown that it has a role in immune evasion during cancer development and progression (D. Jin et al. 2010). As mentioned earlier, enzymatic activity of CD73 results in the production of immunosuppressor adenosine in the extracellular domain. It was demonstrated that coordinated expression of CD39/CD73 on T regulator cells as well as A2A adenosine receptors on activated T effector cells constitutes an immunosuppressive phenotype (Deaglio et al. 2007; Schuler et al. 2014; Gourdin et al. 2018). Further studies have shown that CD73 expressed by both host and tumor cells have distinct functions and collaborate with each other to protect tumors from attacking antitumor cells in the body defense system (Long Wang et al. 2011). Additionally, exosomes secreted by cancer cells are found to be rich in the expression of CD39 and CD73, enzymes involved in the adenosine production from ATP which in turn may negatively regulate the immune response (Clayton et al. 2011). Furthermore, CD73 expression by Th17 cells promotes tumor growth via immunosuppressive fate of Th17 cells (Chalmin et al. 2012).

Pharmacological importance of CD73 in cancer: Owing to the increasing evidences showing the role of CD73 in cancer initiation, progression and metastasis, it has grabbed the attention of scientific community as a novel and promising therapeutic target. Therefore, efforts have already been started to develop effective ways to counter CD73 in order to achieve favorable therapeutic outcomes by developing small chemical inhibitors (Baqi et al. 2010; Iqbal et al. 2013; Rahimova et al. 2018; Dumontet et al. 2018; Ghoteimi et al. 2019) and monoclonal antibodies (Perrot et al. 2019; Hay et al. 2016). Furthermore, CD73 targeting can also be used as a synergistic approach as it enhances the therapeutic activity of anti-PD-1 and anti-CTLA-4 mAbs (Allard et al. 2013). Currently, some mAbs against CD73 and small molecule inhibitors are in different phases of clinical trials in order to evaluate

their efficacy either alone or in combination with other immune checkpoint inhibitors. A complete account of the ongoing clinical trials is outlined in the reviews recently published (Hammami et al. 2019; Boison and Yegutkin 2019). For example, one of the anti-CD73 mAB (MEDI9447; oleclumab) is in the phase 1 multicenter, open label, non-randomized clinical trial to evaluate the clinical outcome of its use alone or in combination with anti-PD-L1 (MEDI4736; durvalumab) in adult patients with selected advanced solid tumors (ClinicalTrials.gov Identifier: NCT02503774) (Hay et al. 2016).

Role of CD73 as a cancer biomarker: Till date, convincing data have been published suggesting the role of CD73 as a potential prognostic as well predictive biomarker (Antonioli et al. 2016; Z. Gao, Dong, and Zhang 2014). Indeed, an overexpression of CD73 has been associated with poor prognosis in cancer patients suffering from different types of cancer including colorectal cancer (Wu et al. 2012; N. Liu, Fang, and Vadis 2012), gallbladder cancer (Xiong et al. 2014), gastric cancer (Lu et al. 2013), prostate cancer (Qing Yang, Du, and Zu 2013), melanoma (H. Wang et al. 2012), breast cancer (Loi et al. 2013), pancreatic ductal adenocarcinoma (PDAC) (L. Zhou et al. 2019) and chronic lymphoblastic leukemia (Serra et al. 2011). However, in certain cases, CD73 does not has a prognostic value (Wieten et al. 2011) or has been associated with a better prognosis (H. K. Oh et al. 2012; Supernat et al. 2012). Absence of CD73 expression in metastatic medulloblastoma cell line (D283) suggests that high expression level could be correlated with poor prognosis in medulloblastoma (Cappellari et al. 2012). Although, different studies have shown contradictory results related to the clinical role of CD73 in ovarian cancers, overexpression of CD73 in high grade ovarian cancer has been shown to be associated with poor prognosis (Turcotte et al. 2015).

Protective roles of CD73 in cancer: A good number of studies has also shown the protective role of CD73 in certain cancer types. For example, CD73 is significantly downregulated in advanced stage endometrial tumors which results in decreased adenosine-mediated actin polymerization and consequently promotes tumor progression (Bowser et al. 2016). However, it is highly expressed in early stage endometrial carcinoma and is associated with a better overall survival (Bowser et al. 2016).

Additionally, CD73 activity is observed to be higher in benign prostatic hyperplasia and lower in poorly differentiated, advanced stage prostatic carcinomas (Rackley et al. 1989). Similar observations were made in laryngeal (Durak et al. 1993) and high grade colon carcinomas (Eroglu et al. 2000). Furthermore, higher CD73 expression is associated with favorable clinicopathological features in patients with nonmuscle-invasive urothelial bladder cancer (Wettstein et al. 2015). Another study examined the correlation between CD73 expression and its clinical significance in breast cancer patients. The results have shown that CD73 expression higher than median value is correlated with longer disease-free and overall survival (Supernat et al. 2012). A study on epigenetic regulation of CD73 expression in malignant melanoma has shown that aberrant methylation in *NT5E* gene downregulates CD73 expression and is associated with a risk of more aggressive and metastatic disease profile (H. Wang et al. 2012). Although the importance of CD73 in cancer is due to its AMPase activity but knocking down CD73 in hepatic stellate cells causes an increase in mRNA expression of tissue non-specific alkaline phosphatase (TNALP), another AMP catalyzing enzyme (Andrade et al. 2011). Therefore, no difference in extracellular adenosine production is observed even in the absence of CD73. However, CD73 knockout caused an increased cell migration and mRNA level of collagen I (Andrade et al. 2011). In ovarian cancer, CD73 is overexpressed in patients with known good prognostic factors like low grade, better differentiation and lower Treg infiltration and, therefore, is associated with an overall better prognosis (H. K. Oh et al. 2012, 73). A recent study has shown that CD73 exhibit a compromised enzymatic activity in human HCC cells (Alcedo et al. 2019).

5'-nucleotidases and hypoxia

Purine nucleotides have shown to be in the central position in response of neuronal cells towards ischemia or hypoxia. Adenosine, for example, is produced and released in CNS under ischemic or hypoxic conditions (Winn, Rubio, and Berne 1981; Zetterström et al. 1982). It has also been shown to play a protective role in neuronal cells during ischemia-induced energy failure (Higgins et al. 1994; Brundege and Dunwiddie 1997). Majority of these neuroprotective functions of adenosine are

executed through abundantly expressed adenosine receptors in the nervous system. Additionally, inosine and guanosine have also been shown to have neuroprotective roles and induce neurite outgrowth under basal as well as certain stress conditions (Benowitz et al. 1998; P. Chen et al. 2002; Gysbers and Rathbone 1996; Jurkowitz et al. 1998). Furthermore, It has been shown that purine nucleosides (adenosine, guanosine and inosine) protect neuronal pheochromocytoma (PC-12) cells against chemical induced hypoxia *i.e.* rotenone via AMPK activation pathway (Tomaselli et al. 2005). Additionally, several studies have shown that adenosine produced by the enzymatic activity of CD73 demonstrates cardioprotective functions under hypoxic stress (Sommerschild and Kirkebøen 2000; Neylon and Marshall 1991). Furthermore, CD73 activity increases under tissue hypoxia and drug-induced ischemic conditions (Minamino et al. 1995; Ueda et al. 1999; Ledoux S. et al. 2002; Eltzschig et al. 2003). An important breakthrough was made by a couple of groups working independently showing that CD73 expression in hypoxia is regulated by HIF-1 α , a transcription factor, and mediates permeability changes in intestinal epithelia both *in vitro* and *in vivo* (Synnestvedt et al. 2002; Thompson et al. 2004b; Ledoux S. et al. 2003). Further studies showed that IFN-beta treatment following the ischemic period upregulates the expression of CD73 and results in improvement of vascular barrier functions (Kiss et al. 2007). Recent study has shown regulatory role of CD73 in peripheral chemoreceptor activity and cardiorespiratory responses to hypoxia *in vitro* (Holmes et al. 2018).

Hypoxia is considered as an important hallmark of solid tumors for the very obvious reason of rapidly proliferating cells and consequently generating a TME void of oxygen and other important nutrients (Mizokami et al. 2006). The first response of cancer cells to this hypoxic microenvironment is the stabilization of otherwise unstable HIF-1 α (Semenza 2012a). On the other hand, convincing number of studies have shown the increased expression of CD73 mediated through HIF-1 α in different cancer cells under hypoxic conditions (X. Li et al. 2006; Eltzschig et al. 2009, 1; Semenza 2012b; Lu et al. 2013; Xinke Zhou et al. 2016; Soleimani et al. 2019; Losenkova et al. 2018). This, in turn, causes an increased production of immunosuppressor adenosine by hydrolyzing of extracellular ATP via CD39/CD73, which

modulates immune response by acting through adenosine receptors on immune cells and finally helping in the favor of cancer cell via immune evasion (Chambers and Matosevic 2019).

On the other hand, the role of cN-II in cellular response towards modulated oxygen concentration has not been well studied. Although, purine nucleosides (inosine and guanosine), preferred substrates of cN-II, have shown protective roles in different neuronal cells in response to hypoxic stress (Tomaselli et al. 2005). However, a couple of studies have shown that cN-II is upregulated in turtle brain in response to long-term anoxia (Storey 2007; Krivoruchko and Storey 2010). Recently, our group has shown that breast cancer cells (MDA-MB-231) with decreased cN-II expression better adapt glucose deprivation under normoxic conditions but are more sensitive under hypoxic stress (Bricard et al. 2017). This shows the involvement of cN-II in cell's response to stress condition like hypoxia. However, other than this, nothing is known about the role of cN-II expression in cancer cells and their behavior to hypoxic culture conditions.

Aims of the PhD Project

Our current knowledge of 5'-nucleotidases (cN-II and CD73) has reached to a point where it has been well established that both cN-II and CD73 can be considered as novel and promising therapeutic targets for cancer treatment and that they are somehow involved in cancer cell biology. Studies have also described them as potential prognostic and predictive biomarkers in certain cancer types and in their treatments. However, there is still an utter need of further exploring their biological roles in cancer, in particular due to the suggested cell-specificity or tissue-specificity of some phenotypes, and to the development of more advanced and sophisticated cellular research approaches. For example, CRISPR/Cas 9 has revolutionized cellular research in recent years by allowing researchers to completely knockout their proteins of interest which will help them to better understand the physiological and pathological roles of these cellular proteins. Before, work related to the roles of cN-II and CD73 in cancer cell biology mostly used transient and incomplete approaches of protein silencing like shRNA or siRNA, or inhibitors of enzymatic activity. Furthermore, despite having published good number of studies highlighting individual cancer-specific roles of either cN-II or CD73, there is not a single study to the best of our knowledge that has used experimental cell models to study their roles together in the same cell. Both cN-II and CD73 hold pivotal positions in nucleotides' metabolism and maintenance of their intracellular and extracellular pools, respectively. Therefore, it would be very interesting to use cell models with altered expression of both cN-II and CD73 in the same cell. Moreover, CD73 has already been shown to have a role in cancer cell's adaptive response towards hypoxia, very little is known about any such role of cN-II in cancer or even in normal cells. Additionally, both cN-II and CD73 have been associated individually with drug resistance in certain cancer types and cytotoxic drugs, their combined effect on new cytotoxic drugs are yet to be explored.

Therefore, during my PhD thesis, we tried to explore individual as well as combined biological effects of cN-II and CD73 in cancer and divided our objectives in two main axes as follows.

Axis 1: Role of 5'-nucleotidases (cN-II and CD73) in cancer cell proliferation and migration

Axis 2: Role of 5'-nucleotidases (cN-II and CD73) in response of cancer cells to cytotoxic drugs and adaptability to stress conditions (nucleotide and hypoxic stress)

My master project and initial PhD project was about the study of the interaction between cN-II and the protein Kidins220. Due to the absence of results within this axis (either for technical reasons or due to no interaction between the proteins), this work was interrupted. A review on Kidins220 was published in *Genes, Chromosomes and Cancer* in 2018 in order to valorize the bibliographic work performed on this protein, and this review is included at the end of the scientific part here after.

Axis 1: Role of 5'-nucleotidases (cN-II and CD73) in cancer cell proliferation and migration

In the first part of our project, we focused on the biological roles of cN-II and CD73 in human breast (MDA-MB-231) and lung cancer (NCI-H292) cell lines as well as mouse breast cancer cell line (4T1). Using CRISPR/Cas9 technique, we developed complete knockout models of these cell lines for either cN-II and/or CD73 expression. These models were then used to study the biological roles of cN-II and CD73 in cancer cell proliferation and migration.

The findings of this study have been submitted for publication and the complete submitted manuscript has been attached hereunder. Results have also been presented at two conferences as follows:

- Cadassou O, Raza MZ, Cros-Perrial E, Armanet C, Gudefin L, Chettab K, Dumontet C & Jordheim LP. Impact of cN-II and CD73 inhibition on cancer cell migration. Oral presentation at 5^{ème} Journée Scientifique du CRCL, Lyon, Juin 2018.
- Cadassou O, Raza MZ, Cros-Perrial E, Armanet C, Gudefin L, Chettab K, Manié S, Dumontet C & Jordheim LP. Impact of cN-II and CD73 inhibition on cancer cell migration. Poster presentation at 109th Annual Meeting of the American Association for Cancer Research, Chicago, IL, USA, April 2018.

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Enhanced migration via COX-2/PGE2/Akt axis in breast and lung cancer cells deficient for cN-II and CD73

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Abstract

Purpose: Purine metabolism involves various intracellular and extracellular enzymes, including cN-II and CD73 that dephosphorylate intracellular and extracellular nucleoside monophosphates into their corresponding nucleosides. We conducted a study to better understand the biological roles of these proteins in cancer cells.

Methods: We abolished cN-II and/or CD73 expression in the triple negative human breast cancer cells (MDA-MB-231), the human lung adenocarcinoma cells (NCI-H292) and in the murine breast cancer cells 4T1, using the CRISPR/Cas9 technique, and evaluated the impact on various cell parameters such as cell proliferation, intracellular nucleotide pools, nucleotide metabolism-related gene expression and cell migration under an extracellular nucleotide stress.

Results: Intracellular nucleotide contents were altered in the modified human breast cell models both at the basal level and after exposure to adenosine or AMP. Altered cN-II and CD73 contents were also associated with cell migration modulations, involving TIMP-2, MMP-2 and MMP-9 expression, as well as an increase in the COX-2/PGE2/Akt pathway.

Conclusion: Our results highlight new and important, although cell-specific, roles of cN-II and CD73 in cancer cell biology and provide insight into the interactions between different intracellular pathways.

Keywords: Nucleotides; cancer cell biology; cN-II; CD73; COX-2

1. Introduction

The purine nucleoside adenosine and its phosphorylated metabolites play major roles in human physiological and pathological conditions. Within a tumor, ATP can for example stimulate the anti-tumoral response of the infiltrated immune system as well as inhibit the growth of cancer cells, whereas adenosine inhibits the immune system and induces apoptotic cell death of cancer cells at higher concentrations [1]. These effects are tissue- and cell-specific and depend on the cellular expression of ATP (P2) and adenosine (P1, ADORA) receptors, as well as the associated intracellular signal transduction machinery. Adenosine can also enter the cell and exert its biological properties after phosphorylation to AMP or ATP [2].

As effects of adenosine are dependent on its concentration, the expression and activities of purine metabolism enzymes within the tumor are expected to play a role in cellular processes. In the extracellular compartment, adenosine is produced from the degradation of ATP by the subsequent actions of CD39 and CD73 [3]. It can thereafter either interact with its receptors, enter the cell through nucleoside transporters or be transformed to inosine by adenosine deaminase (ADA) [3]. Intracellularly, adenosine, that can come either from the extracellular compartment or the hydrolysis of intracellular AMP or S-adenosyl-methionine, is rapidly phosphorylated to AMP by adenosine kinase (ADK), and thereafter to ADP and ATP. Intracellular purine metabolism involves a number of enzymes including ADA and nucleotide degrading enzymes such as cN-I and cN-II [2].

Several of the aforementioned proteins have gained increased attention over the last decade, and we are particularly interested in the 5'-nucleotidases CD73 (NT5E) and cN-II (NT5C2). The latter is an IMP/GMP-preferring enzyme for which a phosphotransferase activity has also been described [4], and both enzymes have demonstrated roles in cancer biology. Indeed, the proliferation of astrocytoma cells (ADF) was increased in case of enhanced cN-II expression [5] whereas its down-regulation did not influence the proliferation of other cell lines of various origins stably expressing cN-II-targeting shRNA [6, 7]. However, in the breast cancer cell line MDA-MB-231, downregulation of cN-II by stable shRNA was associated with an increased adaptability to glucose starvation [8], and in the lung carcinoma cell line A549 its downregulation induced an increased p53 phosphorylation [9] indicating an important role in cancer cell biology. cN-II is also involved in the response to cancer treatments [5, 7, 10]. Concerning CD73, the modulation of its activity by enzymatic inhibitors or of its expression level in cancer cells has allowed to show its involvement in cell proliferation [11–13], cell migration [14, 15] and sensitivity to radiation-based or targeted cancer treatments [16, 17]. Both cN-II [18] and CD73 [19–21] are currently considered as potential targets in oncology.

In the current work, we studied the implication of CD73 and cN-II in cancer cell biology using original models of MDA-MB-231, NCI-H292 and 4T1 cells expressing both CD73 and cN-II, only CD73 or cN-II or none of these. In addition to the study of the models in classical cell culture conditions, we evaluated their response to high concentrations of AMP and adenosine that correspond to those which can be observed in case of extracellular nucleotide stress. Part of this work was performed during the preparation of a PhD and some results were presented in the corresponding thesis [22]

2. Results

2.1. Characterization of cell models

Using the CRISPR/Cas9 technique, we abolished cN-II and/or CD73 expressions in the human triple-negative breast cancer cells (MDA-MB-231) and in the human adenocarcinoma cells (NCI-H292), and cN-II expression in the murine breast cancer cells (4T1). These were validated for their cN-II and CD73 protein expression and hereafter referred to as cN-II⁺/CD73⁺ cells (expressing both cN-II and CD73), cN-II⁺/CD73⁻ cells (expressing cN-II but not CD73), cN-II⁻/CD73⁺ (deficient for cN-II but not for CD73) and cN-II⁻/CD73⁻ (deficient for both cN-II and CD73) (Figure 1). We determined the expression profiles of selected genes coding for adenosine receptors or proteins involved in purine metabolism in the human cell models (Table S1). Among the notable differences in MDA-MB-231 cells, we observed a 6-8-fold decrease in the expression of adenosine receptor A1 in cells without cN-II, a 11-fold increase of ADSL in cN-II⁺/CD73⁻ cells as well as a 1.5-1.7-fold increase in SAMHD1 in cN-II negative cells as compared to their respective controls. For NCI-H292 cells, cN-II⁻/CD73⁻ had a slight decrease in adenosine receptor A2 (1.4-fold) and in RRM1 (2.3-fold) expression.

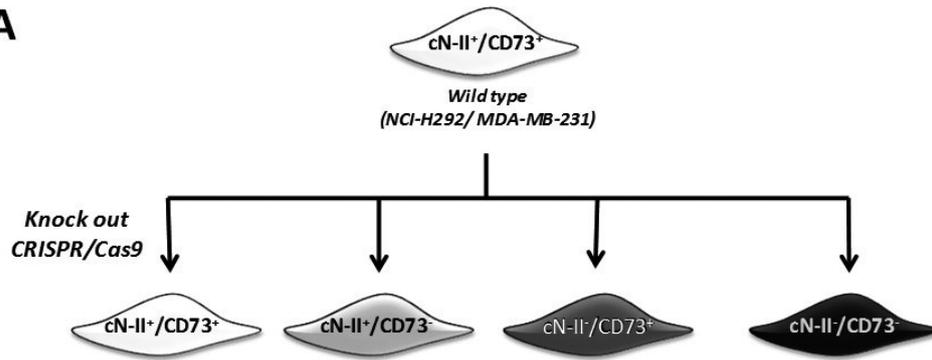
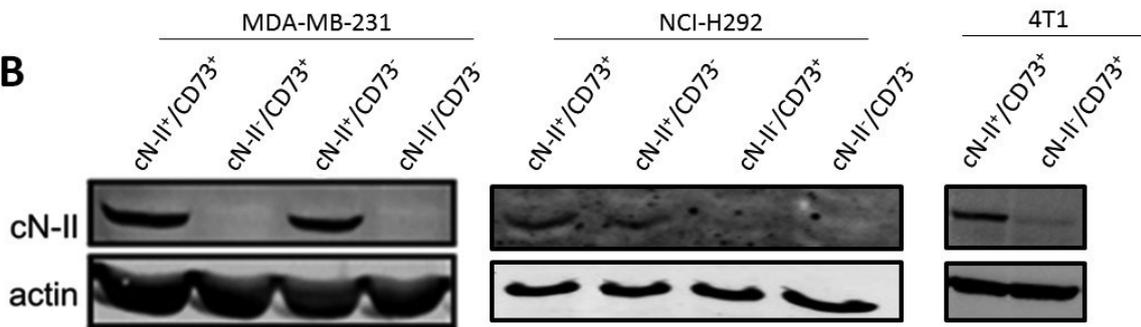
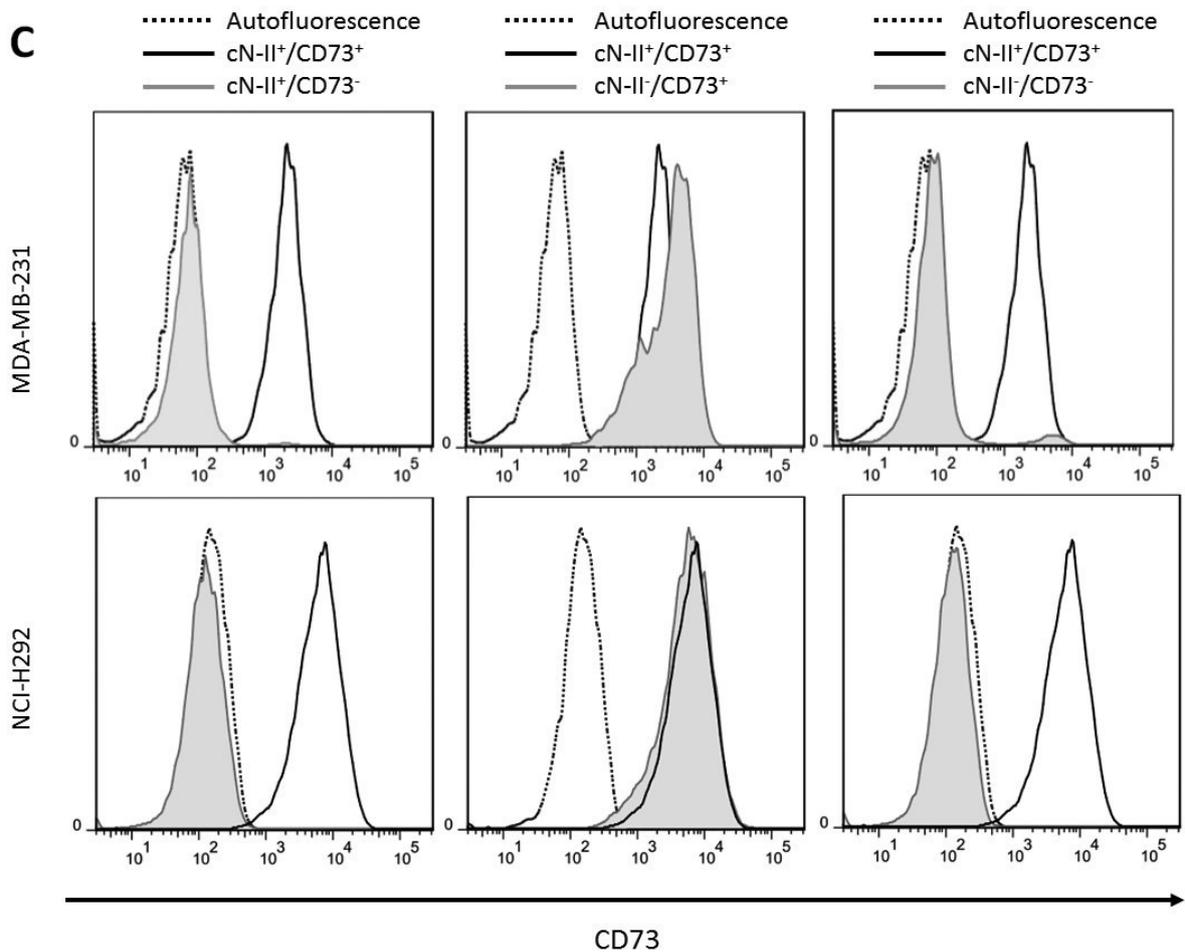
A**B****C**

Figure 1. Characterization of cell models. (A): The CD73 and/or cN-II knockout of MDA-MB-231, NCI-H292 and 4T1 cells were obtained by using CRISPR/Cas9 technology as described in material and methods. (B): cN-II expression in the different cell models as determined by Western blot. Representative image of 3 similar Western blots. (C): CD73 expression in MDA-MB-231 and NCI-H292 cell models as determined by flow cytometry. The image is representative of 4 analyses.

2.2. Extracellular adenosine affects cell proliferation and survival, independently from cN-II and CD73 expression levels

Considering the involvement of nucleotide pools in cell biology, we investigated whether cN-II and/or CD73 knock out impacted cell proliferation and viability. Under classical culture conditions, these knockouts did not influence cell proliferation. CFSE intensity decreased to 23.5-25.4% (MDA-MB-231 cells), 15.0-17.6% (NCI-H292 cells) or 7.1-8.6% (4T1 cells) of the initial value after 3 days for all the cell lines (Figure 2A). In addition, cell viability did not vary between the MDA-MB-231 cells lines after 72 h in culture ($16.2 \pm 7.9\%$ annexin-V positive cells for cN-II⁺/CD73⁺, versus $17.6 \pm 3.5\%$, $15.7 \pm 8.0\%$ and $16.7 \pm 8.3\%$ for cN-II⁻/CD73⁺, cN-II⁺/CD73⁻ and cN-II⁻/CD73⁻ respectively) (Figure 2B). These results indicate that cN-II and CD73 are not involved in cell proliferation or survival in these cells under the applied conditions.

As cN-II and CD73 are involved in nucleotide metabolism, we investigated to what extent these enzymes could modify cellular response to an extracellular nucleotide stress, using the MDA-MB-231 models. To do so, we exposed the cells to high concentrations of AMP or adenosine (1600 μ M) and evaluated their survival after 48 hours. AMP exposure resulted in a strong increase of Annexin-V positive cells in CD73-expressing cells (from $16.2 \pm 7.9\%$ to $41.6 \pm 12.2\%$ for cN-II⁺/CD73⁺, and from $15.7 \pm 8.0\%$ to $32.2 \pm 10.4\%$ for cN-II⁻/CD73⁺ cells). This AMP-induced cell death was totally inhibited by the CD73 inhibitor APCP (adenosine 5'-(α,β -methylene)diphosphate), indicating that CD73 activity, and thus adenosine generation, is required for this effect in these models. Interestingly, cN-II⁻/CD73⁻ cells also showed a slight sensitivity to AMP ($27.8 \pm 4.2\%$ vs $16.7 \pm 8.3\%$ for cN-II⁺/CD73⁺, $p < 0.05$), and this was insensitive to APCP. Overall, exposure to adenosine induced cell death in all cell models. This effect was not significantly different according to cN-II or CD73 expressions, suggesting that these 5'-nucleotidases do not modulate cell survival in the presence of high concentrations of adenosine. When the cells were incubated with adenosine and A2A and A2B antagonists, we did not observe any rescue. Thus, A2A and A2B receptors do not appear to mediate adenosine-induced cell death in these conditions.

Similarly, we evaluated cell proliferation in the presence of high initial concentrations of AMP or adenosine (Figure 2A). In MDA-MB-231 cells, the two purines slowed proliferation down, but adenosine had a stronger effect than AMP ($p < 0.05$ for adenosine, $p > 0.05$ for AMP). Indeed, in presence of this nucleoside, CFSE intensity after 3 days reached 35.3-50.1% of the initial value, versus 23.5-25.4% without adenosine. Here again, no significant difference was observed according to cN-II and CD73 expression between our cell lines. 4T1 cells were also sensitive to adenosine (20.4-23.7% at day 3 versus 7.1-8.6% for control conditions) but not to AMP. Finally, NCI-H292 cell showed no modification in cell proliferation in the presence of high concentrations of either adenosine or AMP (1600 μM), and this was the case for all four models (Figure 2A).

These results show that an extracellular nucleotide stress can affect cell proliferation and cell survival in a cell-specific manner, but independently from cN-II or CD73.

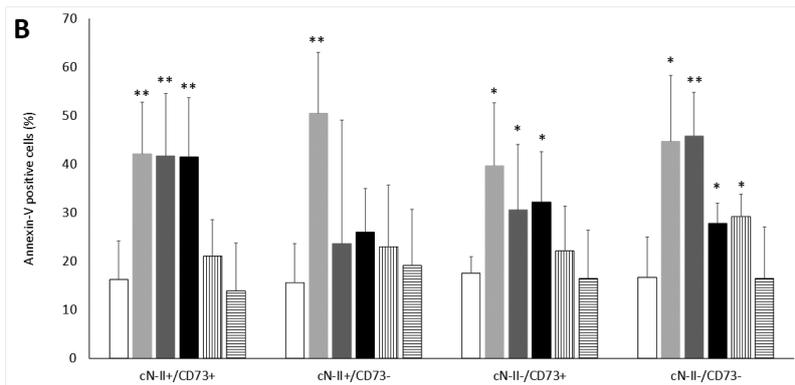
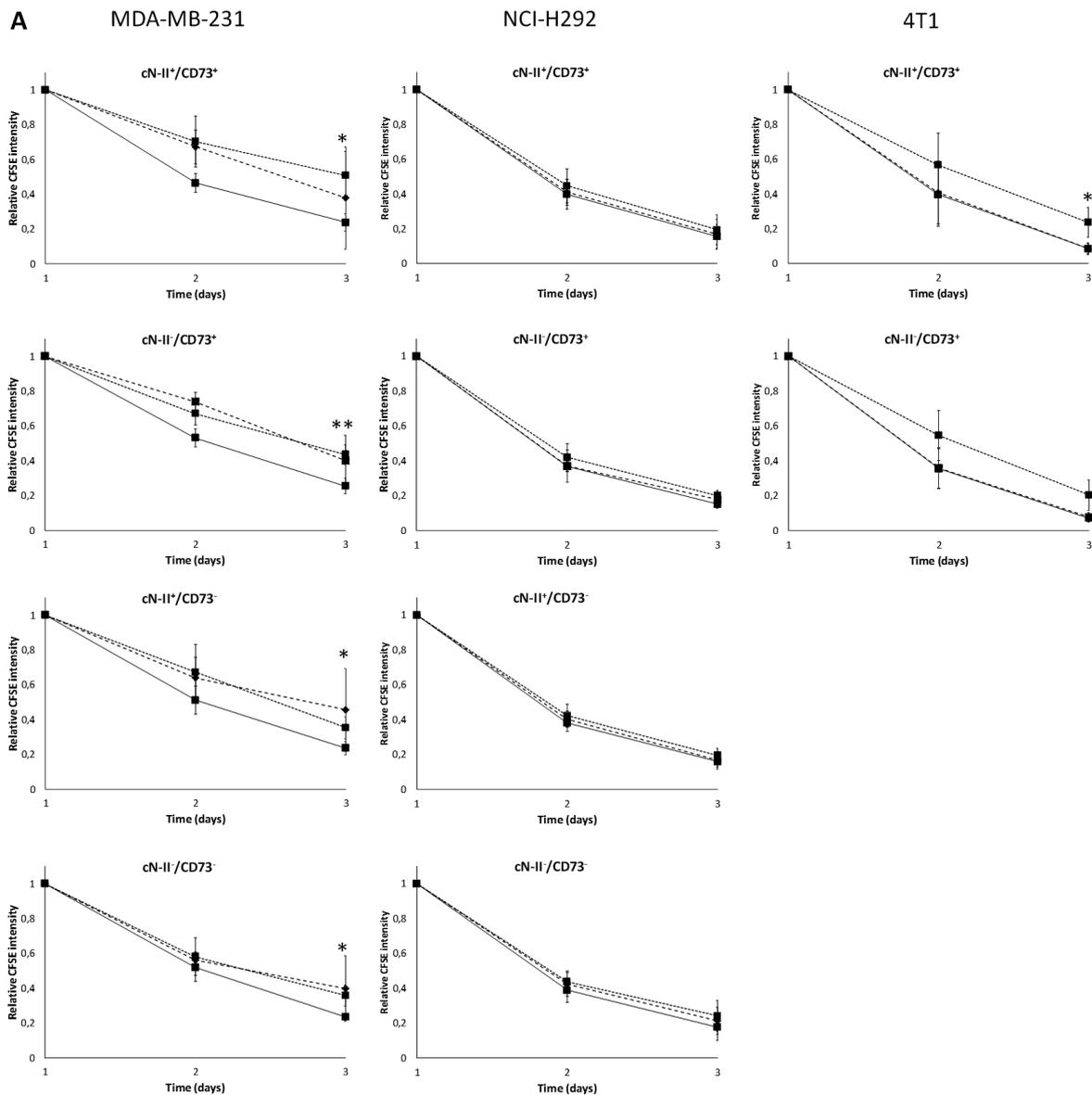


Figure 2. Cell proliferation (A) and survival (B) of cell models. For cell proliferation (A), MDA-MB-231 (left), NCI-H292 (middle) and 4T1 (right panel) cells were stained with CFSE to monitor cell proliferation over 3 days by flow cytometry in the absence (full line) or presence of 1600 μ M adenosine (.....) or AMP (- - - -). The graphs show mean values of 4 independent experiments and error bars are standard

deviations. * = $p < 0.05$; *** = $p < 0.005$ using Students t-test for comparison between adenosine and the control condition. (B): Cell death was evaluated for the MDA-MB-231 cell models in control conditions (white bars) or in presence of adenosine (1600 μM , light grey bars), in presence of adenosine (1600 μM) + the A2A antagonist ZM 241385 (100 nM) + the A2B antagonist PSB 1115 (10 μM) – dark grey bars, in presence of AMP (1600 μM , black bars), in presence of AMP (1600 μM) + APCP (100 μM) – vertical lines or in presence of APCP (100 μM , horizontal lines). The graphs show mean values of 5 independent experiments and error bars are standard deviations. * = $p < 0.05$; ** = $p < 0.01$ using Students t-test in comparison with the control condition.

2.3. Intracellular nucleotide pools in cell models

As indicated in the introduction, both cN-II and CD73 are expected to regulate intracellular nucleotide pools. We measured the pools, with a particular interest in purines, in the MDA-MB-231 models both under baseline conditions and after 1 hour exposure to 400 or 1600 μM adenosine or AMP (Figure 3 and table S2). Exposure to 2-deoxyglucose was used as a control condition inducing major modifications in NTP content. Both ATP and AMP were more abundant in cN-II⁻/CD73⁺ and cN-II⁻/CD73⁻ cells (1.4-1.6-fold) and unmodified in cN-II⁺/CD73⁻ cells as compared to control cells, whereas adenosine and inosine were decreased in both models lacking CD73 (1.4-3.2-fold) and increased in cN-II⁻/CD73⁺ cells (4.6- and 2.1-fold, respectively). Finally, IMP was decreased in cN-II⁻/CD73⁺ (3.4-fold) and increased in cN-II⁻/CD73⁻ cells (5.7-fold). After exposure to adenosine or AMP, control cells had increased content of ATP, AMP, inosine and adenosine. In modified cells, the most striking modifications were the increases of AMP and adenosine in cN-II⁻/CD73⁺ cells and of IMP in cN-II⁻/CD73⁻ cells. The lack of precision in samples exposed to adenosine or AMP resides in the important matrix effect during the LC-MS/MS analysis.

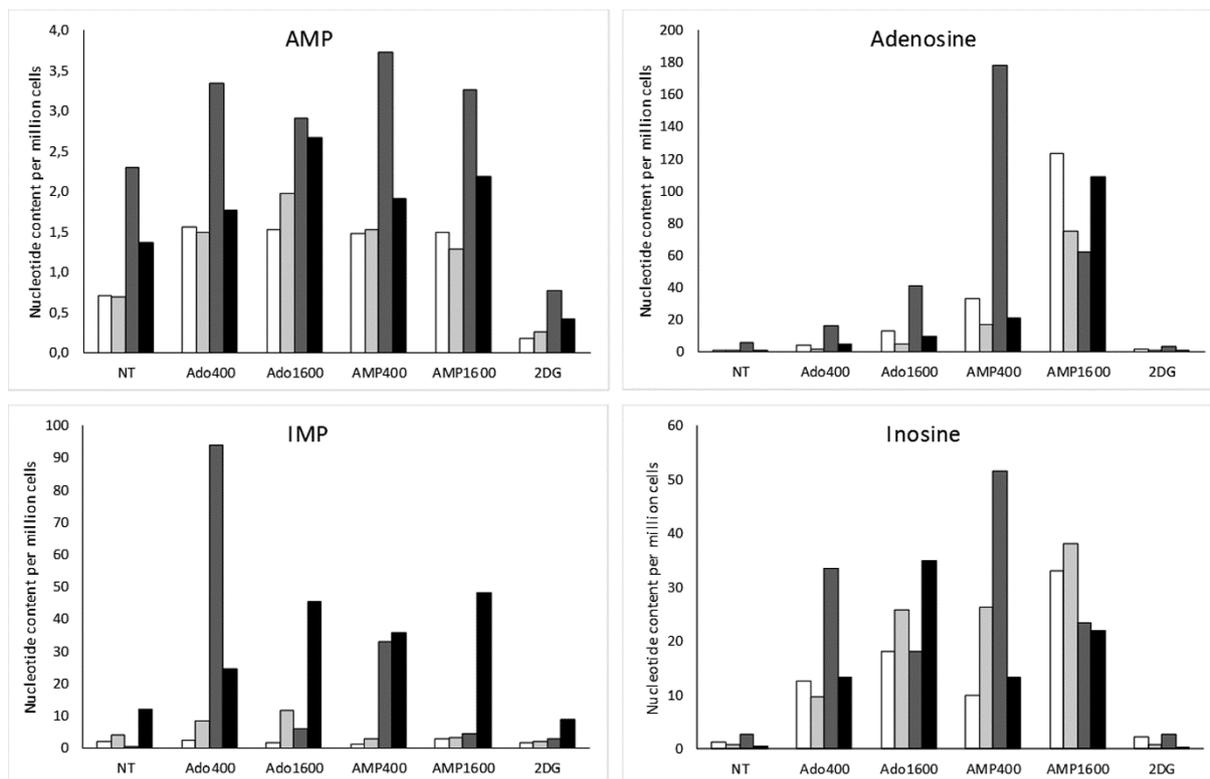


Figure 3. Relative content of intracellular adenosine, inosine, AMP and IMP in the studied MDA-MB-231 cell models, cN-II⁺/CD73⁺ in white, cN-II⁺/CD73⁻ in bright grey, cN-II⁻/CD73⁺ in dark grey and cN-II⁻/CD73⁻ in black. Nucleotides and nucleosides were quantified as indicated in material and methods and are expressed as mean values of the ratios of the surface of the compound of interest / surface of internal standard. The results were further normalized for one million cells and expressed as relative content as compared to unexposed cN-II⁺/CD73⁺ cells in each experiment. Values shown are from three independent experiments. For complete data, see table S2.

2.4.5'-nucleotidase expression and extracellular adenosine modulate cell migration

Cell migration was determined by a wound healing assay and monitored by the IncuCyte[®] device. Under normal cell culture conditions, reduced cN-II expression was associated with enhanced migration in MDA-MB-231 cells (Figures 4A and S1). Indeed, 10 hours after injury, the wound confluence reached 70 to 77% for cN-II⁺/CD73⁺ and cN-II⁻/CD73⁻ cells, whereas it was only 49 to 50% in cN-II⁺/CD73⁻ and cN-II⁻/CD73⁺ cells. For NCI-H292 cells, the deficiency of CD73 expression was associated with a decreased cell migration whereas the modulation of cN-II did not affect this parameter. Indeed, 10 hours after injury, the wound confluence reached 72 to 79% for cN-II⁺/CD73⁺ and cN-II⁻/CD73⁺ cells, whereas it was only 43 to 55% for cN-II⁺/CD73⁻ and cN-II⁻/CD73⁻ cells. In 4T1 cells, cN-II deficiency did not alter cell migration (Figure 4A). Even though these results on migration were repeatedly obtained with the IncuCyte device, the study of cell migration on the xCELLigence device with CIM plates did not give the same results for MDA-MB-231 and 4T1 cells. Indeed, here, we observed that cN-II⁻/CD73⁻ cells grew

faster and cN-II⁺/CD73⁻ cells slower than control cells for MDA-MB-231, and that cN-II⁻/CD73⁺ cells grew faster than control cells for 4T1 (Figure S2). This discrepancy between the two methods could also be due to the fact that the xCELLigence device is taking into account the strength of adherence of the cells in addition to the number of cells, suggesting a difference in the adherence between our models.

We also monitored MDA-MB-231 cell migration in the presence of high initial concentrations of AMP. In cN-II⁺/CD73⁻ cells and cN-II⁻/CD73⁺ cells, AMP delayed migration and this effect was reversed by co-incubation with APCP, suggesting that its conversion into adenosine might be necessary to impact this biological process (data not shown). Again, we observed that AMP could also impact migration in cN-II⁻/CD73⁻ cells, suggesting a CD73-independent AMP effect.

When we realized this assay in the presence of high initial concentrations of adenosine, the nucleoside reduced cell migration in all our cell models, confirming the importance of adenosine production to affect migration (Figure 4B). Interestingly, we observed that cell migration was more affected by adenosine in cN-II-deficient MDA-MB-231 cells when we compared them to their cN-II-proficient counterparts. This was also true at some time-points for NCI-H292 cN-II⁻/CD73⁺ cells but not in the 4T1 model. Figure 4C illustrates this by the differences in wound confluence between unexposed and adenosine-exposed cells at 10 hours. The observed effects were not due to adenosine or AMP-induced cell death as these purines do not significantly affect cell survival at early times (Figure S3).

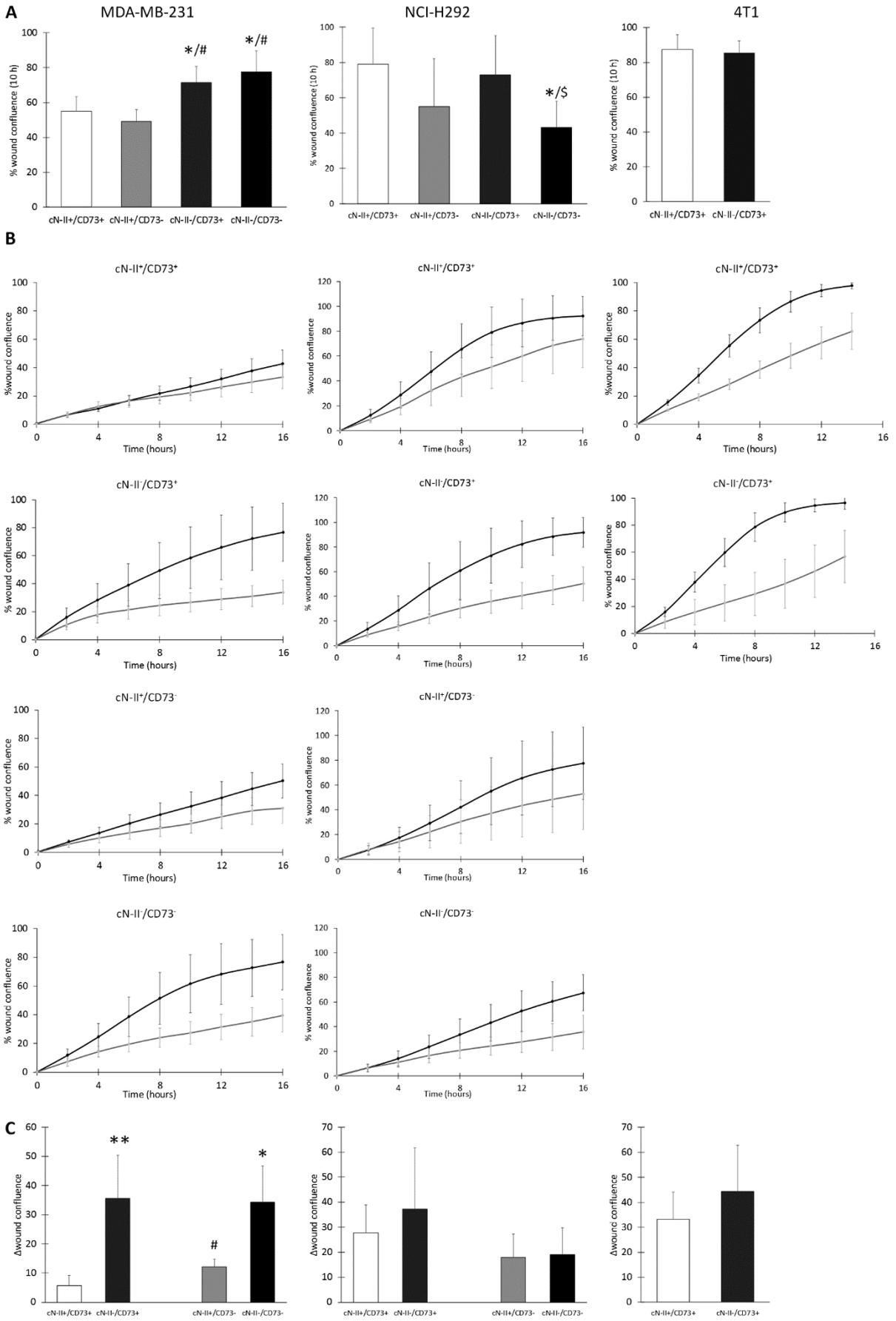


Figure 4. Cell migration of studied cell models. Cell migration assay was performed as indicated in material and methods. **(A)**: Quantification of confluence at 10 h after injury in MDA-MB-231 (left), NCI-H292 (middle) and 4T1 (right) cells. Graphs are mean values of 4 independent experiments performed in triplicate, and error bars are standard deviation. * = $p < 0.05$ using Students t-test in comparison with the cN-II⁺/CD73⁺ cells; # = $p < 0.05$ using Students t-test in comparison with the corresponding cN-II-positive cells, \$ = $p < 0.05$ using Students t-test in comparison with the corresponding CD73-positive cells. **(B)**: Effect of adenosine (bright curves) as compared to unexposed cells (dark curves) over 16 hours after injury in MDA-MB-231 (right), NCI-H292 (middle) and 4T1 (right) cells. Graphs show mean values of 4 independent experiments performed in triplicate. **(C)**: Quantification of the effect of adenosine on cell migration. Δ wound confluence was calculated 10 hours after injury. For each cell line we used Δ wound confluence = $|\% \text{ wound confluence}_{\text{control}} - \% \text{ wound confluence}_{\text{adenosine}}|$. Graphs show mean values of 4 independent experiments performed in triplicate, and error bars are standard deviation. * = $p < 0.05$ and ** = $p < 0.01$ using Students t-test in comparison with the corresponding cN-II-proficient cell line, # = $p < 0.05$ using Students t-test in comparison with the corresponding cN-II⁺/CD73⁺ cells.

2.5. cN-II knock-out is associated with migration-related molecular modifications

In order to find molecular modifications explaining the differences in cell migration between the cN-II-deficient and cN-II-proficient cells, we studied the gene expression of the migration-related genes TIMP-1, TIMP-2, MMP-2 and MMP-9. In accordance with the migration experiments for MDA-MB-231 cells, at the mRNA level, cN-II-proficient cells expressed high levels of TIMP-2, but not TIMP-1, when compared to their cN-II-deficient counterparts (Table 1). Indeed, cN-II knock out was accompanied with a reduction of 34.0 % and 47.9 % of TIMP-2 mRNA expression for cN-II⁻/CD73⁺ and cN-II⁻/CD73⁻ respectively. MMP-2 and MMP-9 were more expressed in the CD73-deficient cells but did not vary according to cN-II expression. This MMP upregulation was not associated with any significant modification in cell migration. For NCI-H292 cells, we observed a slight increase in TIMP-1 expression (1.4-fold) in cN-II⁻/CD73⁺ cells as compared to control cells, and in MMP-2 expression (1.7-fold) in cN-II⁻/CD73⁻ cells as compared to cN-II⁻/CD73⁺ cells.

CD44 is a glycoprotein that promotes migration in cancer cells, in association with MMPs [23]. Thus, we determined its expression at the surface of our cell models and observed that cN-II-deficient MDA-MB-231 cells express less CD44 than their cN-II-proficient counterparts, whereas CD73-deficient NCI-H292 cells express less CD44 than their CD73-proficient counterpart (Figure S4). This result therefore

suggests a link between CD44 expression, cN-II or CD73 expression and cell migration that is cell specific.

Table 1. mRNA expression of cell migration-related and PGE2-related genes in MDA-MB-231 and NCI-H292 models. Values are means \pm standard deviation of four independent experiments, performed in triplicate. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ using Students t-test in comparison with the corresponding cN-II-proficient cell line. # = $p < 0.05$; ## = $p < 0.01$ using Students t-test in comparison with the corresponding CD73-proficient cell line. See legend of table 2 for gene names.

Gene	MDA-MB-231					NCI-H292		
	cN-II ⁺ / CD73 ⁺	cN-II ⁺ / CD73 ⁻	cN-II ⁻ / CD73 ⁺	cN-II ⁻ / CD73 ⁻	cN-II ⁺ / CD73 ⁺	cN-II ⁺ / CD73 ⁻	cN-II ⁻ / CD73 ⁺	cN-II ⁻ / CD73 ⁻
TIMP1	0.65 \pm 0.25	1.48 \pm 0.68	0.89 \pm 0.50	1.18 \pm 0.63	0.34 \pm 0.09	0.51 \pm 0.30	0.49 \pm 0.08*	0.44 \pm 0.05
TIMP2	1.12 \pm 0.15	1.19 \pm 0.07	0.69 \pm 0.21*	0.72 \pm 0.46**	0.25 \pm 0.11	0.41 \pm 0.49	0.19 \pm 0.02	0.16 \pm 0.03
MMP2	0.72 \pm 0.26	1.20 \pm 0.05#	0.78 \pm 0.26	1.15 \pm 0.22	1.08 \pm 0.24	1.01 \pm 0.58	0.93 \pm 0.003	1.62 \pm 0.44#
MMP9	0.88 \pm 0.11	1.62 \pm 0.26##	0.81 \pm 0.31	1.68 \pm 0.26	1.49 \pm 0.54	1.76 \pm 0.57	2.30 \pm 1.69	1.55 \pm 0.43
COX2	0.87 \pm 0.16	1.18 \pm 0.42	21.9 \pm 6.8***	55.2 \pm 33.1*	1049 \pm 396	373 \pm 428	1577 \pm 1271	798 \pm 707
PLA2G4A	1.10 \pm 0.40	79.1 \pm 9.8***	3.14 \pm 1.18	111 \pm 10***	1.37 \pm 0.30	1.42 \pm 0.99	1.29 \pm 0.15	1.44 \pm 0.48

2.6. cN-II modulates the COX-2/PGE2/Akt axis

The COX-2/PGE2/Akt axis can regulate TIMP-2 expression as well as cell migration [24–27]. Indeed, cyclooxygenase 2 (COX-2) participates in prostaglandin E2 (PGE2) production from arachidonic acid in cells. The latter can bind surface G-protein-coupled receptors, leading to the activating phosphorylation of Akt thus promoting cell migration and regulating gene expression. Therefore, we investigated the involvement of this axis in our models.

cN-II deficiency was associated with a 25-47-fold increase in COX-2 expression and a 35-72-fold increase in PGLA2 expression at the mRNA level (Table 1) in MDA-MB-231 cells, and an important increase in COX-2 protein expression both in MDA-MB-231 and NCI-H292 cells (Figure 5A). This was accompanied with a 1.6-2.1-fold higher PGE2 secretion in the cell supernatants of MDA-MB-231 cells (Figure 5B). Interestingly, CD73 silencing alone was not associated with any significant changes but when associated with cN-II silencing in MDA-MB-231 cN-II⁻/CD73⁻, it accentuated COX-2 expression and PGE2 production. COX-2 expression was not modified in 4T1 cN-II⁻/CD73⁺ cells.

In the presence of the COX-2 inhibitor celecoxib, cell migration tended to slow down in the four cell lines (cN-II⁺/CD73⁺ cells were limited to 19.5 ± 9.6% wound confluence after 10 hours, cN-II⁺/CD73⁻ to 21.1 ± 11.7% wound confluence, cN-II⁻/CD73⁺ to 30.6 ± 8.7% wound confluence and cN-II⁻/CD73⁻ to 31.2 ± 14.5% wound confluence under 60 µM celecoxib exposure) (Figure 5C). This was however not observed with other COX-2 inhibitors rofecoxib and valdecoxib (Figure S5A). On the contrary, arachidonic acid-induced PGE2 production slightly enhanced cell migration in cN-II-proficient MDA-MB-231 models, thus confirming that COX-2 activity indeed is involved in this process in these models. Interestingly and similarly as for adenosine, celecoxib was able to more efficiently reduce cell migration in the cN-II-deficient cells, and particularly when it was associated with a CD73-deficiency (Figure 5D). Consistently, arachidonic acid enhanced migration less efficiently in cN-II deficient cells, suggesting that cN-II can be involved in COX-2/PGE2 pathway modulations of cell migration.

AKT activation occurs downstream of prostaglandin receptor activation and is known to promote cell migration [28–31]. Therefore, we evaluated its phosphorylation status, and observed that cN-II-negative cells, and particularly cN-II⁻/CD73⁻ cells showed a stronger basal activation of AKT (Figure 5E). In addition, incubation of the cells with 1600 µM adenosine for 1 hour reduced both COX-2 expression (Figure 5A) and AKT phosphorylation (Figure 5E), which could explain its impact on cell migration. However, cell migration assays in presence of the AKT inhibitor VIII at 10 µM, which did inhibit AKT phosphorylation (data not shown), showed no modification in migration pattern in any of the MDA-MB-231 cell models (Figure S5B). Taken together, these results suggest that the COX-2/PGE2/AKT axis

is reinforced when cN-II is not expressed in MDA-MB-231 cells, and that adenosine can inhibit this axis. Confirmation studies in the 4T1 model showed that celecoxib (Figure S6A) and rofecoxib, and to a less extent valdecoxib (Figure S6B), decrease cell migration in both cell lines, whereas arachidonic acid (Figure S6A) and AKT inhibitor VIII (Figure S6C) do not modify cell migration.

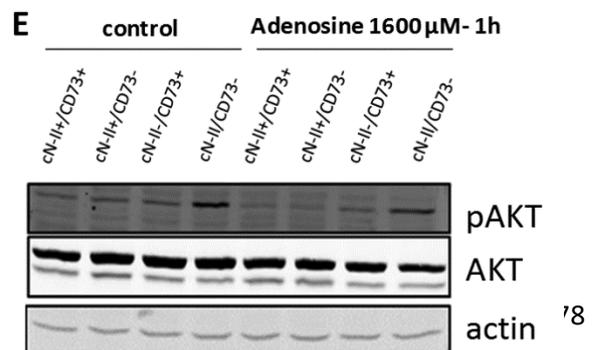
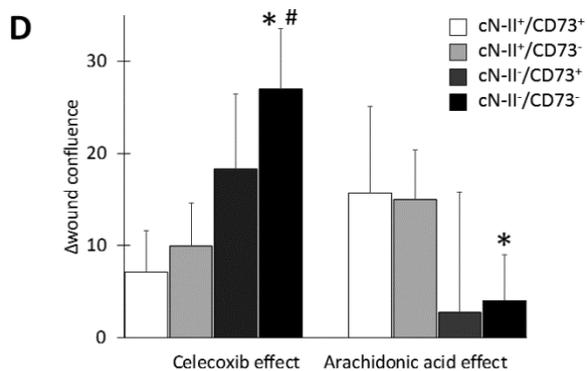
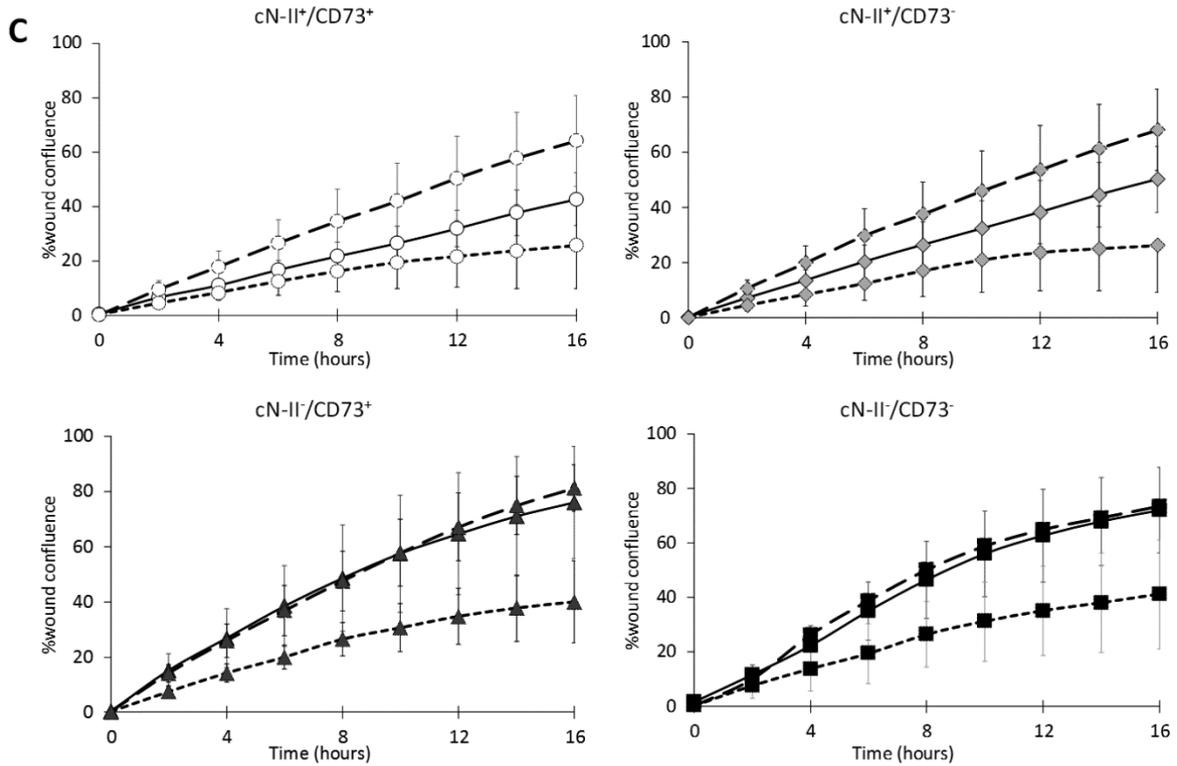
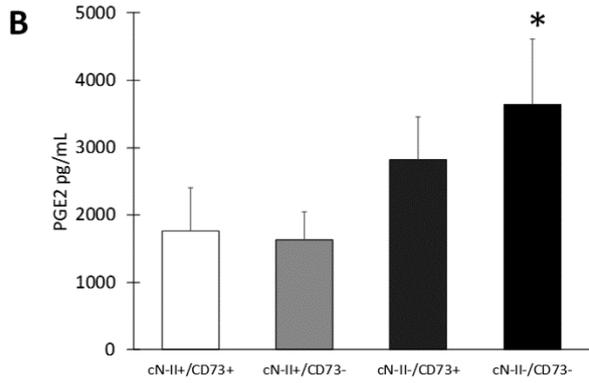
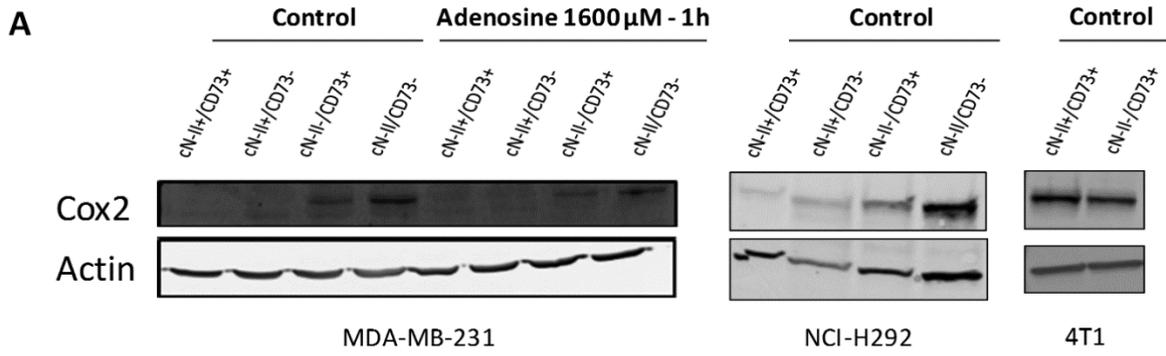


Figure 5. Study of the COX-2/PGE2/Akt axis in cell models. **(A)**: COX-2 protein expression in MDA-MB-231, NCI-H292 and 4T1 cells was determined as indicated in material and methods in control conditions or after one-hour exposure to adenosine (1600 μ M). The blots are representative of 3 experiments. PGE2 secretion **(B)** was quantified in cell supernatants of MDA-MB-231 cells after a 24-hour stimulation with arachidonic acid (15 μ M). Graphs are mean values of three independent experiments performed in triplicate and error bars are standard deviation. * = $p < 0.05$ using Students t-test in comparison with the corresponding cN-II⁺/CD73⁺ cell line. **(C)**: Wound healing curves of MDA-MB-231 cells exposed or not to celecoxib (- - -, 60 μ M) or arachidonic acid (— — —, 30 μ M). Graphs are mean values of three independent experiments performed in triplicate and error bars are standard deviation. **(D)**: quantification of the effect of celecoxib and arachidonic acid on MDA-MB-231 cell migration 10 hours after injury. For each cell line and each condition, we used Δ wound confluence = $|\% \text{ wound confluence}_{\text{control}} - \% \text{ wound confluence}_{\text{celecoxib or arachidonic acid}}|$. Graphs are mean values of four independent experiments performed in triplicate and error bars are standard deviation * = $p < 0.05$ using Students t-test in comparison with the corresponding cN-II-proficient cell line. # = $p < 0.05$ using Students t-test in comparison with cN-II⁺/CD73⁺ cells. **(E)**: AKT expression and phosphorylation in cells exposed or not to adenosine (1600 μ M, 1 hour).

3. Discussion

In this study, we established new cell models that allow a better understanding of the role of 5'-nucleotidases in cancer cell biology. We showed that in MDA-MB-231, NCI-H292 or 4T1 cells, cN-II and CD73 are not necessary for cell proliferation or survival in optimal conditions or under a nucleotide stress. We also confirmed that adenosine impacts cancer cell biology. In the tumor microenvironment, extracellular adenosine concentrations increase because of the inflammatory environment and damaged cells. In this case, the concentrations can reach the micromolar range whereas it is in the nanomolar range under physiological conditions [32]. It is then rapidly degraded by ADA or internalized by the nucleoside transporters. Because of its very short half-life time, we worked with elevated initial concentrations of adenosine (1600 μ M), but these experimental conditions do not allow us to know what concentrations of adenosine remain in the medium at the time points we studied. Although considered as pro-tumoral due to its immunomodulatory effects, we confirmed that adenosine also affects cancer cells themselves by slowing their proliferation and/or migration down and triggering cell death when it is present at very high concentrations, as suggested in previous studies [33, 34]. Nevertheless, cN-II and CD73 do not seem to be involved in these effects.

Little is known about the intracellular nucleoside and nucleotide pools in cells with modified expression of cN-II and CD73. In non-cancerous cells, the overexpression of cN-II was shown to only slightly decrease the NTP [35–37]. No major differences were observed in stably transfected cells with cN-II targeting shRNA in four different models [6], and to our knowledge, no similar data exist for CD73-modified cancer cells. In our models, ATP was increased when either or both cN-II and CD73 were knocked out. We also observed increased IMP and decreased inosine in cN-II⁻/CD73⁻ cells, consistent with the fact that these cells will degrade less IMP into inosine. Whether these differences in nucleotide pools have a role in the phenotypic differences observed between our cell models remains unclear.

Previous studies suggested that CD73 is involved in cancer cell migration by adenosine-dependent and by adenosine-independent mechanisms. Our results support the predominant importance of this nucleoside in MDA-MB-231 and NCI-H292 cells, as adenosine could affect cell migration independently from CD73 expression. cN-II⁻/CD73⁻ cell migration and survival were also sensitive to AMP, independently from its conversion to adenosine. This could be the result of a direct effect of the nucleotide on these cells. Indeed, it has been described that the adenosine receptor A1 can be activated by AMP [38] and can mediate cell death [39]. In our MDA-MB-231 models, A1 is downregulated in cN-II⁻/CD73⁻ cells at the mRNA level and might not be responsible for this effect. To our knowledge, other enzymes such as prostatic acid phosphatase can hydrolyze AMP, independently

from CD73 and mediate cell death by generating adenosine [3]. Thus, their expression could be studied in cN-II⁻/CD73⁻ cells compared to the other models.

Metalloproteinases 2 and 9 are gelatinases that are highly expressed in MDA-MB-231 and regulate cell migration. Their activity depends on their inhibitors TIMP-1 and TIMP-2. High TIMPs expression being associated with low MMP activity and reduced migration, and low TIMPs expression with enhanced MMP activity and migration [27], we evaluated their expression level in our models. We observed higher levels of MMP-2 and MMP-9 mRNA expression under CD73 knockout in MDA-MB-231 cells. This phenomenon is possibly related to purine-dependent signaling. Indeed, by degrading AMP, CD73 promotes high concentrations of adenosine which reduce AKT activation. Downstream of AKT, different gene expressions, including MMPs, are enhanced [40–42]. Thus, in the absence of CD73, we expected reduced pools of adenosine, leading to a better AKT phosphorylation and a higher expression of MMPs, consistently with previous studies that show an inhibiting effect of adenosine on MMPs expression [43, 44]. However, our experiments did not reveal any detectable difference in AKT activation or in migration in the absence of CD73 alone for these breast cancer cells. Notably, in association with cN-II deficiency this AKT activation was more striking. This suggests that MMP-2 and MMP-9 expression modulation upon CD73 silencing is not sufficient to significantly impact cell migration but these alterations interact with cN-II-related modifications to accentuate the pro-migration phenotype induced by cN-II silencing.

Similarly, COX-2 expression can also be promoted by AKT activation [45, 46] and regulated by adenine nucleotides/nucleosides. Lin *et al.* showed that ATP promotes COX-2 expression through NADHP oxidase activity and an increase in ROS production [45, 47]. The triphosphate nucleotide and adenosine tend to have opposite effects on cells and consistently with this notion, in our models, the latter triggered a lower AKT activation and a lower COX-2 expression.

Furthermore, in our MDA-MB-231, cN-II repression was associated with an enhanced constitutive AKT activation that can mediate the observed COX-2 expression. As mentioned above, cN-II can impact intracellular nucleotide pools and its knock-out could lead to an accumulation of nucleotides and a decrease of adenosine levels in the cytoplasm. As adenosine is transferred to the extracellular space through nucleoside transporters, this decrease can also impact extracellular adenosine pools thus resulting in a weaker activation of ADORA receptors, which is not intuitively in accordance with the observed increase in AKT-activation in our models. Nevertheless, we must also consider the expression and activation state of the four PGE2 receptors (EP1, EP2, EP3 and EP4 receptors) on our models. Indeed, these receptors also belong to the GPCR family and are expressed in MDA-MB-231 cells [48]. These receptors share intracellular mediators with ADORA signaling involving cAMP generation and

Akt activation. Simultaneous activation of these pathways can thus lead to different cellular responses according to the panel of receptors expressed on the cells. In addition, our team previously demonstrated that cN-II downregulation with stable shRNAs is accompanied with lower ROS contents [8]. If a complete knockout of this 5'-nucleotidase impacts the cellular ROS contents, that should also act towards a downregulation of COX-2. Adenosine also has dual roles on phospholipase A2 expression and activity, which releases arachidonic acid, the PGE2 precursor [49, 50], thus revealing another link between nucleotide metabolism and COX-2/PGE2 axis. Further studies are needed to decipher whether COX-2 is directly impacted by cN-II and nucleotide metabolism. A recent study demonstrated that cN-II interacts with cytoplasmic proteins, opening the possibility of enzymatic activity-independent effect of the cytosolic 5'-nuceotidase. Indeed, cN-II interacts with the inflammasome protein Ipaf, through its leucin-rich region (LRR) [51], and the absence of this interaction could result in Ipaf oligomerization and thus inflammation. Other members of the inflammasome such as NLRP3 also contain a LLR region and could interact with cN-II, and NLRP3 has been described as regulating COX-2 expression and PGE2 production. With a similar mechanism as for Ipaf, an interaction between cN-II and NLRP3 could consequently impact COX-2 expression as observed in our models.

We showed that the COX-2/PGE2/AKT axis is involved in MDA-MB-231 cell migration and that this axis is reinforced when cN-II is absent. Indeed, the COX-2 inhibitor celecoxib reduced cell migration and, inversely, stimulating PGE2 production with arachidonic acid enhanced cell migration. Nevertheless, cN-II-deficient cells are more sensitive to these effects than their cN-II-proficient counterparts. These observations suggest that cN-II could play a role in migration regulation. The way cN-II is involved in cell sensitivity to agents that can impact on cell migration remains to be studied. This was unfortunately not confirmed with other COX-2 inhibitors, which we only can explain by either a difference in the effective COX-2 inhibition in our cells with the different compounds, or eventually by off-targets effects by celecoxib or by the other two compounds.

We included two techniques for the study of cell migration (wound healing on Incucyte device and CIM-plates on xCELLigence RTCA DP instrument). The results we obtained on the second did not confirm the ones with the first (Figures 4 and S3). There is, to our knowledge, no study comparing directly these two techniques, and we conclude that the molecular mechanisms involved in the migration observed on the different devices are different. Indeed, one is based on the recovery of a two dimensional surface that has already been populated by the cells, whereas the other is based on the migration of a cell from one side to the other on a plate and the signal takes into account both the number of cells and the strength of adherence. Our conclusions on cell migration are therefore limited to the two dimensional recovery.

In conclusion, the generation of cN-II and/or CD73 knockout cells provides new tools that will serve to better understand nucleotide metabolism in cancer, and more precisely, the possible interaction between intra- and extracellular compartments of purines. Indeed, until recently, these compartments have been studied independently and in various pathologies, whereas we know that both direct (nucleoside and nucleotide transports) and indirect (transcriptional regulation through receptors) interplays exist. The model characterization provides further arguments to consider these 5'-nucleotidases as targets to disturb cancer cell biology and reduce their aggressiveness. The differences in behavior between our three cell models (MDA-MB-231, NCI-H292 and 4T1) show that the cN-II and CD73-mediated phenotypes are largely cell specific, and we imagine dependent on the overall nucleotide metabolism in these cells.

4. Material and methods

4.1. Cell culture and transfection

Human triple negative breast cancer cell line MDA-MB-231, human adenocarcinoma cells (NCI-H292) and murine breast cancer cell line 4T1 were obtained from ATCC and cultivated in complete Roswell Park Memorial Institute (RPMI 1640, Gibco) medium supplemented with 10% (v/v) fetal bovine serum (FBS, ThermoFisher Scientific- Courtaboeuf, FRANCE), and fungizone (2 µg/mL), 100 U/mL penicillin, 100 mg/mL streptomycin on collagen-coated flasks in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells were routinely tested for *Mycoplasma* every two weeks. CD73 and/or cN-II knockout cells were generated using CRISPR/Cas9 technology. Oligonucleotides were inserted into pLentiCRISPRv2-blast or pLentiCRISPRv2-puro plasmids (Addgene, Cambridge, MA, USA) using BsmBI (ThermoFisher-Fermentas). Virus were produced using HEK 293T cells and a 24-hours incubation and used for the infection of cells, and stable models were selected with puromycin and/or blasticidine. Target RNA sequences for CD73 and cN-II plasmids were 5'-CCACTAGCATCTCAAATATC-3' and 5'-CTTGTCTTTGACACACTGTA-3' respectively in human cancer cells, and 5'-ACCGTCGAGAAGCCTATCAC-3' for cN-II in 4T1 cells.

4.2. Protein expression

Cells (0.5-1.10⁶ per flask) were seeded and allowed to adhere before being exposed to experimental conditions for indicated times. Cells were then rinsed with PBS and lysed with RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 M DTT, 1 M NaF, protease inhibitor cocktail, phosphatase inhibitors buffer and 100 mM sodium orthovanadate). After centrifugation (15 minutes, 12,000 g, 4 °C), the supernatant was collected and proteins (60 µg per condition) were separated by electrophoresis and transferred to a nitrocellulose membrane with the iBlot™2. TBS or PBS Odyssey Blocking buffer were used to block the membranes and dilute the primary antibodies, and PBS or TBS to rinse the membranes and dilute the secondary antibodies. The following antibodies were used: anti-cN-II (H00022978-M02, Novus Biologicals, Lille, FRANCE: 1/500), anti-pAkt (4060, Cell Signaling, Saint Quentin Yvelines, FRANCE : 1/500), anti-Akt (5239, Cell Signaling: 1/500), anti-COX-2 (12282S, Cell Signaling: 1/500), anti-actin (A5441, Sigma, Saint-Quentin Fallavier, FRANCE: 1/5000), anti-murine antibody (IRDye® 800CW, 1/5000; LI-COR Biosciences, Bad Homburg, GERMANY) or anti-rabbit antibody (IRDye® 680, 1/5000; LI-COR Biosciences). Protein expression was visualized using the Odyssey infrared system (LI-COR Biosciences).

Surface CD73 and CD44 expression were evaluated by flow cytometry using anti CD73 FITC-labeled (561254, BD Biosciences - Le Pont de Claix, FRANCE: 1:100) and anti CD44 APC-labeled (A10193, BD

Biosciences: 1/100) antibodies. Cells were harvested, pelleted and washed in PBS before a 30-minute staining with the antibodies or control isotypes (IgG1 FITC-labeled: 555748, BD Biosciences: 1:100 and IgG1 APC-labeled: 555751, BD Biosciences: 1:100).

4.3. Gene expression

The mRNA extraction was performed on pellets of 10^6 cells using the Qiagen (Les Ulis, FRANCE) column extraction kit, following the manufacturer's protocol. One microgram of mRNA was used for reverse transcription with M-MLV reverse transcriptase (InVitrogen, Cergy Pontoise, FRANCE). The cDNA was then diluted, and relative gene expression was determined by PCR in a final volume of 5 μ L with Takyon NO ROX SYBR Mmix dTTP blue mix (Eurogentec, Angers, FRANCE). Runs were performed on a Lightcycler (LC480, Roche Life Science). Quantification was performed by the $\Delta\Delta$ CT method using 28S mRNA expression as a housekeeping gene. Primers used for each gene are indicated in table S3.

4.4. Intracellular nucleotides

Cells ($2 \cdot 10^6$ per flask in 25 mm² flasks) were seeded, adhered for 24 hours and incubated 1 hour in presence or absence of 400 or 1600 μ M adenosine (Sigma) or AMP (Adenosine 5'-monophosphate sodium salt, Sigma). Then, cells were rinsed three times with cold PBS and nucleotides were extracted with a cold mixture of methanol/water (70/30, v/v). Extracted nucleosides and nucleotides were quantified using a validated on-line extraction coupled with LC-MS/MS method as described elsewhere [52]. The nucleotide content of each sample was calculated as the peak area of the compound of interest divided by the peak area of the corresponding internal standard, further divided by the number of cells as determined in a flask containing cells cultured under the same conditions. For each nucleoside and nucleotide, internal standards were corresponding labelled nucleotides except for IMP for which we used GTP.

4.5. Proliferation assay

Cells were trypsinized and rinsed with PBS to be stained with a solution containing 10 μ M Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) in PBS-0.1% BSA. After labeling, the cells were rinsed with culture medium and seeded in 6-well plates (200,000 cells/well) and allowed to adhere before being exposed to adenosine or AMP. Cells from one well were scratched for every time point, to evaluate CFSE fluorescence by flow cytometry on the BD FACSCalibur.

4.6. Cell survival

Cells (50,000 per well in 24 well-plates) were cultured in presence or absence of the indicated compounds. At indicated times, cells were trypsinized, washed and stained with an anti-Annexin V-FITC labeled antibody and propidium iodide, from the Annexin-V FLUOS kit (11 988 549 001, Roche; Mannheim, GERMANY). The associated fluorescence was measured by flow cytometry on the BD LSR-II Flow Cytometer. Annexin-V and/or PI-positive cells were considered as dead or dying cells.

4.7. Cell migration

For Incucyte analysis, cells (50,000 per well for MDA-MB-231 and 70,000 cells per well for 4T1) were seeded in an ImageLock 96-well plate (Essen BioScience, Welwyn Garden City, Hertfordshire, UK) and cultured to confluence. A wound was generated, using a Woundmaker 96 (Essen BioScience). The cells were then rinsed with PBS and incubated under the indicated conditions. The wound closure was monitored every 2 hours with the IncuCyte® device.

For xCELLigence analysis, cells (30,000 per well for MDA-MB-231 and 40,000 cells per well for 4T1) were seeded in serum-free media on CIM-Plate 16 in which complete media was added in the lower chambers following the manufacturer's instruction (ACEA Biosciences, San Diego, CA, USA). Cell index was recorded for at least 24 hours on the xCELLigence device.

4.8. PGE2 quantification

Cells (500,000 per well) were seeded and allowed to adhere before being exposed to fresh culture medium with or without 15 μ M arachidonic acid (Abcam; Paris, FRANCE). PGE2 quantification was performed on cells supernatants after a 24-hour incubation, using the Parameter™ Prostaglandin E₂ assay from R&D Systems®, according to the manufacturer's protocol.

4.9. Statistical analysis

The statistical analysis was performed using unpaired Student's t-test with Microsoft Excel. $p < 0.05$ was considered statistically significant. No correction for multiple analysis was performed.

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Supplemental material

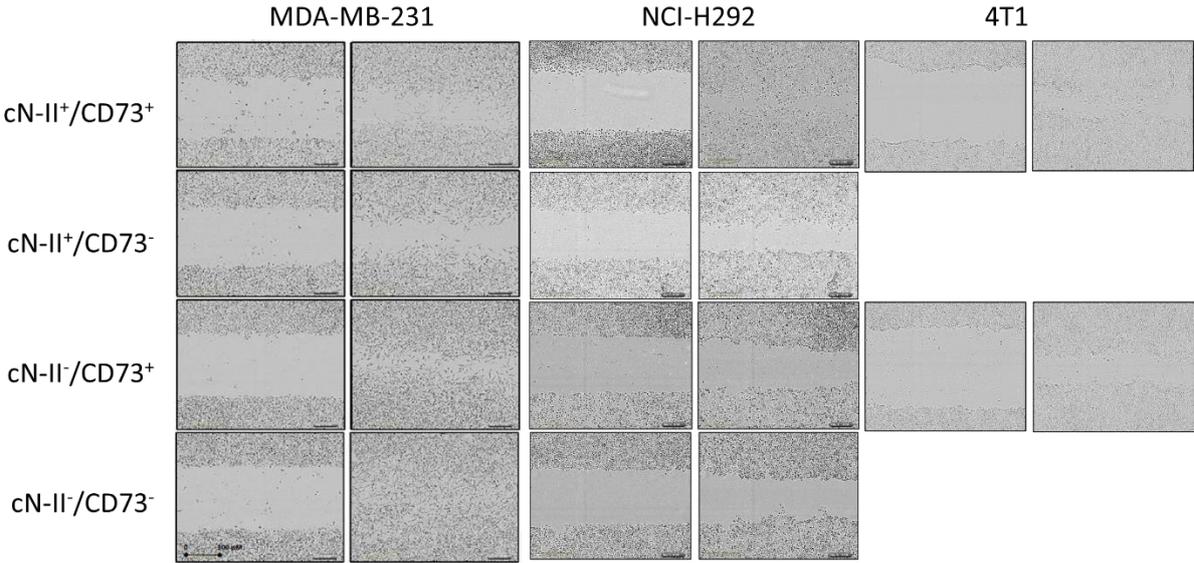


Figure S1. Images of cell confluence during cell migration studies just after (0 h) and 10 h after injury in MDA-MB-231 cells (left), NCI-H292 (middle) and 4T1 (right) cells.

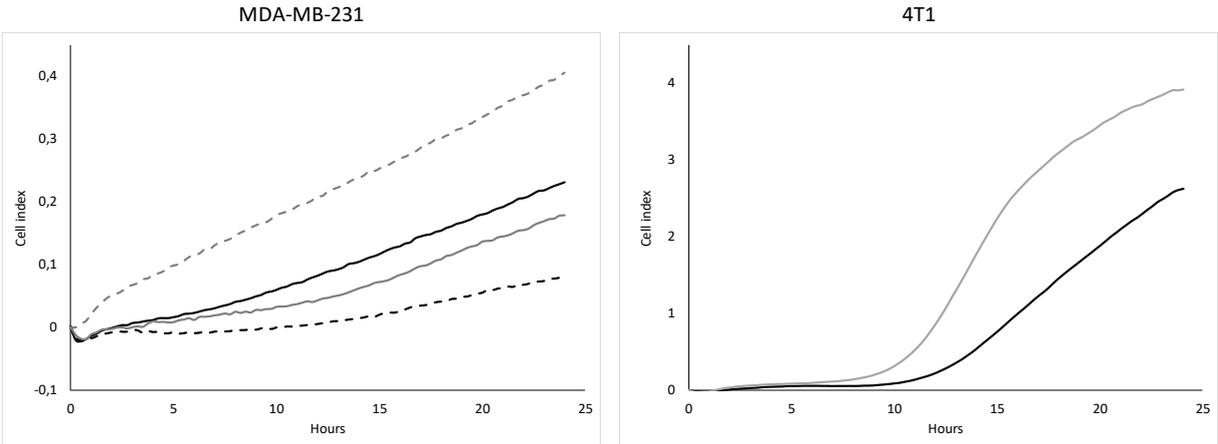


Figure S2. Cell migration using the xCELLigence device and CIM plates. Figures are representative of three independent experiments for MDA-MB-231 cells (left) and 4T1 cells (right). For MDA-MB-231 cells, black full line is for cN-II+/CD73+ cells, black dotted line for cN-II-/CD73- cells, grey full line for cN-II+/CD73- cells and grey dotted line for cN-II-/CD73+ cells. For 4T1 cells, black line is for cN-II+/CD73+ cells and grey line is for cN-II-/CD73+ cells.

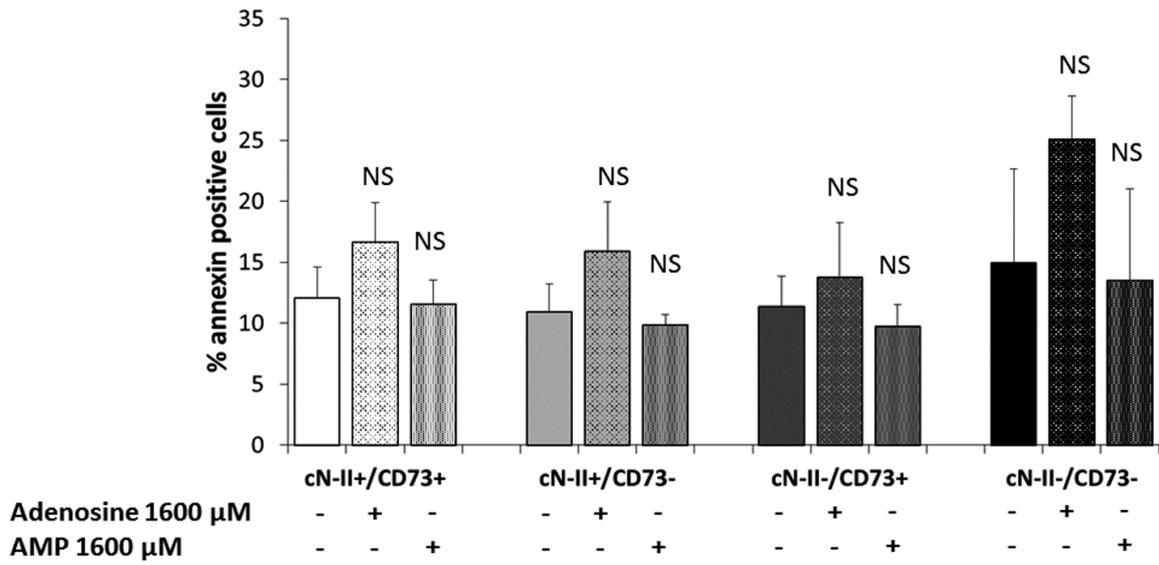


Figure S3: cell death after a 10-hour exposure to adenosine (1600 μM) or AMP (1600 μM). Cell death was assessed by flow cytometry with Annexin-V and PI labelling. Graphs are mean values of three independent experiments performed in duplicate and error bars are standard deviation. Students t-test was used to compare exposed cells to controls.

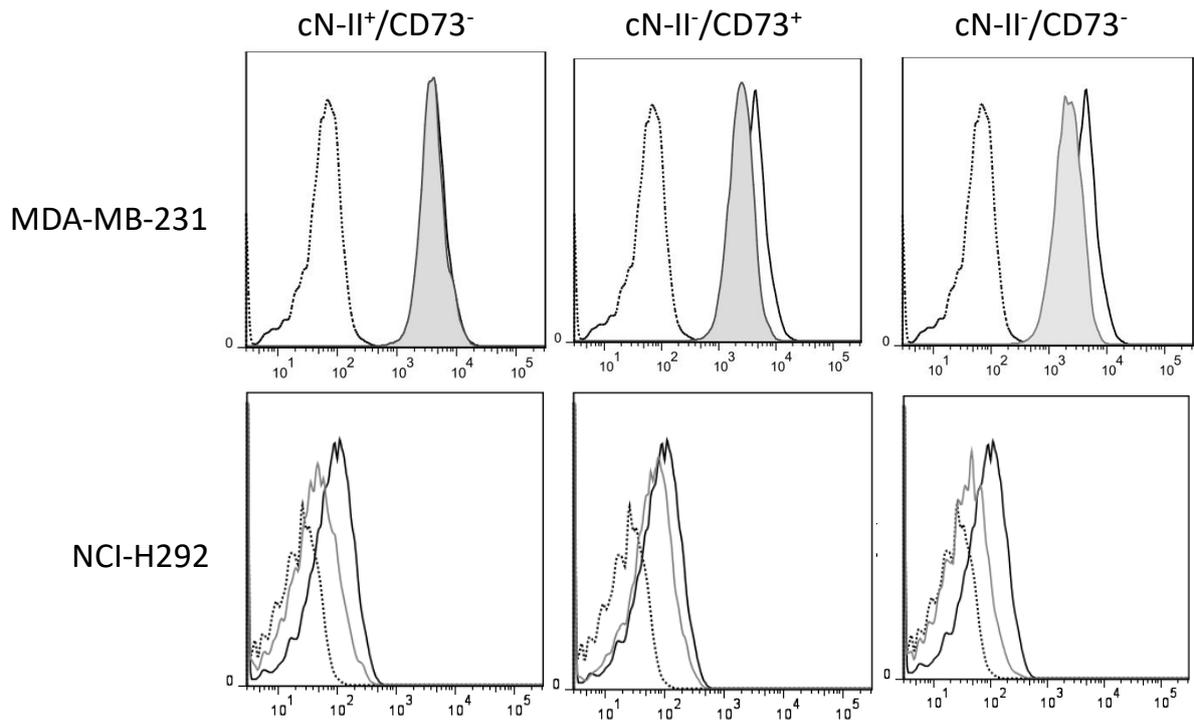


Figure S4. CD44 protein expression at cell surface of MDA-MB-231 cells and NCI-H292 cells. CD44 expression was evaluated by flow cytometry as indicated in material and methods. Dotted lines are for autofluorescence, black lines for the cN-II⁺/CD73⁺ cells, and grey lines for the indicated cell line.

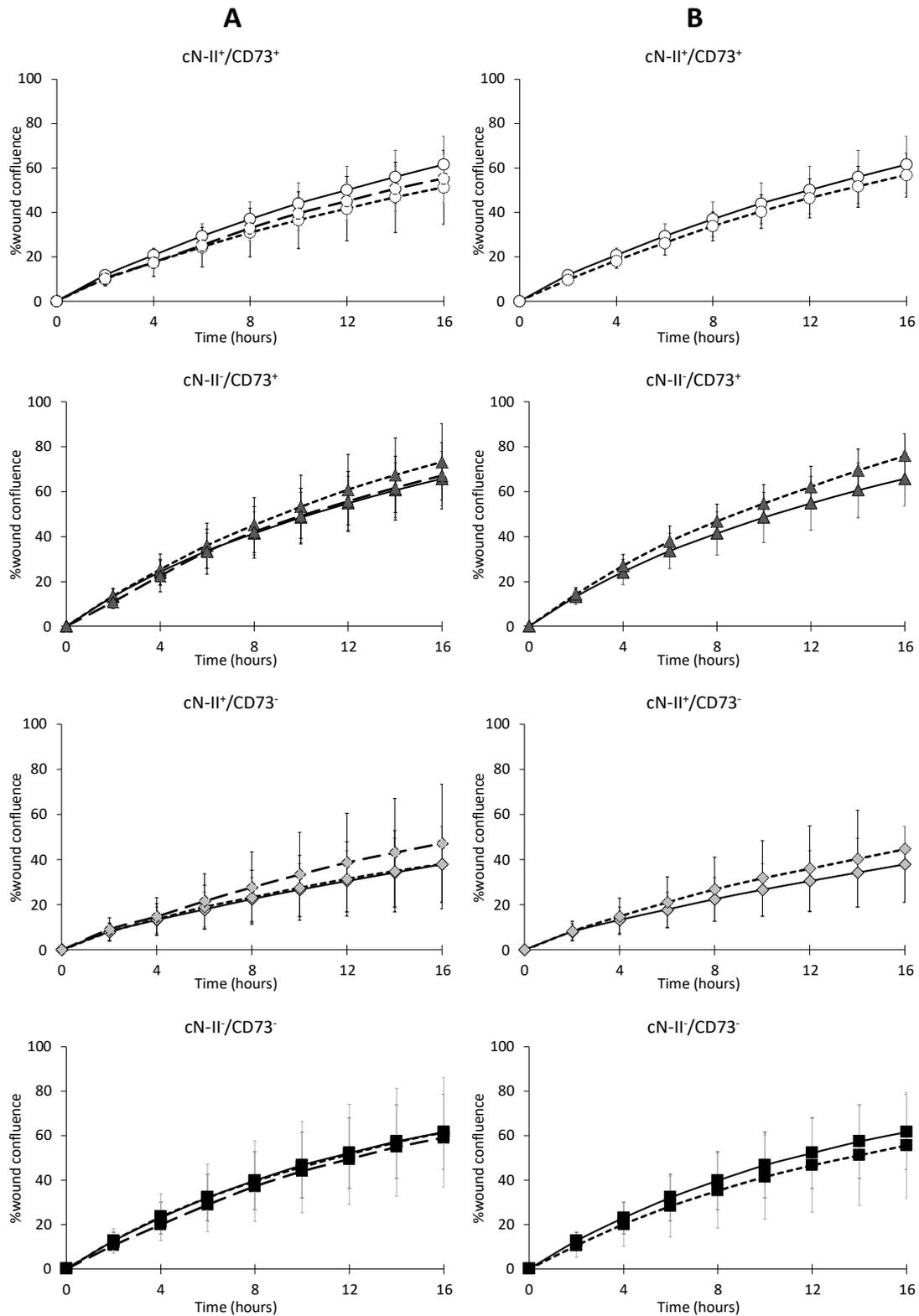


Figure S5. Additional conditions for the study of the COX-2/PGE2/Akt axis in cell migration in MDA-MB-231 cells. Wound healing curves of MDA-MB-231 cells exposed or not to (A) valdecoxib (---, 100 μ M) or rofecoxib (---, 100 μ M) or to (B) AKT inhibitor VIII (---, 10 μ M). Graphs are mean values of four independent experiments performed in triplicate and error bars are standard deviation.

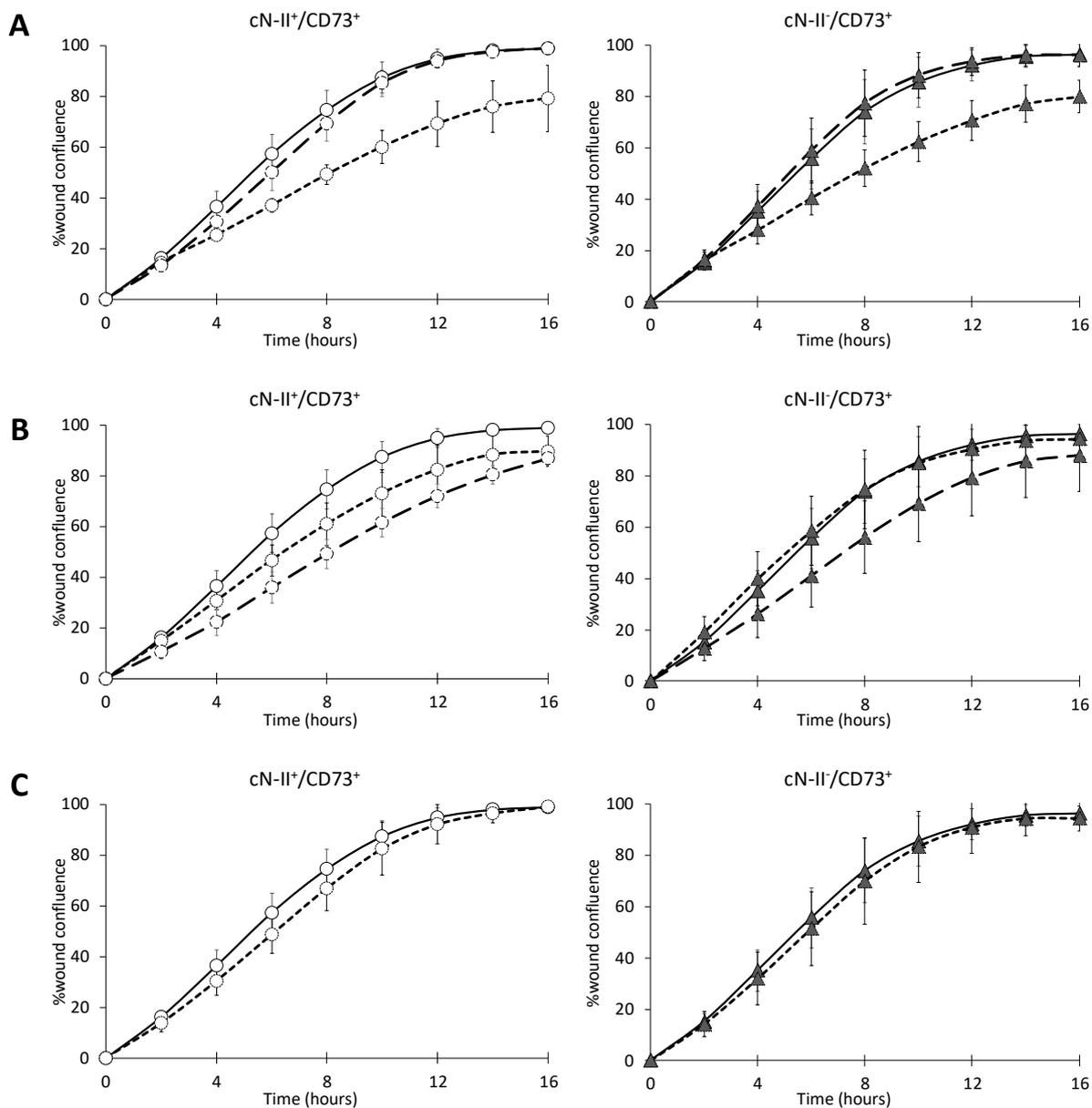


Figure S6. Additional conditions for the study of the COX-2/PGE2/Akt axis in cell migration in 4T1 cells. Wound healing curves of 4T1 cells exposed or not to (A) celecoxib (---, 60 μ M) or arachidonic acid (— — —, 30 μ M), to (B) valdecoxib (---, 100 μ M) or rofecoxib (— — —, 100 μ M) or to (C) AKT inhibitor VIII (---, 10 μ M). Graphs are mean values of four independent experiments performed in triplicate and error bars are standard deviation.

Gene	MDA-MB-231				NCI-H292			
	cN-	cN-	cN-II ⁻	cN-II ⁻	cN-	cN-	cN-II ⁻	cN-II ⁻
	II ⁺ /CD73 ⁺	II ⁺ /CD73 ⁻	/CD73 ⁺	/CD73 ⁻	II ⁺ /CD73 ⁺	II ⁺ /CD73 ⁻	/CD73 ⁺	/CD73 ⁻
A1	0.74 ±	0.98 ±	0.12 ±	0.16 ±	2.03 ±	1.89 ±	1.96 ±	2.02 ±
	0.19	0.13	0.03 ***	0.07	0.38	1.01	0.45	0.58

A2A	0.84 ±	0.78 ±	0.69 ±	0.78 ±	0.046 ±	0.120 ±	0.037	0.033
	0.17	0.04	0.22	0.34	0.010	0.182	±	±
							0.010	0.005*
A2B	5.40 ±	5.92 ±	9.58 ±	6.37 ±	3.05 ±	8.88 ±	5.87 ±	7.43 ±
	3.93	4.08	6.51	3.26	1.79	6.10	5.22	5.77
ADA	0.85 ±	0.90 ±	0.72 ±	1.19 ±	5.47 ±	5.11 ±	5.33 ±	6.07 ±
	0.12	0.06	0.12	0.11 **	0.68	2.52	0.67	1.52
AdK	0.97 ±	1.03 ±	0.88 ±	0.82 ±	0.74 ±	0.82 ±	0.79 ±	0.71 ±
	0.09	0.10	0.12	0.07 *	0.14	0.19	0.15	0.08
AMPD3	1.14 ±	1.21 ±	1.56 ±	1.55 ±	3.07 ±	2.28 ±	3.13 ±	2.66 ±
	0.26	0.27	0.36	0.28	0.93	0.67	0.89	0.85
ADSL	0.95 ±	11.01 ±	0.85 ±	0.92 ±	0.72 ±	0.79 ±	0.77 ±	0.79 ±
	0.04	0.02 *	0.07	0.10	0.10	0.15	0.12	0.13
ADSS	1.40 ±	1.93 ±	1.79 ±	1.72 ±	1.48 ±	1.62 ±	1.26 ±	1.61 ±
	0.33	0.37	0.22	0.26	0.32	0.10	0.59	0.39
ENT-1	1.94 ±	2.02 ±	1.59 ±	1.61 ±	0.48 ±	0.86 ±	0.52 ±	0.61 ±
	0.68	0.82	0.98	0.47	0.07	0.57	0.17	0.22

dCTPPP1	1.06 ±	1.10 ±	1.14 ±	1.35 ±	2.13 ±	2.14 ±	2.29 ±	2.23 ±
	0.11	0.10	0.06	0.20 *	0.37	0.49	0.25	0.45
GMPR	1.12 ±	1.57 ±	0.82 ±	1.09 ±	0.052	0.190 ±	0.051	0.041
	0.08	0.02 ***	0.1 **	0.20	0.011	0.317	±	±
							0.033	0.016
GMPS	1.11 ±	1.37 ±	1.16 ±	1.12 ±	1.49 ±	1.53 ±	1.79 ±	1.69 ±
	0.10	0.17 *	0.11	0.15	0.20	0.14	0.27	0.29
IMPDH1	1.12 ±	1.24 ±	1.16 ±	1.46 ±	2.01 ±	1.87 ±	1.72 ±	2.03 ±
	0.13	0.10	0.22	0.48	0.75	0.39	0.54	0.44
IMPDH2	1.13 ±	1.16 ±	1.03 ±	1.01 ±	1.03 ±	1.18 ±	1.02 ±	0.98 ±
	0.18	0.09	0.06	0.09	0.09	0.20	0.10	0.13
ITPA	0.96 ±	1.06 ±	1.18 ±	1.59 ±	0.45 ±	0.67 ±	0.43 ±	0.43 ±
	0.04	0.17	0.24	0.21	0.13	0.34	0.11	0.12

PNP	1.05 ±	0.72 ±	1.19 ±	1.55 ±	0.93 ±	1.01 ±	0.90 ±	0.75 ±
	0.14	0.06 **	0.21	0.26 *	0.16	0.58	0.27	0.19
PPAT	2.13 ±	1.72 ±	2.19 ±	2.40 ±	1.98 ±	2.57 ±	2.39 ±	2.20 ±
	0.79	0.41	0.40	0.64	1.15	0.86	0.28	0.54
RRM1	1.19 ±	1.12 ±	0.87 ±	0.92 ±	1.93 ±	2.07 ±	1.01 ±	0.84 ±
	0.17	0.44	0.60	0.76	0.40	0.82	0.71	0.60*
SAMHD1	1.43 ±	1.61 ±	2.10 ±	2.70 ±	0.89 ±	1.31 ±	0.97 ±	0.78 ±
	0.36	0.15	0.24 *	0.36 **	0.29	0.94	0.09	0.10

Table S1. mRNA expression of nucleotide metabolism-related genes and ADORA receptors in MDA-MB-231 and NCI-H292 models. Values are means ± standard deviation of four independent experiments, performed in triplicate. * = p < 0.05, ** = p < 0.01 and *** = p < 0.001 using Students t-test in comparison with the corresponding cN-II⁺/CD73⁺ cell line. See legend of table 2 for gene names.

Cell model	Experiment al condition	ATP	AM P	Adenosin e	GTP	IMP	dAT P	dGT P	UT P	CT P	Inosin e
cN- II ⁺ /CD7 3 ⁺	-	93 ±	72 ±	123 ± 52	97 ±	202 ±	95 ±	91 ±	93	96	132 ±
		9	34		4	202	6	11	±	± 7	85
	Adenosine	143	156	410 ±	113	252 ±	129	96 ±	10	12	1255
	400 μM	± 23	± 90	243	± 32	229	± 14	12	3 ±	6 ±	± 239
									26	31	
	Adenosine	126	152	1320 ±	113	154 ±	104	98 ±	94	13	1805
	1600 μM	± 17	± 86	864	± 7	146	± 13	15	± 9	8 ±	± 920
										19	
	AMP 400	162	147	3342 ±	105	115 ±	155	97 ±	86	10	999 ±
	μM	± 32	± 51	4450	± 3	92	± 33	7	± 7	4 ±	1293
									24		
AMP 1600	149	150	12310 ±	118	304 ±	135	104	10	13	3311	
μM	± 20	± 79	16966	± 10	375	± 10	± 5	7 ±	0 ±	±	
								9	23	4229	
2-deoxy-	38 ±	19 ±	172 ± 86	51 ±	154 ±	16 ±	21 ±	32	29	223 ±	
glucose	3	10		12	115	4	9	±	±	166	
								22	18		
cN- II ⁺ /CD7 3 ⁻	-	77 ±	69 ±	75 ± 28	88 ±	417 ±	73 ±	57 ±	72	77	92 ±
		11*	40		15	150	20*	18*	±	±	49
								*	10	8*	
									*	*	

Adenosine	116	149	184 ±	111	845 ±	102	75 ±	75	10	981 ±	
400 μM	± 18	± 83	181	± 39	902	± 7*	12	± 6	6 ±	609	
									22		
Adenosine	125	198	480 ±	111	1155	94 ±	52 ±	73	11	2596	
1600 μM	± 29	±	197	± 39	±	21	46	±	7 ±	±	
		141			1284			21	44	1343	
AMP 400	111	153	1688 ±	88 ±	278 ±	102	59 ±	64	79	2633	
μM	± 27	± 51	2295	24	291	± 39	12*	±	±	±	
							*	26	29	4146	
AMP 1600	99 ±	129	7512 ±	90 ±	317 ±	92 ±	60 ±	70	88	3805	
μM	16*	± 38	10505	18	251	18*	7*	±	±	±	
								26	35	6165	
2-deoxy-	25 ±	25 ±	48 ± 1	40 ±	207 ±	10 ±	14 ±	19	21	76 ±	
glucose	10	22		18	64	7	10	±	±	48	
								14	15		
cN-II⁻	-	146	230	568 ±	144	60 ±	114	73 ±	12	15	276 ±
/CD73⁺		±	±	384	±	52	± 33	28	8 ±	3 ±	137
		35*	149		23*			40	49		
		*	*						*		
Adenosine	204	334	1629 ±	173	9409	143	89 ±	14	17	3363	
400 μM	± 61	±	1163	± 59	±	± 52	28	4 ±	1 ±	±	
		148			1623			68	56	2757	
					6						

Adenosine	212	291	4160 ±	188	627 ±	133	73 ±	13	21	1827	
1600 μM	±	±	1175	±	659	± 23	17	5 ±	4 ±	±	
	22*	101		19*				34	20	1378	
	*			*				*			
AMP 400	204	373	17812 ±	204	3316	155	83 ±	11	16	5158	
μM	± 29	±	24391	±	±	± 52	24	9 ±	4 ±	±	
		174		45*	4537			52	74	1799*	
										*	
AMP 1600	208	326	6231 ±	174	449 ±	157	80 ±	13	17	2354	
μM	±	±	7962	±	496	± 51	24	8 ±	8 ±	±	
	6**	121		8**				19	40	3093	
2-deoxy-	71 ±	77 ±	336 ±	94 ±	289 ±	13 ±	19 ±	57	63	278 ±	
glucose	16*	44	140	35	35	5	19	±	±	249	
								43	42		
cN-II⁻	-	127	138	38 ± 1*	142	1206	107	77 ±	10	11	55 ±
/CD73⁻		±	± 70		± 53	±	± 47	39	3 ±	4 ±	43*
		31*				835*		24	23		
Adenosine	157	176	527 ±	176	2453	105	71 ±	94	13	1335	
400 μM	± 33	± 83	677	± 79	±	± 44	48	±	3 ±	±	
					667*			13	42	1907	
Adenosine	163	266	981 ±	182	4528	84 ±	75 ±	94	16	3512	
1600 μM	± 25	±	1258	± 90	±	31	47	±	2 ±	±	
		216			3702			26	76	4013	

AMP 400	140	192	2136 ±	152	3600	101	69 ±	94	11	1329
μM	± 34	±	2972	± 49	±	± 40	32	±	9 ±	±
		108			2686			10	16	2200
AMP 1600	153	219	10889 ±	167	4836	118	67 ±	84	11	2199
μM	± 36	±	15279	± 62	±	± 56	33	±	8 ±	±
		137			5475			17	14	3582
2-deoxy-	62 ±	42 ±	30 ± 1	111	902 ±	18 ±	15 ±	29	35	35 ±
glucose	23	52		± 63	401	13	19	±	±	24
								16	22	

Table S2. Complete data for intracellular nucleotides and nucleosides in studied cell models. Nucleotides and nucleosides were quantified as indicated in material and methods and are expressed as mean values of the ratios of the surface of the compound of interest / surface of internal standard. The results were further normalized for one million cells and expressed as relative content as compared to unexposed cN-II⁺/CD73⁺ cells in each experiment. Values are means of three independent experiments ± standard deviations. * = p < 0.05; ** = p < 0.01 using Student's t-test in comparison with unexposed cN-II⁺/CD73⁺ cells.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
28S	CGATCCATCATCCGCAATG	AGCCAAGCTCAGCGCAAC
COX2	TTATCTACACGGCCCCCTCC	CCAGGGCACGATGAAGTCAC
PLA2G4A	CATGCCCAGACCTACGATTT	CCCAATATGGCTACCACAGG
MMP-2	CCCATGAAGCCCTGTTCCACC	CGGTCGTAGTCCTCAGTGGT
MMP-9	GGAGGCGCTCATGTACCCTA	GGACCATAGAGGTGCCGGAT
TIMP-1	GCTGGAAAACCTGCAGGATGGA	GTCCGTCCACAAGCAATGAGT
TIMP-2	TTATCTACACGGCCCCCTCC	CCAGGGCACGATGAAGTCAC
A1	GAGCCGGAGGACTATGAGC	CCTGGAAAGCTGAGATGGAG
A2A	TCTTCAGTCTCCTGGCCATC	TCCAACCTAGCATGGGAGTC
A2B	CTCCATCTTCAGCCTTCTGG	CATGCACAGGTAACCAGCAC
ADA	AGCCCAAAGTAGAACTGCAT	CAAACCTGGCCAGGAAGTCT
ADK	TCACCCAAGGGAGAGATGAC	ATAGTGGCCAGCACGGATAC
AMPD3	CACATCCTGGCTCTCATCAC	GGATGTGTGTGTCCACCTTT
ADSL	ACAGCTACCGCTCACCTCTT	GCCGCCATGTCCGGAATTTA
ADSS	GCAGAGCAGTTCAGTTCGCT	GGCCGCAATCGCCGTT
ENT-1	GCTGGGTCTGACCGTTGTAT	CTGTACAGGGTGCATGATGG
dCTPP1	AAATGGACATCAACCGGCGA	AGTCACAGGGAATGTCCGCA
GMPR	ACGGAGTGTGCTGGAGAAGT	CCTCCTGCGTGCTTGTTCAT
GMPS	AAGGTTGTGGCACGTTCTGG	AGGGTGGAAGTGTGCTCCAT
IMPDH 1	GCCCTAGATTGGACCTCGCT	ATCAGGTAGTCCGCCATGCT
IMPDH 2	GAGGCAATGTGGTCACTGCT	GATGGAGCCACTTCCCATGC
ITPA	TCCCCGGCCCCTACATAAAG	GCTGACTTGTCTCGAACCC
PNP	TGAAATCCCCAACTTTCCCC	AATGTCACCTCCAGAGTGGG
PPAT	ATCACACAAGGGAATGGGTC	ACAGACCAATACCATG ACGC

RRM1	GCAGCTGAGAGAGGTGCTTT	CAGGATCCACACATCAGACA
SAMHD1	CCCAAAGTTTGCTCGACGTGA	TGCATTCCATAATCCATGTTG

Table S3: COX-2: cyclooxygenase 2; PLA2G4A: phospholipase A2; MMP-2: matrix metalloproteinase 2 ; MMP-9 : matrix metalloproteinase 9 ; TIMP-1 : tissue inhibitor of metalloproteinases 1 ; TIMP-2 : tissue inhibitor of metalloproteinases 2; A1: adenosine receptor 1; A2A: adenosine receptor 2A; A2B: adenosine receptor 2B; ADA: adenosine deaminase; ADK: adenosine kinase; AMPD3: AMP (adenosine monophosphate) deaminase 3; ADSL: adenylosuccinate lyase; ADSS: adenylosuccinate synthetase; ENT-1: equilibrative nucleoside transporter 1; dCTPP1: dCTP (deoxycytidine triphosphate) pyrophosphatase 1; GMPR: GMP (guanosine monophosphate) reductase; GMPS: GMP synthetase; IMPDH 1: IMP (inosine monophosphate) dehydrogenase 1; IMPDH 2: IMP (inosine monophosphate) dehydrogenase 2; ITPA: Inosine triphosphate pyrophosphatase; PNP: purine nucleoside phosphorylase; PPAT: amidophosphoribosyltransferase; RRM1: ribonucleoside-diphosphate reductase large subunit; SAMHD1: deoxynucleoside triphosphate triphosphohydrolase

Axis 2: Role of 5'-nucleotidases (cN-II and CD73) in response of cancer cells to cytotoxic drugs and adaptability to stress conditions (nucleotide and hypoxic stress)

In the second part of our project, we aimed to decipher the role of cN-II and CD73 in cells response to different stress conditions. Here we use all four phenotypes of human lung adenocarcinoma cell lines (NCI-H292) used in the first part of the project. The stress conditions used in this study were nucleotides stress (adenosine 1600 μ M, AMP 1600 μ M), hypoxic stress (1% oxygen), CoCl₂-induced stabilization of HIF-1 α as well as several cytotoxic drugs. Under the said stress conditions, we observed cell proliferation, migration, sensitivity both *in vitro* and *in vivo*, cell behavior (cell index) and tumor growth *in vivo*.

The findings of this study have been submitted for publication and the complete submitted manuscript has been attached hereunder. Results have also been presented at two international conferences as follows:

- Raza MZ, Cadassou O, Cros-Perrial E, Dumontet C & Jordheim LP. 5'-nucleotidases are involved in the biology of human lung cancer cell lines. Oral presentation at 18th International Symposium on Purine and Pyrimidine Metabolism in Man, Lyon, June 2019.
- Raza MZ, Cadassou O, Cros-Perrial E, Puisieux A, Dumontet C & Jordheim LP. 5'-nucleotidases are involved in the biology of human lung cancer cell lines. Poster at 110th Annual Meeting of the American Association for Cancer Research, Atlanta, GA, USA, March-April 2019.

CD73 and cN-II regulate the cellular response to chemotherapeutic and hypoxic stress in lung adenocarcinoma cells

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Abstract

Cytosolic 5'-nucleotidase II (cN-II) and ecto-5'-nucleotidase (ecto-5'-NT, eN, CD73) are enzymes involved in the nucleotide metabolism by dephosphorylating nucleoside monophosphates. Both enzymes have been shown to be involved in cancer by modifying anticancer drug activity, cancer cell biology and immune modulation. We have modified the lung cancer cell models (NCI-H292) to become a complete knockout of either or both of these enzymes using the CRISPR/Cas9 technique, and used these models to study the implication of these enzymes in the cellular response to different stress condition *i.e.* chemotherapeutic agents, hypoxia and nucleotide stress using different techniques (CFSE staining, MTT assay, IncuCyte RTCA confluence assay, wound healing assay, Caspase 3/7 assay, Flow cytometer, xCELLigence and *in-vivo* mice experiments). Our results show that there is no significant role of these enzymes in cell proliferation under hypoxic stress (1% oxygen or CoCl₂-mediated stabilization of HIF-1 α). Similarly, cN-II and CD73 are not shown to be involved in cell migration under CoCl₂-mediated HIF-1 α stabilization. Furthermore, our results show that CD73-deficiency is associated with increased apoptosis in response to 1600 μ M adenosine, decreased sensitivity to mitomycin C (IC₅₀ value of $3.3 \pm 0.6 \mu$ M in CD73-deficient cells as compared to $1.2 \pm 0.15 \mu$ M in control cells, $p < 0.05$) and enhanced sensitivity to vincristine. Our study gives new insights into the biological roles of cN-II and CD73 under stress conditions in this particular cancer cell line. Further experiments will help deciphering the molecular mechanisms underlying the observed differences.

Key-words: 5'-nucleotidase; cancer cells; hypoxia; cancer drugs; cellular response

Introduction

Nucleotides are important players in the functioning of cellular machinery. They have strong strategic positions in intracellular as well as extracellular activities. Inside the cells, they serve as building blocks for nucleic acids, currency notes for cell's powerhouse, regulators for several metabolic enzymes and mediators for hormonal activities. Nucleotides also hold an inevitable position in a wide range of extracellular processes, both physiological and pathological. Examples include but are not limited to cell growth and expansion, neurotransmission, platelet aggregation, blood vessels' tone, regulation of immune system, purinergic signaling and homeostasis of nucleotide pools on both sides of the cell membrane. Many cell types release nucleotides under quiescent conditions [1, 2] as well as in response to varied stress signals such as mechanical stimulation [3–6], pharmacological compounds like bradykinin, serotonin and acetylcholine [7] and via regulated exocytosis [8–10]. A large number of nucleotides' functions is directly or indirectly linked to their overall metabolism that includes 5'-nucleotidases along with nucleotide kinases, deaminases, transporters and other enzymes. Eight different types of 5'-nucleotidases have been reported, including six cytoplasmic (cN-IA, cN-IB, cN-II, cN-IIIA, cN-IIIB, cdN), one membrane bound (CD73) and one mitochondrial (mdN). In the field of cancer, cN-II and CD73 have been identified as role players both for cancer cell biology and for response to cancer chemotherapy, and hence been studied more extensively as compared to other members of 5'-nucleotidases in this regard.

Cytosolic 5'-nucleotidase II (cN-II, NT5C2) is a ubiquitously expressed, structurally conserved and highly regulated bifunctional enzyme present in cytoplasm, and catalyzes the dephosphorylation of its preferred substrates, IMP and GMP [11]. Because of its enzymatic activities, cN-II plays a vital role in maintaining the balance of intracellular pools of purine compounds [12]. Studies have shown that downregulation of cN-II is associated with an increased, whereas its overexpression is related to a decreased, intracellular concentration of all triphosphorylated purine and pyrimidine nucleosides [13–16]. Free nucleosides generated intracellularly by the enzymatic activity of cN-II can cross the cell membrane according to their concentration gradient and can have biological effects when in extracellular space. Adenosine, for example, through its interaction with adenosine receptors, is involved in activation of mitogen activated protein kinases (MAPK) [17], modulation of neuronal signaling and neurodevelopment [18] and regulation of immune system [19]. Furthermore, a large number of studies have shown the role of cN-II in cancer, mainly hematological malignancies but also in some solid tumors [20]. Altered expression (at mRNA, circRNA and protein level) or enzymatic activity obtained by biomolecular approaches or due to certain mutations in cN-II are associated with an impact on tumor aggressiveness, relapse and response to purine nucleoside analogs [21–27].

CD73 (ecto-5'-nucleotidase, encoded by *NT5E*) is an extracellular glycosylphosphatidyl inositol (GPI)-anchored 5'-nucleotidase which plays a vital role in maintaining the extracellular pool of the nucleosides and nucleotides by dephosphorylating in particular adenosine monophosphate (AMP), its preferred substrate. CD73 is variably expressed in different tissues. However, its expression and function can be regulated by different factors including hypoxia [28–30], inflammatory mediators, cell exposure to certain metabolites [31–33], chemical inhibitors [34, 35] and monoclonal antibodies [31, 36–39]. Along with its enzymatic activity, CD73, in collaboration with tenascin C (TnC), constitutes an adhesion complex which plays a key role in cell migration, invasion and *in fine* in cancer progression [40]. Thus, CD73 has a significant role in many pathologies, but it has gained attention from cancer researchers in recent years as a potential checkpoint for cancer immunotherapy [41]. The reason being the major role played by adenosine, catalyzed from AMP by CD73, in tumor immune evasion and angiogenesis.

As indicated here over, nucleotides hold a paramount position in cell response mechanism to stress conditions, and cN-II and CD73 along with having a direct effect on these nucleotides, also have a role in cancer. We and others have previously studied the cellular roles of these enzymes using either pharmacological inhibitors of CD73 [34, 35, 42–47] and cN-II [48–53] or their partial knock-down by siRNA or shRNA [14, 21]. Here, we aimed to decipher the role of cN-II and CD73 in the response of human lung cancer cells to different stress conditions. We used four different cell phenotypes obtained by knocking out either one or both 5'-nucleotidases in the human lung adenocarcinoma cell line NCI-H292 using CRISPR/Cas9 technique.

Materials and methods

Cell models and cell culture procedures

Human lung cancer cell line NCI-H292 was obtained from the American Type Culture Collection (ATCC). Cells were cultured in complete Roswell Park Memorial Institute (RPMI 1640, Gibco) medium supplemented with 10% (v/v) fetal bovine serum (FBS, ThermoFisher Scientific- Courtaboeuf, France), 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 µg/mL fungizone on collagen-coated flasks at 37 °C with 5% CO₂. A routine test for *Mycoplasma* was conducted twice a month. CD73 and/or cN-II knockout NCI-H292 cells were generated by using CRISPR/Cas9 technology as earlier described (Cadassou et al., submitted manuscript). Oligonucleotides were inserted into pLentiCRISPRv2-blast or pLentiCRISPRv2-puro plasmids (Addgene, Cambridge, MA, USA) using BsmBI (ThermoFisher-Fermentas). Virus were produced using HEK 293T cells and a 24-hours incubation and used for the infection of cells, and stable models were selected with puromycin (0.5 µg/mL) and/or blasticidine (10 µg/mL). Target RNA sequences for CD73 and cN-II plasmids were 5'-CCACTAGCATCTCAAATATC-3' and 5'-CTTGTCTTTGACACACTGTA-3' respectively.

Cell proliferation by CFSE staining

Cells were trypsinized, centrifuged and incubated with 10 μ M CFSE (Cell Trace CFSE, INVITROGEN) for 10 minutes at 37 °C with 5% CO₂ and 21% oxygen. After adding 5 mL cold complete RPMI medium, cells were incubated in ice for 5 minutes. Then, cells were washed twice with complete RPMI medium before being seeded at 200,000 cells per well in 6 well plates and incubated in hypoxic conditions (37 °C, 5% CO₂, 1% oxygen) until the end of experiment. When using cobalt chloride (CoCl₂, Sigma-Aldrich) to stabilize Hypoxia-Inducible Factor-1 α (HIF-1 α), the same protocol was followed except that the cells were incubated in normoxic conditions (37 °C, 5% CO₂, 21% oxygen) and CoCl₂ (100 μ M) was added every 24 hours post incubation until the end of experiment. Intensity of CFSE stain in the cultivated cells was analyzed once a day for 3 days using BD LSRII Multi-color Flow Cytometer.

Cell proliferation, adherence and sensitivity with xCELLigence

Background noise was calculated with 50 μ L RPMI medium in all 16 wells of E-plates 16 (ACEA Biosciences) before cells (3000 cells per well in 100 μ L RPMI) were added. Finally, respective experimental compounds (in 100 μ L RPMI) were added in the wells and plates were fixed in the xCELLigence RTCA DP device (ACEA Biosciences) after a short incubation of 30 minutes at room temperature. Cell index was determined every 15 minutes using using RTCA software 2.0.

Protein expression

Cells were lysed on ice with complete RIPA lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium-desoxycholate, 1 M DTT, 1 M NaF, Protease Inhibitor Cocktail, phosphatase inhibitors buffer and 100 mM sodium-orthovanadate) for one hour followed by centrifugation at 12,000 g for 15 min at 4 °C to extract proteins. Bradford's Method was then used for quantification of proteins. Gel electrophoresis was performed with 100 μ g of proteins from each sample on BIO-RAD precast 4-15% Mini-PROTEAN TGX gels, proteins were transferred to PVDF membranes using iBlot 2 gel transfer device (Invitrogen), and the membranes were blocked using Odyssey[®] Blocking Buffer (TBS) (LI-COR, Inc.). This was followed by an overnight incubation with 1/500 dilution of primary antibody for cN-II (Abnova Mouse mAb #H00022978-M02 and Abcam rabbit mAb #ab96084), HIF-1 α (Cell Signaling Rabbit mAb #36169S) and (β -Actin: Sigma Mouse mAb #A5441), and then by a 1-2 hours incubation of secondary antibody ("Goat anti-mouse IgG H&L: IRDye[®] 800CW- LI-COR: 926-32210" and "Goat anti-rabbit IgG H&L: (IRDye[®] 680D #926-68071") diluted in Odyssey Blocking Buffer in TBS (LI-COR). Finally, the membranes were scanned at 800 or 700 nm on Li-Cor Odyssey IR scanner (LI-COR 9120 Odyssey IR system) or Ozyme c500 (Azure biosystems).

For the expression of membrane bound proteins (CD73 and CD44), harvested cells were labeled with corresponding antibodies (FITC mouse anti-human CD73 mAb: BD Pharmingen #561254 and APC anti-human CD44 antibody: BD Pharmingen #559942) and isotypes (IgG1 FITC-labeled: 555748, BD

Biosciences: 1:100 and IgG1 APC-labeled: 555751, BD Biosciences: 1:100) for 30 minutes at 4 °C. Labelled cells were analyzed on BD LSRII flow cytometer and data processed with BD FACSDiva (BD Biosciences, San Diego, CA, USA) and FlowJo (Tree Star, Ashland, OR, USA) softwares.

Wound healing assay

Cell migration was analyzed using the IncuCyte® Scratch Wound Assay (Essen BioScience, Ann Arbor, Michigan, USA). Cells (60,000 cells/well) were seeded in RPMI using IncuCyte® ImageLock 96-well Plates and incubated for 24 hours before a wounding procedure was performed with WoundMaker™. After washing the wells with PBS, cells were incubated in control conditions or with CoCl₂ 100µM (added every 24 hours) and the images of the wounds were acquired every 2 hours. The IncuCyte ZOOM® 96-Well Scratch Wound Cell Migration data processing software was used to measure the spatial cell density in the wound area relative to the spatial cell density outside the wound area that is representative for cell migration.

MTT-based drug sensitivity assay

For MTT assays, cells (3000 per well in triplicate) were seeded in 96 well plates, adhered for 24 hours, and incubated with varying concentrations of different drugs. Plates were incubated at 37 °C with 5% CO₂ for 72 hours. MTT reagent (Sigma) was added (1 µg/well), and after incubation at 37 °C with 5% CO₂ for 1-2 hours, the medium was replaced by 100 µl isopropanol/H₂O/HCl 90/9/1 v/v/v. Plates were then incubated for 15 minutes at room temperature and absorbance of all wells was measured at 570/690 nm using Multiskan® EX (Thermofischer) bench-top microplate photometer and Ascent software for the photometer. IC50 values were calculated using Compusyn version 1.0 (ComboSyn, Inc).

IncuCyte-based drug sensitivity assay

Sensitivity to certain drugs was measured using IncuCyte® device. Cells (5000 cells/well in triplicate) were seeded in RPMI media using 96 well plates. Cells were incubated in control or experimental conditions 24 hours following seeding. Relative confluence of the wells was measured using IncuCyte ZOOM® data processing software which produces automated collection of time-lapse images every 2 hours. The relative percentage of well confluence as a function of time was used as parameter for cell survival and proliferation, and thus the comparison between different conditions used as a parameter for sensitivity.

Apoptosis assays

The activity of caspase-3/7 was measured using the IncuCyte® Caspase-3/7 apoptosis assay reagent (Essen BioScience, Ann Arbor, Michigan, USA). After 24 hours of seeding the cells in a 96 wells plate (5000 cells per well), experimental compounds (AMP/adenosine/cytotoxic drugs) along with the caspase 3/7 reagent (with a final concentration of 5 µM) were added directly to the wells. Kinetic

activation of caspase-3/7 was measured using live cell imaging, and quantified using the IncuCyte™ ZOOM basic analyzer. Green signals indicating activated caspase 3/7 were calculated using formula (green object confluence (percent) / phase object confluence (percent)). The values obtained were normalized with the initial values and plotted on graph as shown in the corresponding figures.

***In-vivo* tumor growth and drug sensitivity**

In-vivo experiments were performed using female SCID CB17 mice (Charles River Lab). All mice were raised in SPF environment with free access to standard food and water. This study was approved by the Animal Ethics Committee of the University Claude Bernard of Lyon. First, cell suspensions of exponentially growing NCI-H292 cells were prepared in 0.2 mL buffer (10% FBS, 1% PBS). Then, these cells (5 millions per mice) were injected subcutaneously into the right flank of mice. To study the *in vivo* tumor growth and *in vivo* sensitivity of cell models towards cytotoxic drugs, random groups (3-5 mice per group) were made. For experiments related to *in vivo* sensitivity of cell, the first dose (0.2 mg/kg vincristine and 1.5 mg/kg mitomycin C) was administered on the same day of injecting cells followed by a frequency of three injections per week. Once tumor volumes reached 200 mm³, it was measured every 3 days (length and width) with a caliper using the formula $\frac{4}{3} \times \pi \times [(l + w)/2]^3$.

Results

Validation of cell models

The cN-II and CD73-positive human lung adenocarcinoma cell line (NCI-H292) was used to develop four different cell models by knocking out cN-II and/or CD73 expression using CRISPR/Cas9 technique as explained in Material and Methods. These cell models were validated for their cN-II and CD73 expression using western blot and flow cytometry respectively, and already used for a series of experiments (Cadassou et al., submitted manuscript). Hereafter, these cells will be referred to as cN-II⁺/CD73⁺ cells (expressing both cN-II and CD73), cN-II⁺/CD73⁻ cells (expressing cN-II but not CD73), cN-II⁻/CD73⁺ (expressing CD73 but not cN-II) and cN-II⁻/CD73⁻ (deficient for both cN-II and CD73).

Cell proliferation in hypoxic conditions or under nucleotide stress is independent of cN-II and CD73

We studied the role of cN-II and CD73 in cell proliferation under hypoxic conditions (1% oxygen supply), during four days. The results showed no significant difference in cell proliferation between all four phenotypes as CFSE intensity decreased to 10.8-15% of its initial value over the experimental period (Figure 1).

We further studied the role of cN-II and CD73 in cell proliferation under nucleotide stress (high initial concentration of adenosine or AMP) in hypoxic environment. Adenosine reduced the rate of cell proliferation in all four phenotypes independently of 5'-nucleotidases expression (Figure 1). Indeed, there was a decrease of only 53.1-54.6% of initial CFSE intensity in adenosine-exposed cells as compared to 68.2-71.1% decrease in control cells after 24 hours of incubation and 14.8-18.3% as compared to 10.8-15.0% after 3 days. On the other hand, no significant difference in proliferation rate was observed with AMP, neither in comparison with control conditions nor within four phenotypes. Indeed, the relative decrease in CFSE intensity in all four phenotypes after 24h of AMP incubation (68.8-72.0%) is comparable with the decrease in control conditions (68.2-71.1%). After three days of hypoxic incubation, relative CFSE staining of all four phenotypes of NCI-H292 cells decreased to a range of 11.5-13.9% in response to AMP stress. Overall, these results indicate that 5'-nucleotidases, under applied conditions, are not involved in proliferation of NCI-H292 cells in either hypoxic and/or nucleotides stress environment.

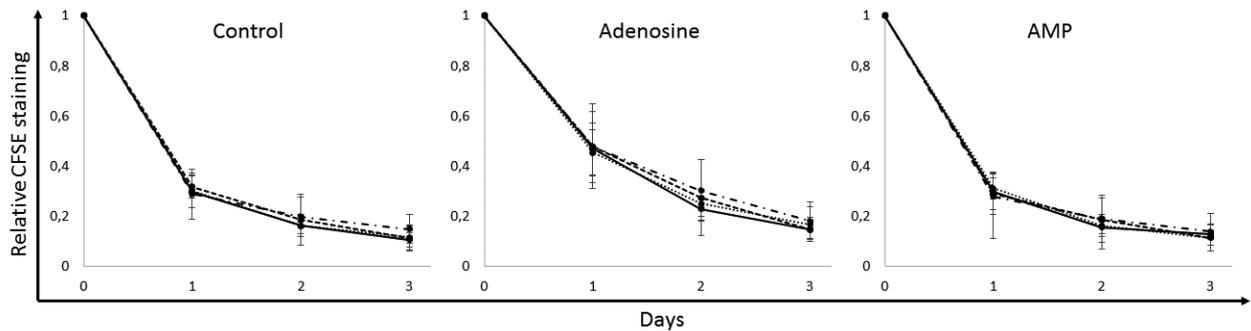


Figure 1. Proliferation of NCI-H292 cell models (— cN-II⁺/CD73⁺, cN-II⁻/CD73⁺, - - - cN-II⁺/CD73⁻, · · · · cN-II⁻/CD73⁻) under hypoxic conditions (1% oxygen supply). The graphs show mean values of relative CFSE-staining of at least three independent experiments and error bars are standard deviations.

CD73-deficient cells show a decreased cell index under hypoxic conditions

Using xCELLigence technique, we analyzed the role of cN-II and CD73 in cell proliferation and cell adhesion under hypoxic condition with or without nucleotides stress. These two parameters are presented as cell index which increases with cell proliferation and adhesion to the surface of culture plates. Interestingly, we observed that CD73-deficient cells (cN-II⁺/CD73⁻ and cN-II⁻/CD73⁻ cells) clearly showed lower cell index as compared to their control cells in hypoxic conditions (Figure 2). However, when exposed to adenosine 1600 μ M, a general decrease in the cell index was observed in all four cell phenotypes whereas AMP 1600 μ M preferentially delayed the increase of cell index in CD73 expressing cells. Indeed, under control conditions, after 94 hours all the cells reached their maximum cell index *i.e.* 6.1 for cN-II⁺/CD73⁺, 6.7 for cN-II⁻/CD73⁺, 3.1 for cN-II⁺/CD73⁻ and 2.4 for cN-II⁻/CD73⁻. On the other hand, under AMP stress the peak was observed at 150 hours for CD73 expressing cells (5.7 for cN-II⁺/CD73⁺ and 5.7 for cN-II⁻/CD73⁺) whereas it remained similar for CD73 deficient cells (89 hours and 3.1 for cN-II⁺/CD73⁻; 104 hours and 2.4 for cN-II⁻/CD73⁻). This decreased cell index in CD73-deficient cells under control hypoxic conditions, can be best explained as a difference in their adhesion because we observed no difference in proliferation using CFSE staining. CD44 is a transmembrane single chain glycoprotein involved in maintaining organ and tissue structure by facilitating cell adhesion with surrounding cells and matrix [54], and could therefore represent an explanation for this difference. However, our cell lines had similar expression patterns for CD44 (Supplemental figure 1).

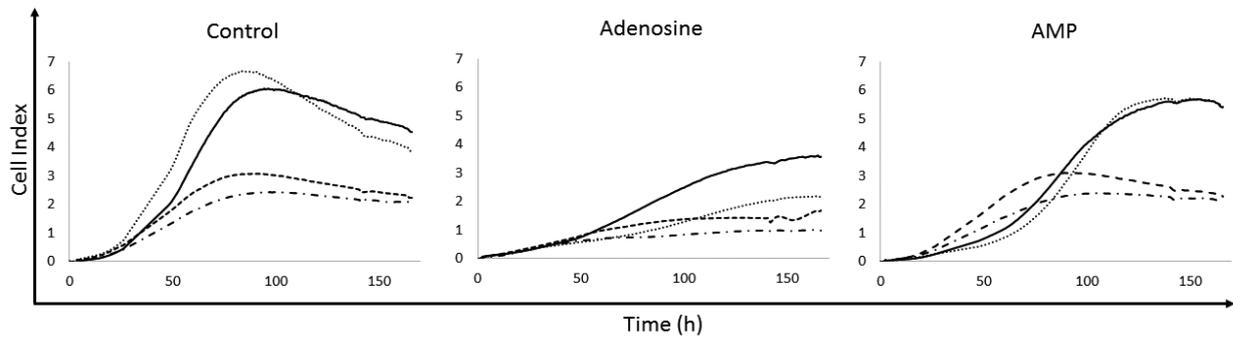


Figure 2. Cell index measured by xCELLigence RTCA under hypoxic stress in the presence or absence of nucleotide stress in NCI-H292 cells. Graph shows the cell index of NCI-H292 cells (— cN-II⁺/CD73⁺, cN-II⁻/CD73⁺, - - - cN-II⁺/CD73⁻, · · · · · cN-II⁻/CD73⁻) measured by xCELLigence RTCA under hypoxic incubation.

HIF-1 α is not upregulated in NCI-H 292 cells under hypoxic stress

It has been shown that under limited oxygen supply, cells undergo gene expression remodeling process to better adapt the stress condition. The changes in genes expression are primarily regulated by HIF-1 α [55]. Based on this, and the previous observation showing that upregulation of CD73 expression in hypoxic stress could be mediated through HIF-1 α [28], we studied the expression of HIF-1 α in our cell models incubated in hypoxic conditions. The results have shown that there is no upregulation of HIF-1 α in all four phenotypes of NCI-H292 cells after 12 hours of incubation in hypoxic condition, whereas it was increased in other cell lines in the same condition (Figure 3A, 3B). This result not only validates our hypoxic conditions but also shows that HIF-1 α stabilization as a result of hypoxia is cell dependent. Furthermore, as all NCI-H292 cell models are insensitive to hypoxia-induced HIF-1 α expression, the delayed increase of cell index observed in xCELLigence assay for CD73 expressing cells cannot be explained by this mechanism. In parallel to HIF-1 α expression, we also analyzed the expression of CD73 in cells incubated in hypoxic conditions for 12 hours, and this remained also insensitive to hypoxia (Supplemental figure 2). Using CoCl₂, another stabilizer for HIF-1 α [56], we showed that all our cell models express this protein, and that CD73-deficiency is associated with a lower HIF-1 α expression (Figure 3C). In a kinetic study, HIF-1 α expression was high after 12 hours culture, and low at 24, 36 and 48 hours (Figure 3D).

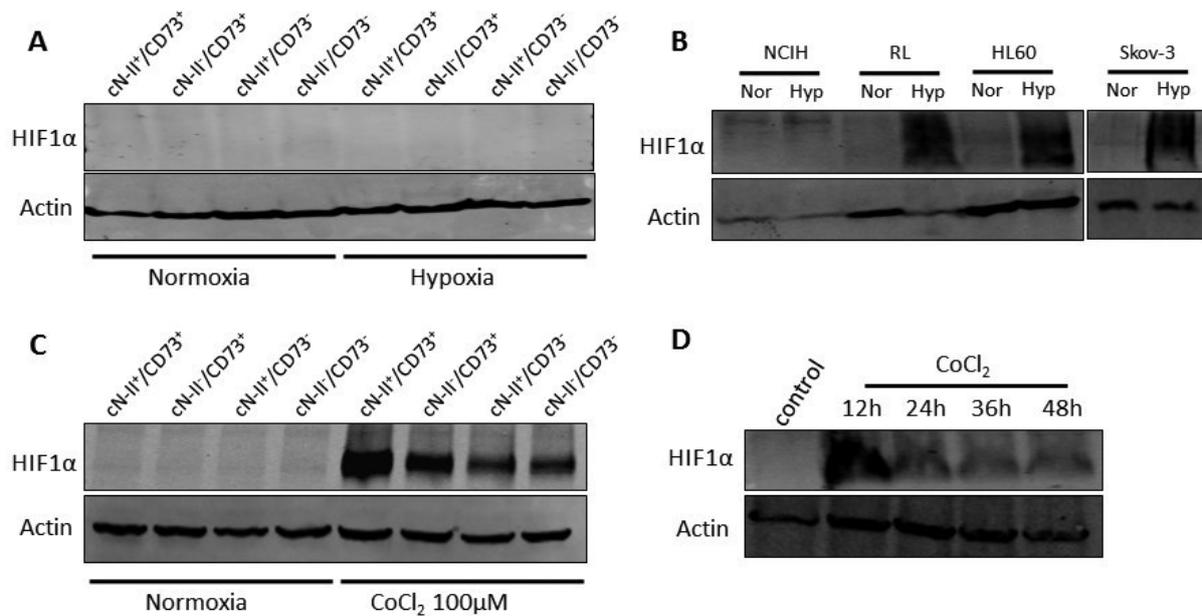


Figure 3. Expression of different proteins in cells under low oxygen hypoxic models and CoCl₂-induced HIF-1 α expression. **(A)** Expression of HIF-1α and actin in NCI-H292 cell models after incubation in hypoxic conditions for 12 hours. **(B)** Expression of HIF-1α in NCI-H292 and other cells lines after incubation for 12 hours in hypoxia. **(C)** Expression of HIF-1α in NCI-H292 cell models after 12 hours of incubation with CoCl₂ 100 μM. **(D)** Expression of HIF-1α over time after incubation with CoCl₂ 100 μM.

cN-II and CD73 are not involved in proliferation, adherence or migration in HIF-1α expressing cells

As our hypoxic conditions did not cause an overexpression of HIF-1α in our cell models, we continued our study of our cell models in stress-conditions with CoCl₂ in order to evaluate the effect of HIF-1α expression on different phenotypes in our models. Using this chemical hypoxia model in the CFSE-based proliferation assays, we showed that there is no difference in cell proliferation between four cell models suggesting that neither cN-II nor CD73 has a role in cell proliferation under the applied conditions (Figure 4A). Indeed, CFSE intensity decreased to 8% of its initial value simultaneously in all four phenotypes after three days of incubation in 100 μM CoCl₂. This result was perfectly in line with our observation made in 1% oxygen condition. To ensure long-time HIF-1α expression, CoCl₂ was added every 24 hours.

When we studied cell index with the xCELLigence device with exposure to CoCl₂, cells reached their peak cell index 100-125 hours after the first dose of CoCl₂ (Figure 4B). Indeed, maximum cell index for cN-II⁺/CD73⁺ was observed as 2.4 after 123 hours, 1.9 for cN-II⁻/CD73⁺ after 125 hours, 1.6 for cN-II⁺/CD73⁻ after 100 hours and 2.6 for cN-II⁻/CD73⁻ after 120 hours. Similar to the observation made in

hypoxic stress (1% oxygen), cN-II⁺/CD73⁻ cells showed decreased cell index as compared to CD73 expressing cells, although it was not as pronounced as in hypoxic condition. However, double knockout cells didn't show a decreased cell index when treated with CoCl₂ 100 μM.

We also studied cell migration of our cell models exposed to CoCl₂, with the wound healing assay using IncuCyte Live Cell Analysis System (Essen Bioscience). The results have shown that there is no significant difference in the cell migration rate between four different cell phenotypes suggesting that there is no role of cN-II and CD73 in cell migration with exposure to CoCl₂ (Figure 4C). Indeed, cells behaved similarly in unexposed and CoCl₂-exposed cells, except for a minor effect in cN-II⁺/CD73⁻ cells.

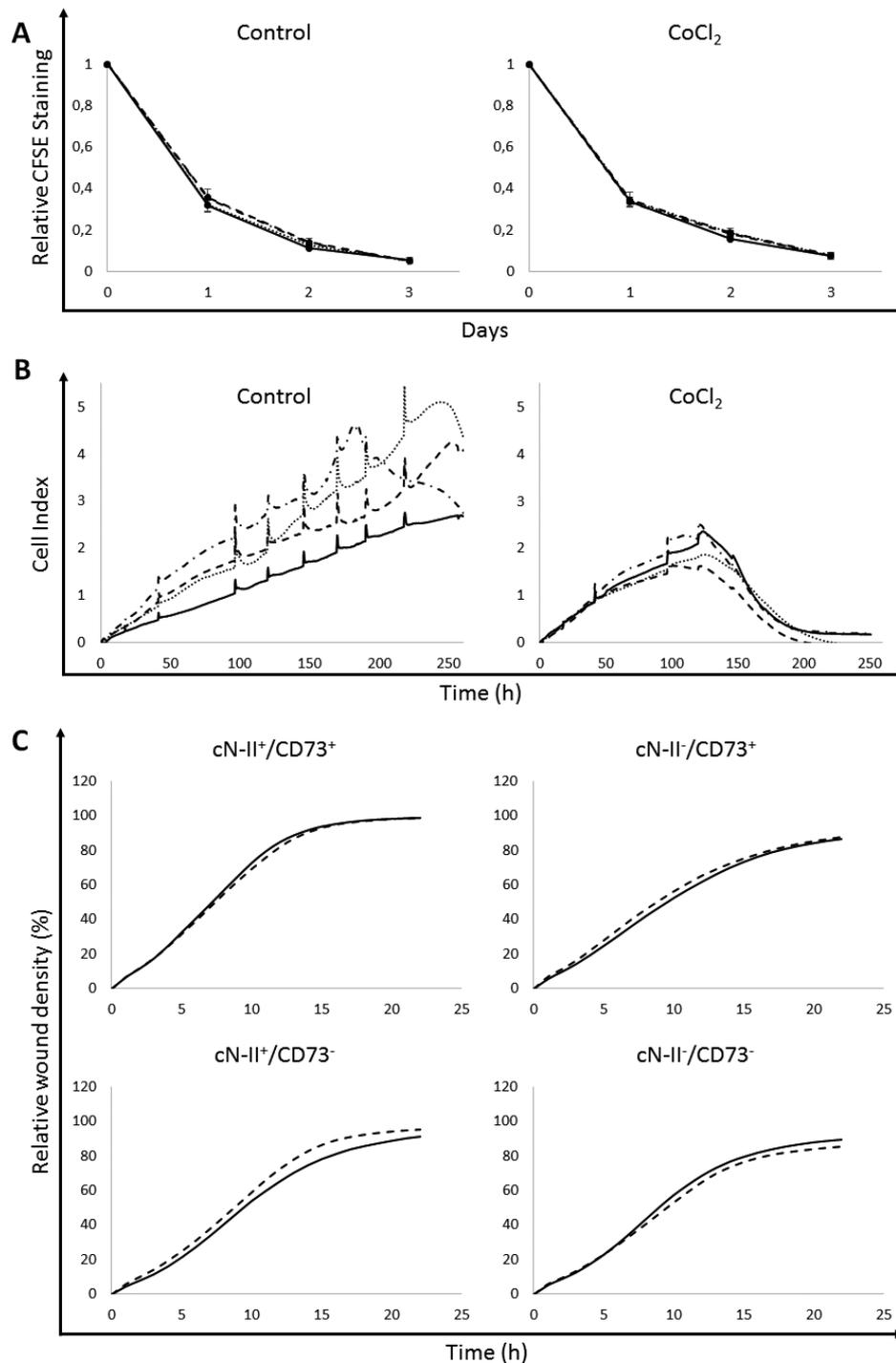


Figure 4. Cell proliferation, adherence and migration of NCI-H292 cell models in presence and absence of CoCl₂. **(A)** Proliferation rate of unexposed and CoCl₂-exposed cells (— cN-II⁺/CD73⁺, cN-II⁻/CD73⁺, - - - cN-II⁺/CD73⁻, ····· cN-II⁻/CD73⁻). The graphs show mean values of relative CFSE-staining of two independent experiments and error bars are standard deviations. **(B)** Cell index measured by xCELLigence RTCA in unexposed and CoCl₂-exposed cells (— cN-II⁺/CD73⁺, cN-II⁻/CD73⁺, - - - cN-II⁺/CD73⁻, ····· cN-II⁻/CD73⁻). The graphs are representative of two independent experiments. **(C)** Migration of unexposed (—) and CoCl₂ (- - -) exposed cells.

Adenosine induces apoptosis in CD73 deficient cells

Using IncuCyte RTCA and caspase 3/7 assay, we observed that high concentrations (1600 μM) of adenosine caused an increase in the caspase 3/7 cascade in CD73 deficient cells with a more pronounced effect in cN-II⁺/CD73⁻ cells than in cN-II⁻/CD73⁻ cells (Figure 5). The results have also shown that the activation of Caspase 3/7 cascade started within the first 10 hours of exposure to adenosine, whereas as cN-II⁻/CD73⁺ cells become positive after about 40 hours. All four cell models were virtually insensitive to high concentrations of AMP.

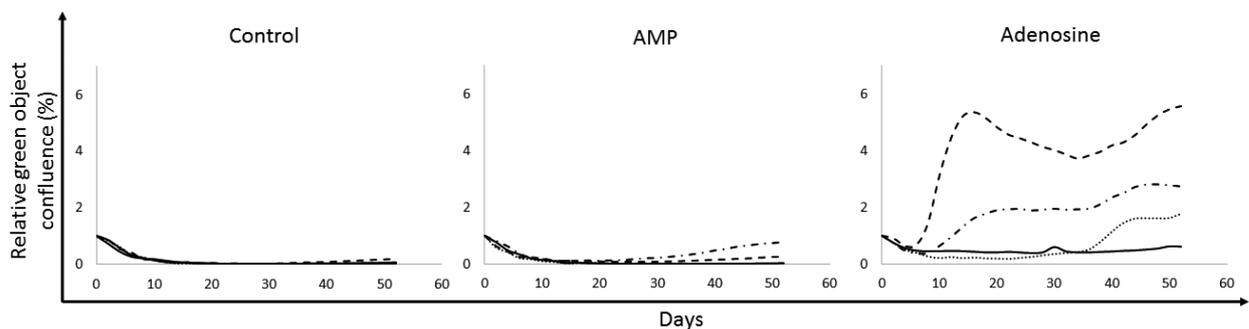


Figure 5. Effect of nucleotide stress on viability of NCI-H292 cells (— cN-II⁺/CD73⁺, cN-II⁻/CD73⁺, - - - cN-II⁺/CD73⁻, · · · · · cN-II⁻/CD73⁻) via caspase 3/7 assay. The figures show relative green object confluence (%) of all four cell models as a function of time in cells cultured in control conditions, with adenosine (1600 μM) or with AMP (1600 μM). The graphs are the representative of two independent experiments performed in triplicates.

CD73-deficient NCI-H292 cells are resistant to mitomycin C and sensitive to vincristine

Previously, it has been shown that 5'-nucleotidases are associated to the sensitivity to certain cytotoxic drugs and thereby potentially to the clinical outcome of the patients treated with these drugs. We hypothesized that knocking out one or both of the 5'-nucleotidases (cN-II and CD73) might effect sensitivity of lung cancer cells towards various cytotoxic drugs. Data in table 1 show that there is no major involvement of these proteins in the sensitivity to the tested drugs. However, cN-II⁺/CD73⁻ cells are significantly more resistant to mitomycin C than cN-II⁺/CD73⁺ cells ($3.3 \pm 0.6 \mu\text{M}$ versus $1.2 \pm 0.15 \mu\text{M}$, $p < 0.05$). In addition, cN-II⁻/CD73⁻ showed a 4.1-fold statistically non-significant increase in IC50 for this drug, reinforcing the role of CD73 in the sensitivity of these cells to mitomycin C. On the other hand, IC50 of daunorubicin decreased 2-fold in cN-II⁻/CD73⁺ cells as compared to control cells. Additionally, other drugs also showed increased or decreased IC50 values in knockout models,

however these variations were not statistically significant as compared to control cell lines as exemplified by the 1.3-fold higher sensitivity of cN-II⁺/CD73⁻ cells as compared to control cells.

In order to further confirm the results obtained from MTT assays, we used IncuCyte RTCA technique and observed the sensitivity of cells towards these drugs by evaluating the relative well confluence over time (Figure 6). Under control conditions, relative confluence of all four cell models increased at a similar rate further validating the results previously obtained using CFSE proliferation assay (Cadassou et al., submitted manuscript). When incubated with vincristine 0.01 μ M, a universal decrease in the proliferation rate was observed in all cells as none of them reached a relative confluence value of 2 after 50 hours as compared to 4 for unexposed cells. However, cN-II⁺/CD73⁻ cells showed a more important decrease than other cell models, and this was confirmed in an independent caspase 3/7 assay (Supplemental figure 3). On the contrary, in response to 10 μ M mitomycin C, CD73 deficient cells showed increased confluence rate as compared to their counterparts reaching 2.2 after 50 hours as compared to 1.5 for cN-II⁺/CD73⁺. This confirmed the observations made in MTT assay that CD73 deficient cells are resistant towards mitomycin C and sensitive towards vincristine. For the other drugs evaluated, it is notable that deficit of cN-II and/or CD73 sensitizes slightly to carboplatin, and that CD73-deficiency is associated to a lower sensitivity to gemcitabine.

Table 1. IC50 values of different cytotoxic drugs in our four cell models. The values are means of at least three independent experiments \pm SEM. *: $p < 0.05$ as compared to cN-II⁺/CD73⁺ cells as determined with Student's t-test.

Drug	cN-II ⁺ /CD73 ⁺	cN-II ⁻ /CD73 ⁺	cN-II ⁺ /CD73 ⁻	cN-II ⁻ /CD73 ⁻
Gemcitabine (nM)	14 \pm 1	19 \pm 5.5	21 \pm 3.5	48 \pm 19.5
Vincristine (nM)	0.87 \pm 0.3	0.77 \pm 0.1	0.66 \pm 0.05	1.4 \pm 0.2
Vinblastine (nM)	1.52 \pm 0.11	1.57 \pm 0.16	1.31 \pm 0.34	1.24 \pm 0.10
Carboplatin (μM)	49.4 \pm 5	48.0 \pm 4.6	41.0 \pm 6	65.5 \pm 14.15
Mitomycin C (μM)	1.2 \pm 0.15	1.5 \pm 0.2	3.3 \pm 0.6*	5.1 \pm 1.4
Doxorubicin (μM)	0.20 \pm 0.03	0.15 \pm 0.02	0.17 \pm 0.02	0.14 \pm 0.02
Daunorubicin (μM)	0.12 \pm 0.02	0.06 \pm 0.01*	0.09 \pm 0.02	0.10 \pm 0.02
Mitoxantrone (μM)	1.03 \pm 0.20	0.96 \pm 0.24	0.98 \pm 0.20	0.55 \pm 0.09
Bleomycin (μM)	29.23 \pm 4.05	23.76 \pm 4.62	31.31 \pm 6.52	23.74 \pm 6.53

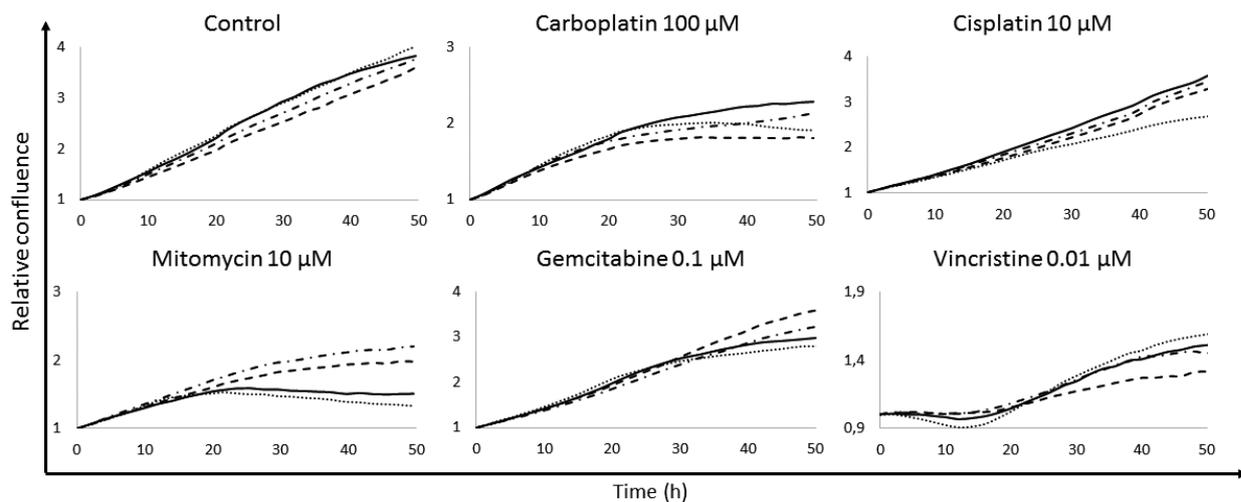


Figure 6. Relative confluence of NCI-H292 cells (— cN-II⁺/CD73⁺, cN-II⁻/CD73⁺, --- cN-II⁺/CD73⁻, · · · · cN-II⁻/CD73⁻) incubated alone or in presence of cytotoxic drugs. The relative confluence was assessed by IncuCyte Live Cell Analysis technique and is used as a surrogate for cell survival.

cN-II and CD73 knockout cells showed higher sensitivity towards Mitomycin C and vincristine *in vivo*

Based on the results obtained from *in vitro* sensitivity assays explained above, we performed mice experiments to observe the role of 5'-nucleotidases in sensitivity towards cytotoxic drugs *in vivo*. Three groups of 5 SCID CB17 mice each were treated with either vincristine (0.2 mg/kg), mitomycin C (1.5 mg/kg) or PBS, starting on the same day of injecting the cancer cells. As shown in figure 7, cN-II negative tumors seem to grow faster than their corresponding controls, whereas CD73-deficiency does not seem to influence tumor growth. Further, the treatments were suboptimal in mice bearing cN-II⁺/CD73⁺ cells, whereas there was trends to activity in mice with cN-II⁺/CD73⁻ cells for vincristine and in mice with cN-II⁻/CD73⁺ and cN-II⁻/CD73⁻ with both drugs.

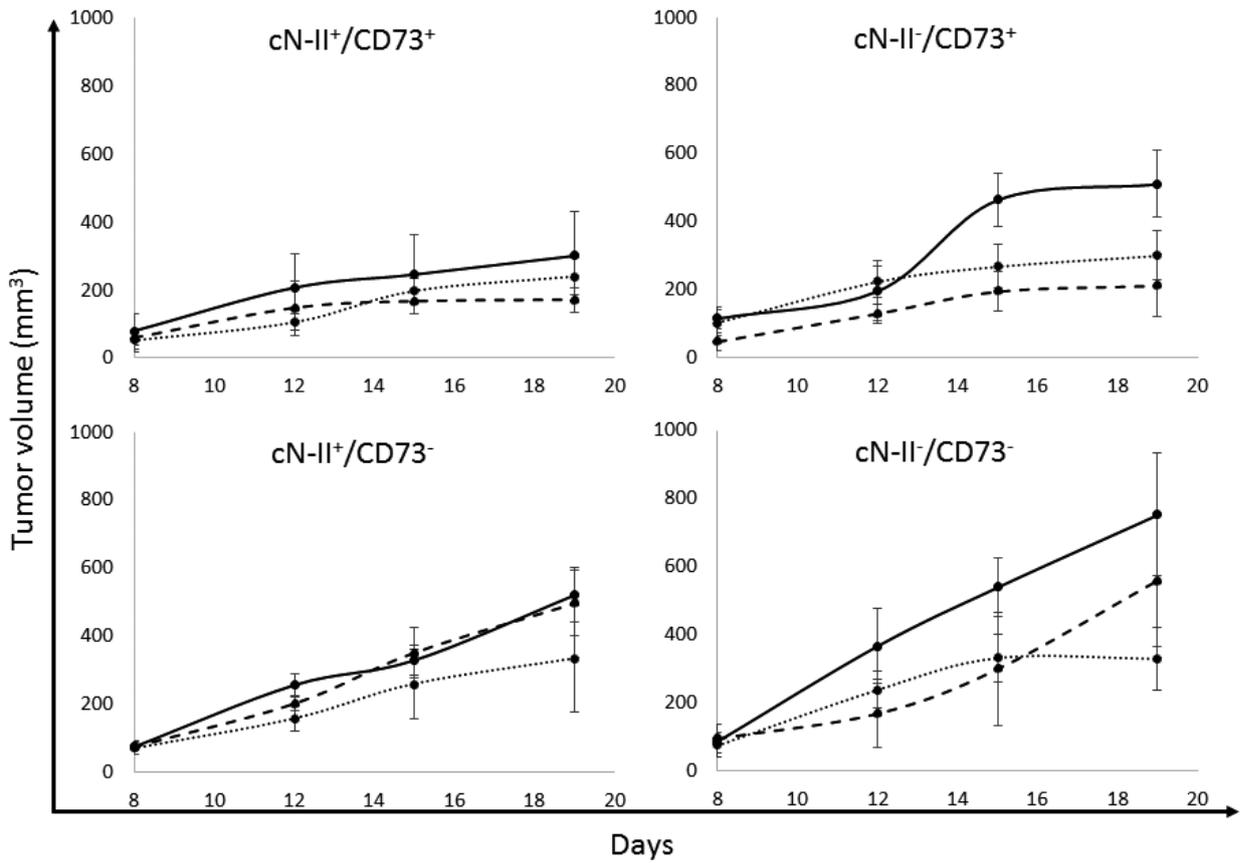


Figure 7. Effect of cN-II and CD73 in sensitivity towards mitomycin C and vincristine in vivo. The graphs show tumor growth in female SCID CB17 mice receiving corresponding treatments (— PBS, mitomycin C 1.5 mg/kg, - - - vincristine 0.2 mg/kg). These graphs are mean values of tumor growth in five mice of the same treatment group and error bars are standard deviation.

Discussion

As indicated in the introduction, there are growing evidences that the 5'-nucleotidases cN-II and CD73 play important roles in cancer cell biology and in cancer pharmacology. It is also known that cells behave differently under different stress conditions. This change in behavior depends upon many factors and are not limited to type of cell, stress and duration of stress. Apparent morphological changes as well as phenotypes are associated with underlying molecular and genetic changes. Stress conditions have an obvious impact on the way cells respond to internal as well as to external stimuli. It can turn cells into an ultimate situation where they can either initiate defensive or suicidal responses. These modifications in response to such stress are part of the adaptability or plasticity of cancer cells. Cellular stresses include mechanical stress, chemical stress, oxidative stress, replication stress and others. As cancer is characterized by rapid and uncontrolled cell growth, it is obvious that soon after its initiation, cancer cells would be competing not only for essential nutrients and oxygen but also have to cope with other internal and external self-protective or self-destructive mechanisms. This causes a shear imbalance in supply-demand chain and cancer cells enter a stressed phase. Previously, we have shown the that in particular cN-II is involved in the metabolic plasticity of cancer cells. Indeed, using the breast cancer cell MDA-MB-231, we showed that cells with shRNA-mediated decrease in cN-II expression adapt better than control cells to glucose deprivation, and that this is linked to the regulation of reactive oxygen species [14]. We have also showed that the same cells with CRISPR/Cas9-mediated knocked-out cN-II expression have increased migration and do not respond in the same way as control cells to adenosine (Cadassou et al., submitted manuscript). In this study, we were interested to know whether 5'-nucleotidases play a protective or destructive role under stress conditions in human lung adenocarcinoma cell lines (NCI-H292). We used different types of stress conditions *i.e.* hypoxic stress (1% oxygen concentration), CoCl₂-mediated HIF-1 α stabilization, nucleotide stress (adenosine 1600 μ M and AMP 1600 μ M) and cytotoxic drugs stress.

We first confirmed that cN-II or CD73 have no role in cell proliferation under hypoxia as well as under combined hypoxic and nucleotide stresses. Hypoxia is well-known as a hallmark of cancer whereas adenosine is a key facilitator for immune evasion in tumor microenvironment. Discovery of defensive mechanism adapted by cancer cells to cope with hypoxic microenvironment lead Dr Gregg Samanza and others to win 2019 Nobel prize in physiology and medicine [57]. They showed that hypoxia causes stabilization of transcription factor HIF-1 α in cells which then regulate the expression of different proteins needed in hypoxic stress. Later, it was shown that HIF-1 α causes an increase in the expression of CD73 which then helps in different protective physiological functions mainly mediated through production of extracellular adenosine [28, 58]. We were therefore first interested in how cells with and without CD73 expression would behave in hypoxic conditions, asking the question on the

functionality of CD73 regulation by HIF-1 α during hypoxia. As cells with low cN-II expression showed a higher sensitivity to glucose deprivation in hypoxic conditions (Bricard G Oncotarget 2017), we also wanted to increase the knowledge on the behavior of cN-II-deficient cells in hypoxic and HIF-1 α expressing conditions. In this study, we first showed, using CFSE assay and xCELLigence, that only CD73-deficient cells had a modified phenotype with the xCELLigence technique. It is noteworthy that cell index cannot be taken as a parameter for cell proliferation as it not only considers well confluence *i.e.* area occupied by the cells in a well but also the strength with which the cells attach to the surface of the xCELLigence plate. Therefore, cell index is a measure of both cell number as well as cell adherence, and since our models behave the same in the CFSE assay, this indicate a lower adherence for CD73-negative cells. We used flow cytometer to observe the expression of CD44 in our cell models and observed no differential expression of CD44 in our cell models. CD44 is a transmembrane single chain glycoprotein involved in maintaining organ and tissue structure by facilitating cell adhesion with surrounding cells and matrix [54]. Interestingly, we have observed that adenosine causes a decreased cell proliferation in all cell models either through action on adenosine receptors or by entering the cells. However, under AMP stress, as expected, AMP did not influence the cell index of CD73-deficient cells, whereas it delayed the increase in cell index for CD73-proficient cells.

Concerning limitations being faced during these experiments, it is important to mention that hypoxic condition (1% oxygen) didn't induce the stabilization of HIF-1 α in NCI-H292 cells whereas it did in other cells (validating our installation). We therefore also used CoCl₂, known as a HIF-1 α stabilizer, to evaluate the effect of HIF-1 α -expression in our cell models [56]. Interestingly, we observed that CoCl₂-mediated HIF-1 α expression is higher in CD73-expressing cells as compared to CD73-deficient cells, suggesting a potential regulation not only of CD73 by HIF-1 α , but also the other way around. Further experiments are needed to decipher this potential regulation.

Next, we evaluated the effect of CoCl₂ (and thus HIF-1 α -stabilization) over time and showed first that CoCl₂-mediated HIF-1 α expression is time dependent and experiments therefore require regular addition of CoCl₂. Again, we performed CFSE assay and observed that even an increased HIF-1 α expression is not related to any difference in cell proliferation in all four cell phenotypes verifying that both cN-II and CD73 are not involved in cell proliferation under hypoxic stress in NCI-H292 cells. Previously, we have shown that CD73 regulates cell migration in NCI-H292 and cN-II in MDA-MB-231 cells under normoxic conditions (Cadassou et al., submitted manuscript). Here, using CoCl₂ mediated hypoxia model, we performed migration assay and observed that cN-II and CD73 are not involved in cell migration. Again, further studies are warranted in order to understand more precisely the role of cN-II and CD73 in hypoxic cancer cells.

It is also known that nucleotide stress can affect cell survival, proliferation and migration. Our team has shown that higher extracellular concentration of AMP causes an induction of apoptosis in CD73 expressing cells using MDA-MB-231 cells as experimental models (Cadassou et al., submitted manuscript). We observed that in these cells apoptosis is due to enzymatic conversion of AMP into adenosine by CD73. Using same cell models, we also observed that higher adenosine concentration caused cell death in all cell models independent of the expression of either cN-II or CD73. Additionally, the apoptotic effect of adenosine was not rescued even with the use of adenosine receptors antagonists. In the current study, we observed that adenosine 1600 μ M causes an activation of caspase 3/7 apoptotic pathway in CD73 deficient cells, but not in CD73-expressing cells. This suggest either a different expression of adenosine receptors between CD73 positive and CD73 negative cells, or a different metabolism for intracellular adenosine making these cells more sensitive to adenosine stress.

Finally, we studied the role of cN-II and CD73 in cell sensitivity towards a panel of cytotoxic drugs. Previously, it has been shown by several studies that both cN-II and CD73 are involved in cell sensitivity towards cytotoxic drugs. In particular, downregulation of cN-II in cell models of haemetological malignancies is associated with increased sensitivity to purine nucleoside analogues [21], whereas in glioblastoma cells it is associated to a resistance to cisplatin [15]. For CD73, its expression on breast cancer cells is associated for example with a resistance to doxorubicin [59] in addition to be a regulator for the environmental immune system. In this study, we showed that CD73 deficient cells are more resistant towards mitomycin C (alkylating agent) and sensitive towards vincristine (vinca-alkaloid). We further showed that double knockout cell models become even more sensitive towards vincristine in the presence of high extracellular adenosine stress. This was confirmed by several techniques.

Our mice experiment first showed us that cN-II deficient cell grow faster than cN-II proficient NCI-H292 cells *in vivo* whereas there was no evident effect of CD73 expression. This confirms the observation on cN-II we previously observed with shRNA-mediated cN-II knock-down in the same cells [14]. The role of CD73 on tumor growth has already been shown to be cell-line dependent, and also often more important in immunocompetent mice models. The *in vivo* drug-sensitivity data we obtained are not confirming our *in vitro* data, suggesting an important involvement of the tumor microenvironment in the response of the tumor to this treatment and warranting further investigations for the mechanisms involved in these responses.

In conclusion, we present data on a series of original cell models allowing to ask important question about the role of 5'-nucleotidases in cancer cells. Our results add to the growing knowledge both on cN-II and CD73 that remain of great interest in both fundamental and translational cancer research. Further work could allow identifying the molecular mechanisms involved in the observed phenotypes.

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Supplemental material

Figure S1. Expression of CD44 by NCI-H292 cells incubated in hypoxic conditions for 18 hours (upper panel) or exposed to CoCl₂ 100 μM for 48 hours (lower panel).

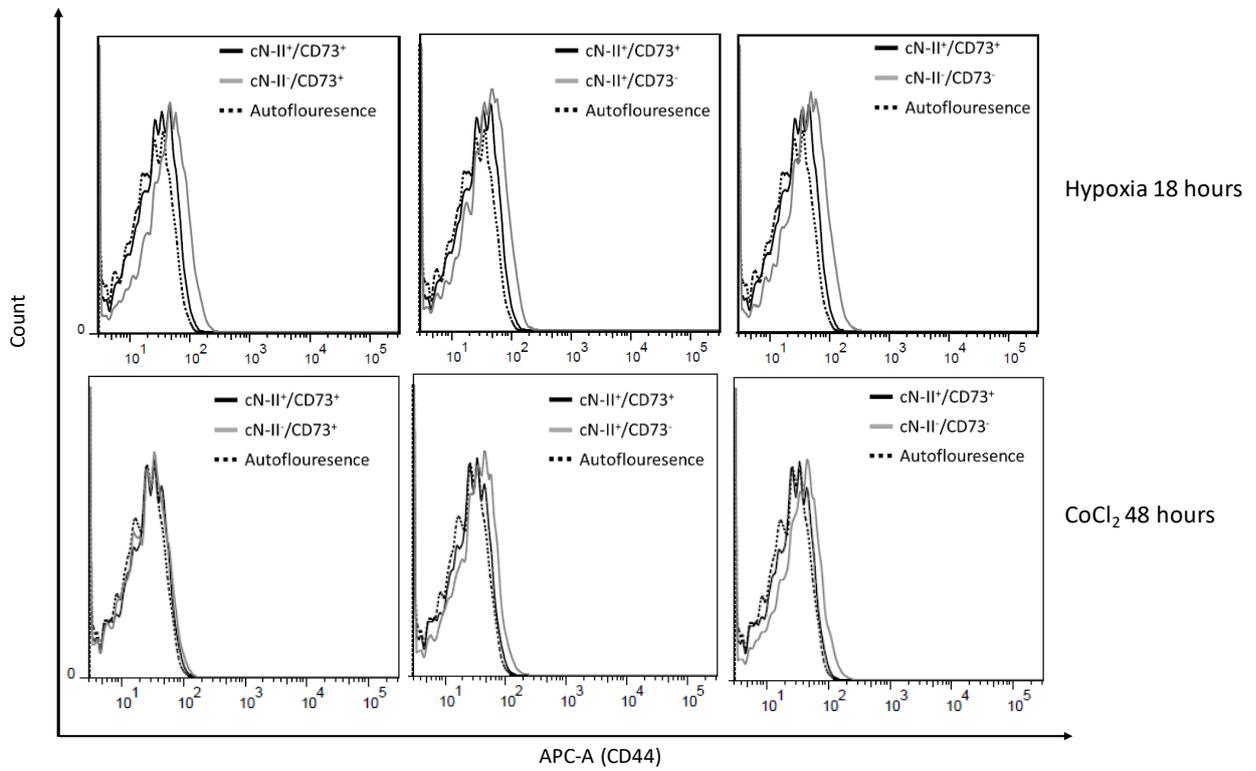


Figure S2. Expression of CD73 by NCI-H292 cells incubated in normoxia (black graphs) or hypoxia (grey graphs) for 18 hours.

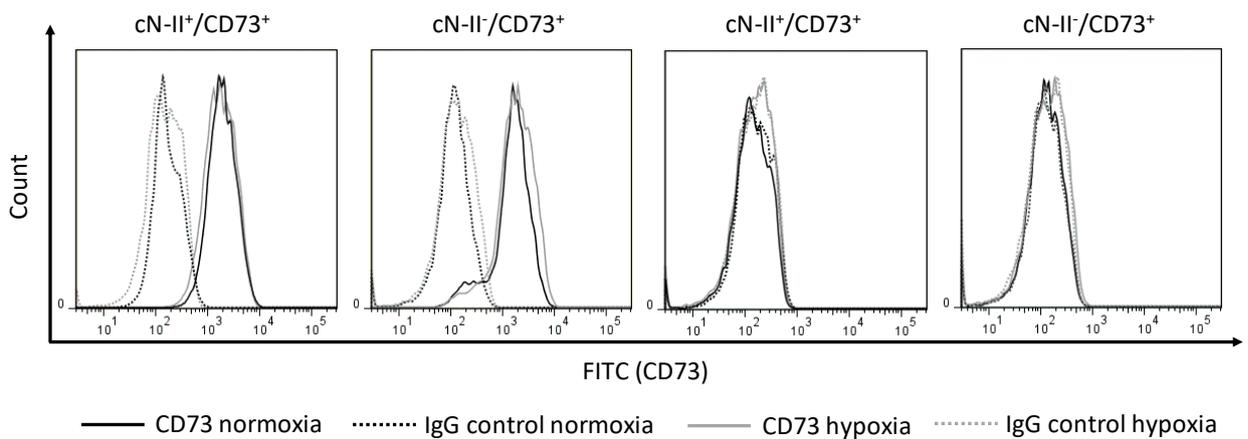
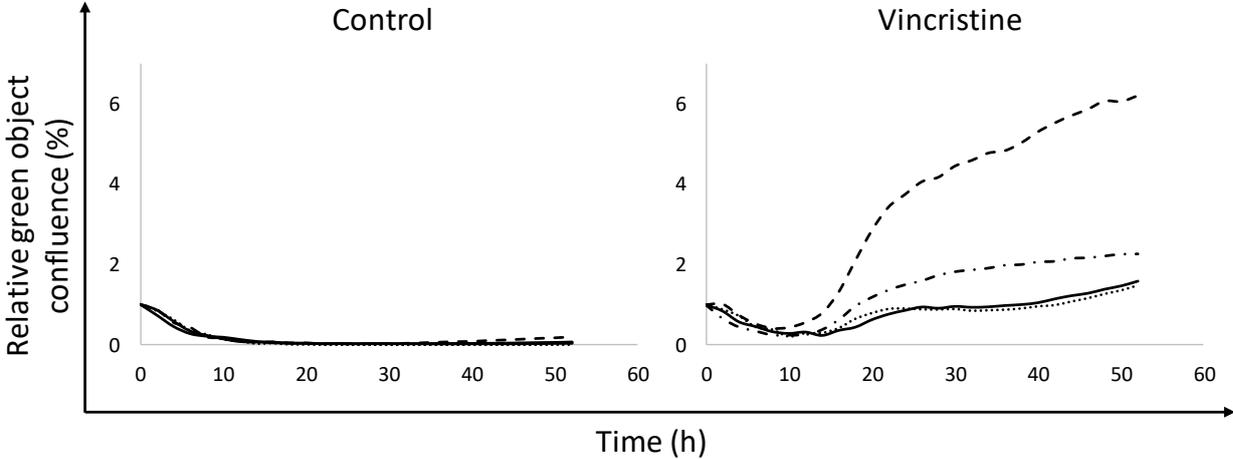


Figure S3. Effect vincristine 0.01 μ M on NCI-H292 cells (— cN-II⁺/CD73⁺, cN-II⁻/CD73⁺, - - - cN-II⁺/CD73⁻, ······ cN-II⁻/CD73⁻) as determined by caspase 3/7 assay. The graphs are the representative of two independent experiments performed in triplicates.



Review Article: Functions of the multi-interacting protein KIDINS220/ARMS in cancer and other pathologies

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REVIEW ARTICLE

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Functions of the multi-interacting protein KIDINS220/ARMS in cancer and other pathologies

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Abstract

Development of an organ and subsequently the whole system from an embryo is a highly integrated process. Although there is evidence that different systems are interconnected during developmental stages, the molecular understanding of this relationship is either not known or only to a limited extent. Nervous system development, amongst all, is maybe the most crucial and complex process. It relies on the correct distribution of specific neuronal growth factors and hormones to the specific receptors. Among the plethora of proteins that are involved in downstream signalling of neuronal growth factors, we find the kinase-D interacting substrate of 220 kDa (KIDINS220), also known as ankyrin-rich repeat membrane spanning (ARMS) protein. KIDINS220 has been shown to play a substantial role in the nervous system and vascular system development as well as in neuronal survival and differentiation. It serves as a downstream regulator for many important neuronal and vascular growth factors such as vascular endothelial growth factor (VEGF), the neurotrophin family, glutamate receptors and ephrin receptors. Moreover, activation and differentiation of B- and T-cells, as well as tumour cell proliferation has also shown to be related to KIDINS220. This review comprehensively summarises the existing research data on this protein, with a particular interest in its role in cancer and in other pathologies.

1 | INTRODUCTION

Interaction of different proteins plays an important functional role in the development of interconnected systems of all organisms. In human beings, nervous system development and differentiation is one of the most sophisticatedly regulated mechanisms. Among many neurotrophic factors, a group of closely related compounds called neurotrophin (NT) has a crucial role in controlling the number, growth, and differentiation of neurones, the functional unit of the nervous system. These neurotrophic factors bind to high-affinity tropomyosin receptor kinases (Trks) and/or the low-affinity pan-neurotrophin nerve growth factor receptor (NGFR) present on neuronal cells surface. Binding of NTs to these receptors initiates specific intracellular signalling cascades resulting in many important functions such as neuronal survival and proliferation, growth and differentiation of axon and dendrite, and the expression and function of important proteins like ion channels and neurotransmitter receptors.¹⁻³ Synaptic strength and plasticity are also regulated by Trk receptors in the adult nervous systems, and the activation of cell surface receptors leads to one of many intracellular signalling pathways. The specificity of the receptors to NTs and the corresponding

intracellular signalling cascade depends on the expression of Trk/NGFR on cell surface and availability of signalling intermediates inside the neuronal cell. At the intracellular level, activated Trk/NGFR provide docking sites at phosphorylated tyrosine residues for different adapters present in the cytoplasm depending on cell type. The most important initial adapter proteins involved in the corresponding intracellular signalling events are RAS protein family, Extracellular regulated kinases (ERK or MAPK), phospholipase-C γ (PLC- γ), phosphatidylinositol-3 kinase (PI3-K), AKT, and KIDINS220^{4,5} (Figure 1). Among these, KIDINS220 was successfully cloned and identified in 2000 as the first physiological substrate of protein kinase-D (PKD).⁶ It is a transmembrane protein phosphorylating proteins at serine and threonine residues. At the same time, it was also identified as a downstream signalling target of neurotrophin receptor tyrosine kinase NTRK⁷ and named ARMS (ankyrin-rich repeat membrane spanning) owing to its characteristic structural features. Later studies revealed that it is a hub for several cellular interactions of paramount importance. Through protein-protein interactions, it plays a major role in the development of various systems.^{8,9} This is exemplified by nervous system processes such as neuronal outgrowth,¹⁰ neuronal polarity¹¹ and differentiation,¹²

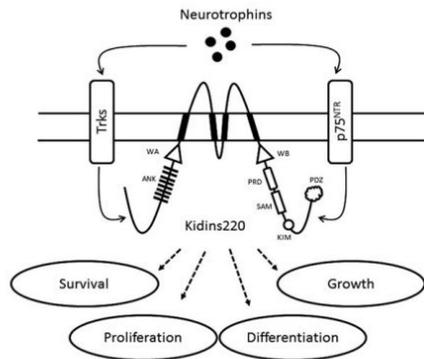


FIGURE 1 Structural features of KIDINS220 and signaling pathways of neurotrophins through KIDINS220 inducing cancer-related cellular processes. The pathways are multistep and involve several cellular proteins as indicated in the text and in references. ANK: Ankyrin repeats; WA: Walker A domain; WB: Walker B domain; PRD: Proline-rich Domain; SAM: Sterile Alpha Motif; KIM: Kinesin-1 binding motif; PDZ: PDZ-binding Motif; Trks: Tropomyosin Receptor Kinases

neuronal survival,¹³ synaptic transmission,¹⁴ vascular system development,¹⁵ neuroblastoma cell proliferation,¹⁶ UV irradiation-induced apoptotic cell-death in melanoma,¹⁷ and B and T-cells activation and differentiation.^{18,19} KIDINS220 also forms a ternary complex with Trk and NGFR²⁰ and plays an important role in human immunodeficiency virus type-1 Tat-induced microglial activation.²¹ Finally, a recent study has shown that KIDINS220 acts as an interactional partner of synembrin-B and thus plays a role in the NGF-induced cellular secretions.²² Recent reviews on KIDINS220 have been published and summarise the available data on the history, structure and associated functions of this protein.^{5,9} Therefore, we will focus on the functions and importance of KIDINS220 in the development of human pathologies, with a particular interest in cancer in this review.

2 | STRUCTURAL INSIGHT OF KIDINS220

KIDINS220 is a large protein with 1771 amino acids and an apparent molecular weight of 220 kDa, encoded by the human gene *KIDINS220* situated at 2p25.1. The amino acid sequence of KIDINS220 can be divided into several different structural domains including protein interaction domains, transmembrane domains and phosphorylation site. The central part (amino acids 500–706) has four transmembrane domains whereas both N- and C-terminals are cytoplasmic. The cytoplasmic N-terminal domain harbours 11 ankyrin repeats (amino acids 37–402) that serve as a docking site for different protein partners.¹⁰ Next to this region (amino acids 467–474) is a consensus motif ATP/GTP binding site (P-loop, AQWGSQKS). Due to the presence of nucleotide-binding Walker A and Walker B motifs in juxtamembrane regions of KIDINS220, it has been suggested as P-loop nucleotide phosphatases of the “KAP Family”, named after KIDINS220/ARMS and PIFA.⁹

However, the functional role of these latter motifs in KIDINS220 is still unclear. At its C-terminal end, KIDINS220 harbours several other important interacting domains. Some of these domains include; KIM region (amino acids 1356–1395) for binding of KIDINS220 with Kinesin-1,¹² proline-rich region (amino acids 1080–1092) that binds the adapter protein CRKL,^{9,23} a sterile alpha motif (SAM; amino acids 1231–1300) and a PDZ-binding motif (PSD-95/Disc large/Zonula occludens-1) which comprises of the last four amino acids of the KIDINS220 sequence.^{7,24}

3 | SEQUENCE EVOLUTION OF KIDINS220

KIDINS220 protein or orthologues thereof are found in a large selection of diversely evolved organisms (Table 1). The analysis of the sequence evolution of KIDINS220 shows more than 90% homology and identity within higher mammals. For avian and reptilian proteins, sequence similarities with the human protein decrease to between 80 and 90%. Orthologues of KIDINS220 are found in several metazoans like *Caenorhabditis elegans* (26.4% identity, 42.7% homology) and *Drosophila melanogaster* (28.6% identity, 42.0% similarity).^{6,7} This highly conserved protein sequence from nematodes to humans strongly suggests that KIDINS220 plays evolutionarily conserved functions, which is confirmed in particular by the protein–protein interactions (see hereunder). Overall, the N-terminal region of KIDINS220 is highly conserved, unlike the more divergent C-terminal region, suggesting a differential evolutionary importance of properties associated with N- or C-terminal domains of the protein.

Presence of KIDINS220 in cells of different tissues and its role in different systems, like immune system,^{18,19,25,26} the vascular system,^{8,15} nervous system, and pathological conditions like tumour proliferation and neural disorders, can also be a consequence of the evolutionary divergence that some parts of KIDINS220 have underwent.

4 | KIDINS220/ARMS AND HUMAN DISEASES

In addition to its involvement in cancer (see next paragraph), KIDINS220 has been associated with different pathological situations in humans (Figure 2).

NGF is considered to be an important player in bronchial hyper-responsiveness and inflammation in asthma patients.^{27,28} Recent studies revealed that KIDINS220 is overexpressed in the lung, spleen, and peripheral blood of BALB/c mice after allergen challenge.^{29,30} On the other hand, a significant reduction in the expression of KIDINS220 occurs after intra-nasal administration with anti-NGF antibodies to mice, suggesting that KIDINS220 is regulated by NGF signalling in asthma.³⁰ Interestingly, the ovalbumin-induced expression of NFKB, IL1B, IL-4, and TNF in ovalbumin-sensitized mice was limited by the treatment with anti-KIDINS220 antibodies. Based on these findings, it is clear that NGF/NTRK1-KIDINS220-ERK signalling pathway holds a major position in the pathogenesis of allergic asthma²⁹ and, if validated

TABLE 1 Percentage of protein homology and identity between Kidins220 from human and different species

Taxon	Species	Biological nomenclature	Identity	Homolog
Mammalia	Monkey	<i>Macaca mulatta</i>	99.1%	99.5%
	Sumatran orangutan	<i>Pongo abelii</i>	98.4%	98.6%
	Dog	<i>Canis lupus familiaris</i>	96.9%	98.3%
	Cattle	<i>Bos Taurus</i> (isoformX7)	93.6%	95.9%
	Rat	<i>Rattus norvegicus</i>	93.1%	95.9%
	Mouse	<i>Mus musculus</i>	92.9%	95.8%
	Elephant	<i>Loxodonta africana</i>	92.9%	94.4%
	Rabbit	<i>Oryctolagus cuniculus</i>	83.4%	86.7%
	Brandt's bat	<i>Myotis brandtii</i>	74.4%	77.3%
Aves	Chicken	<i>Gallus gallus</i>	86.4%	91.9%
	Zebra finch	<i>Taeniopygia guttata</i>	86.1%	91.8%
Reptilia	Lizard	<i>Anolis carolinensis</i>	85.0%	91.4%
	Green sea turtle (Reptiles)	<i>Chelonia mydas</i>	83.8%	89.2%
Amphibia	Frog	<i>Xenopus tropicalis</i>	79.2%	87.6%
Actinopterygii	Zebrafish	<i>Dario rerio</i>	70.0%	79.5%
	Tilapia	<i>Oreochromis niloticus</i>	69.7%	79.5%
	Puffer fish	<i>Tetraodon nigroviridis</i>	60.8%	69.1%
Insecta	Wasp	<i>Nasonia vitriopennis</i>	34.2%	48.9%
	Pea aphid	<i>Acyrtosiphon pisum</i>	34.2%	50.2%
	Red Beetle	<i>Tribolium castaneum</i>	33.2%	47.3%
	Buff-tailed bumble	<i>Bombus terrestris</i>	31.2%	45.9%
	Common bumblebee	<i>Bombus impatiens</i>	31.1%	45.9%
	Carpenter ant	<i>Camponotus floridanus</i>	29.5%	43.6%
	Yellow fever mosquito	<i>Culex quinquefasciatus</i>	29.5%	43.6%
	Malaria mosquito	<i>Anopheles gambiae</i>	28.9%	42.8%
	Fruit fly	<i>Drasophila melanogaster</i>	28.6%	42.0%
Chromadorea	Pig roundworm	<i>Ascaris suum</i>	27.1%	43.2%
	Round worm	<i>Burgia malayi</i>	26.4%	43.8%
Secernentea	Worm	<i>Caenorhabditis briggsae</i>	26.1%	4.8%
	Worm	<i>Caenorhabditis elegans</i>	26.4%	42.7%
Chromadorea	Eye worm	<i>Loa loa</i>	25.8%	42.1%
	Sea squirt	<i>Oikopleura dioica</i>	25.1%	37.7%
Hydrozoa	Hydra	<i>Hydra vulgaris</i>	20.1%	33.7%
Adenophorea	Pork worm	<i>Trichinella spiralis</i>	11.7%	17.6%

Sequences were obtained from PubMed and compared for alignment using EMBOS Needle nucleotide alignment (EMBL-EBI website).

in humans, KIDINS220 can serve as a new therapeutic approach towards the allergic diseases of the airway.^{30,31}

KIDINS220 has also shown its pivotal role in HIV-associated neurocognitive disorders (HAND). HIV infects central nervous system-resident immune cells, microglia, which in turn releases proinflammatory

molecules causing HANDS.^{32,33} KIDINS220 has shown to be expressed in microglial cells of central nervous system and to be involved in the activation of NFKB.²¹ A study showed that production of tumour necrosis factor alpha and activation of NFKB falls down remarkably when KIDINS220 is downregulated.³⁴

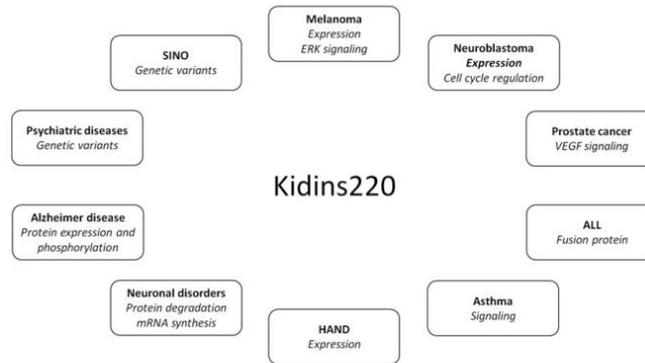


FIGURE 2 Known or suggested associations between KIDINS220 and human pathologies. Proven molecular mechanisms or cellular modifications are indicated in italic. See text for abbreviations and explanations

It is well established that KIDINS220 holds a pivotal position in neuronal disorders through its role in the signalling of neurotrophins. It has also been shown that KIDINS220 expression is regulated by certain pathological conditions e.g. cerebral ischemia. The underlying mechanism of this behaviour relies on *N*-methyl-D-aspartate receptors (NMDAR) excitotoxicity because of the overproduction of glutamate during ischemic conditions.¹³ Hyperactivation of NMDARs causes an increased influx of Ca^{2+} in the post-synaptic neurons.^{35,36} This influx of Ca^{2+} causes downregulation of KIDINS220 in two different ways which are either calpain-dependent (cleavage of C-terminals of KIDINS220 and NR2A/NR2B subunits of NMDARs) or calpain-independent (transcriptional inhibition of *KIDINS220* gene).¹³ These findings suggest that neuronal degeneration induced by cerebral ischemia might be due to cleavage of PDZ-binding domain on C-terminal of KIDINS220, which is responsible for the downstream signalling during neuronal differentiation and survival.

Unlike its reduction in cerebral ischemia, KIDINS220 has shown an enhanced expression in Alzheimer's disease (AD) where it correlates with TAU.³⁷ TAU is a microtubule-associated protein which is hyperphosphorylated in AD.^{38,39} Necropsies from AD patients with different progression stages showed that KIDINS220 expression levels are increased in human brain owing to the increased resistance to calpain cleavage associated with a hyperphosphorylation of KIDINS220.³⁷ Potential mechanisms of the involvement of KIDINS220 in the AD have been suggested elsewhere.⁴⁰ Based on all this data and a short study on KIDINS220 expression in AD brain and cerebrospinal fluid using an anti-KIDINS220 antibody, it has been suggested that KIDINS220 can serve as a novel biomarker for AD neurodegeneration.⁴¹ Further studies, however, would have a far-reaching importance concerning regulation of KIDINS220 expression in other similar pathologies like hypoxia, acute trauma and other neurodegenerative diseases.

Still, in relation to neurological diseases, it was shown that genetic variation in the signalling pathways of neurotrophins, and thereby in *KIDINS220*, was associated with increased risk of schizophrenia and psychosis. These include A557V, H1085R and A1299G mutations in

patients with schizophrenia-related psychosis, and the mutations are probably modifying the interaction with CRKL and the subsequent MAPK signalling, or the SAM-domain-related properties.^{42,43} Although rare, such genetic variants in *KIDINS220*, together with other genes, can explain certain cases of psychiatric disorders.⁴⁴

Three childhood cases of a new syndrome named SINO have been associated with variants of KIDINS220 with premature stop codons.⁴⁵ SINO syndrome is characterized by spastic paraplegia, intellectual disability, nystagmus and obesity. The variants of KIDINS220 responsible for this sort of syndrome were closely related to an isoform of KIDINS220 with alternative splicing and present only in adult tissues.⁴⁶ The study has shown the importance of the delicate balance of KIDINS220 isoforms' expression during the developmental phases of an embryo.⁴⁵ Other genetic variants were found in unborn fetuses corresponding to a premature termination codon in exon 25 resulting in nonsense-mediated mRNA decay and absence of protein.⁴⁷ The fetuses had enlarged cerebral ventricles and limb contractures that suggested the association between the phenotype and KIDINS220.

5 | KIDINS220 AND CANCER

There is currently a growing amount of published data showing the involvement of KIDINS220 in various cancers (Figure 2). Here, we discuss the link between KIDINS220 and cancer by separating what has been shown directly with this protein (table 2), what can be suggested from indirect studies, and how these data constitute the basis for new therapeutic approaches.

5.1 | Direct roles of KIDINS220 in cancer

First of all, KIDINS220 is clearly involved in cell proliferation and survival, as KIDINS220 knock out mice have a large rate of increased apoptotic cells as compared to control mice.⁵ Melanoma, a tumour ontogenetically originating from neural crest, highly expresses KIDINS220 which protects tumour cells from stress-induced apoptotic

TABLE 2 Cancer-related studies on Kidins220

Cancer type	Sample/model	Observation	Reference
Melanoma	Melanoma cell lines and 100 clinical samples	Kidins220 is overexpressed in melanoma samples and high expression of Kidins220 is associated with poor survival in patients. siRNA-mediated inhibition of Kidins220 is associated with decreased cell proliferation, colony formation, cell migration, <i>in vivo</i> growth and formation of metastasis, and with enhanced sensitivity to UVB radiations.	17,48
Neuroblastoma	Neuroblastoma cell line	shRNA-mediated inhibition of Kidins220 is associated with decreased cell proliferation due to longer G1 phase	16
Neuroblastoma	Neuroblastoma cell lines and 62 clinical samples	Protein expression in cell lines and mRNA expression in patient samples siRNA-mediated inhibition of Kidins220 is associated with a lower pERK response to NGF and morphological changes, but is not associated with cell migration or sensitivity to cancer drugs	49,50
Prostate cancer	Prostate cancer cell lines and 48 clinical samples	shRNA-mediated inhibition of Kidins220 is associated with decreased cell proliferation, vascular development and <i>in vivo</i> tumor growth, and with increased apoptosis. The effects of Kidins220 in prostate cancer cells seems to be regulated by miR-4638-5p	51
Acute lymphoblastic leukemia	One clinical sample	A fusion protein PAX5-Kidins220 is potentially associated with a lack of differentiation of B cells and an increased proliferation of leukemic cells.	52

death by activation of ERK signalling pathway.¹⁷ KIDINS220-depleted melanoma cell lines expressed a significant inhibition of anchorage-independent growth in soft agar and an extended cell death by UVB-induced apoptosis. It's downregulation also decreased the tumorigenicity in severe combined immunodeficient mice, strongly evidencing the functional role of the protein in this disease.¹⁷ This was validated in another study by the same authors, together with the finding of a correlation between KIDINS220 protein expression and clinical outcome for melanoma patients.⁴⁸ Indeed, there was a better overall survival of patients with a negative KIDINS220 expression as compared to those with weak, moderate, or strong expression.

Another study showed that an increased expression of KIDINS220 stimulated cell proliferation in human neuroblastoma cells, another neural crest tumor.¹⁶ The underlying mechanism of this function suggested that the downregulation of KIDINS220 is associated with a decreased level of cyclin D1/CDK4 during the G1 phase of cell cycle. CCND1/CDK4 is a protein frequently overexpressed and associated with tumorigenesis in many cancers.⁵³ Decreased KIDINS220 also upregulates P21 (a cyclin-dependent kinase inhibitor, CDKN1A) which in turn reduces the hyperphosphorylation of RB1, a tumour suppressor protein, by suppressing the kinase activity of CCNG1/CDK complexes.¹⁶ KIDINS220 is expressed in different neuroblastoma tumours and cell lines from these tumours in which its downregulation results in a decreased NGF induced-MAPK signalling. However, its downregulation has no effect on BDNF induced-MAPK signaling.⁴⁹ Depletion of KIDINS220 in neuroblastoma cell lines has also been associated with the neural to Schwann like transition.⁵⁰ Quantification and presence of Schwannian cells in the peripheral neuroblastic tumours is one of the markers for favourable

prognosis,⁵⁴ and is being used by the International Neuroblastoma Pathology Classification (INPC) guidelines to stage these tumors.⁵⁵ Thus, KIDINS220 promoting the decrease of these cells is thus associated with a bad prognosis.

In prostate cancer, KIDINS220 is involved in angiogenesis and castration resistance.⁵¹ KIDINS220 binds with VEGFR2 and VEGFR3 which act as co-receptors for the interaction between VEGF and VEGFR. This leads to the activation of VEGF/PI3K/AKT signalling pathway, which in turn promotes proliferation and vasculogenic mimicry formation. It also impairs apoptosis of prostate cancer cells as well as increases the expression level and secretion of VEGF to stimulate angiogenesis in an autocrine manner. The micro-RNA miR-4638-5p targets KIDINS220 3'-UTR sequence and thereby downregulates its expression in prostate cancer cells, which is associated with an increased sensitivity to castration. This is hypothesized to be the functional explanation of the tumour suppressor activity of this micro-RNA.

A fusion protein between KIDINS220 and paired box-5 (PAX-5) has been described in acute lymphoblastic leukaemia (ALL).⁵² This protein corresponds to the 306 first amino acids of PAX5 and the 901 last amino acids of K220 (871–1171). PAX-5 is a transcriptional regulator that ensures the commitment to B-lymphoid lineage and maintenance of its normal phenotype.^{56,57} Any tempering in the functions of protein because of fusions at genetic level causes an abnormal deviation from the development and differentiation of B-cells at an uncommitted progenitor stage. It precludes to the development of B-cell ALL.^{58,59} The fusion protein between KIDINS220 and PAX5 contains the N-terminal region of PAX5 which incorporates its DNA-binding domains, and the C-terminal region of KIDINS220.⁵² This latter is therefore still able to interact with other proteins through the proline-rich domain.

KIDINS220 has also shown interaction with B-cell antigen receptor (BCR) in unstimulated B lymphocytes. However, B-cells stimulation and Src-independent signalling enhance this interaction.¹⁹ Studies have shown that KIDINS220 expression is essential for bracketing BCR to the Ca²⁺ and RAS-ERK pathways and hence plays a vital role in BCR-mediated cellular activation and B cell development in bone marrow.

A detailed characterization of gene expression and protein localization of KIDINS220 during early phases of embryogenesis has shown that it is dynamically regulated during development and therefore has an imperative role in distinct spatiotemporal differentiative events.⁶⁰ The nervous system, eye, branchial arches, heart, and somites are different embryonic regions expressing KIDINS220 mRNA from neurula to larval stage. Protein expression has also shown a similar behavior, with embryonic expression in central nervous system, cranial nerves, motor nerves, intersomitic junctions, retinal ganglion cells, lens, otic vesicle, heart, and branchial arches. As compared with the earlier examined stages, embryos during stage 42 displayed a differential localization of KIDINS220 protein in some regions like retina and somites.⁶⁰ Aberrant re-expression of embryonic and developmental genes, eventually by epigenetic modifications, has been repeatedly shown to be associated with cancer progression.⁶¹ Given the described roles for KIDINS220 in both development and cancer, this could also be a potential explanation of its role in certain cancers.

In summary, the available data clearly suggests that KIDINS220 is directly associated with cancer growth and development. For the moment, no clear and single molecular mechanism is identified as being responsible for this, and as shown in the previous paragraphs, multiple cancer types have been identified as being influenced by KIDINS220. Future work should therefore be performed in order to confirm both involvement in identified cancers and the underlying mechanisms, and to elucidate whether this complex protein can be considered an oncogene, used as a biomarker and if its cancer-related properties are related to its phosphorylation by protein kinase D (PKD) or not (see hereafter).

5.2 | Indirect roles of KIDINS220 in cancer

In addition to studies focusing directly on KIDINS220, data exists on the potential involvement of this protein in cancer through its protein partners. First, KIDINS220 has been shown to interact with VEGFR2 and the intracellular signalling of VEGF in some conditions.¹⁵ Thus, if this is also the case in tumours, KIDINS220 can be involved in the angiogenic activity of VEGF and thus the cancer progression.

Protein kinases are enzymes responsible for catalyzing the phosphorylation of different proteins to modulate the activity inside the cell in response to activated membrane receptors by certain stimuli. They play major roles in cancer and have become an attractive target for cancer treatment.⁶² PKD is a PKC-like serine/threonine kinase that phosphorylates the synthetic peptide Syntide 2, but, as compared with PKC, only very weak physiological substrates have been evaluated.⁶³ KIDINS220, and more precisely its serine 919, was thus identified as the first physiological substrate of PKD upon its cloning and initial characterization.⁶ Since then, several substrates for PKD have been

described, including cancer-related proteins. The phosphorylation of SSHL1 by PKD, for example, causes a cofilin-mediated decrease in the migration of breast cancer cells.^{64–66} PKD is also involved in the suppression of epithelial-to-mesenchymal transition through phosphorylation of the transcription factor Snail.⁶⁷ Moreover, PKD-HDAC5 pathway plays a vital role in VEGF regulation of gene transcription and angiogenesis.⁶⁸ Considerable evidence suggests PKD be a potential therapeutic target owing to its effective role in different types of cancers.⁶⁹ The phosphorylation of KIDINS220 by PKD has been shown to regulate the secretion of neurotensin.⁷⁰ In addition, studies have shown that loss of KIDINS220 and PKD1/2 have a similar phenotype *i. e.* multiple axons and aberrant dendrites,⁷¹ underlying the functional importance of the interaction between these two proteins. Therefore, it could be possible that some of the cancer-related properties of PKD could be mediated by KIDINS220. Additional work should be performed in order to clarify this association. Finally, the involvement of KIDINS220 in cancer could be related to Trks or to NGFR. There is indeed an increasing amount of data in the literature demonstrating the role of these receptors in cancer. Both receptors were recently shown to be highly expressed at the protein level in thyroid cancer and their targeting by siRNA decreased the cell viability.⁷² Increased expression of NGFR or both NGFR and Trks was also shown in ovarian cancer,⁷³ neuroblastoma,⁷⁴ renal cell carcinoma⁷⁵ and Schwannomas,⁷⁶ whereas NGFR expression has been shown to be decreased in both gastric cancer⁷⁷ and hepatocellular carcinomas.⁷⁸ In addition, the possibility to target neurotrophin receptors was shown in several of the cited here over as well as for melanomas⁷⁹ and pancreatic cancer cells.⁸⁰ The potential role of KIDINS220 was not evaluated in any of these studies on neurotrophin-receptors and cancer, but due to its central position in neurotrophin signalling, we imagine that it's involved as well. The precise role of KIDINS220 in this phenomenon should, of course, be confirmed by additional studies.

5.3 | Targeting KIDINS220 in cancer

Based on the existing data reviewed here, we believe that upcoming research will validate KIDINS220 as a potential target for cancer therapy. Various strategies could be used to target this large multifunctional protein based on the molecular mechanism involved in the tumour development. As no enzymatic activity of the protein is known, its inhibition would be based on less classical approaches. As several of its activities are based on the physical interaction with other proteins, small molecules inhibiting these interactions could be used to target a given effect mediated by a specific interaction. This approach has been successful in cancer as exemplified for example by compounds targeting the interaction between TP53 and MDM2.⁸¹ This strategy would, however, need a better knowledge about the tridimensional structure of either the complete KIDINS220 protein or the targeted domain to use bioinformatics tools to search for inhibitors. An enhanced understanding of the molecular mechanisms involved in each cancer type and for each protein interaction is also a prerequisite to this approach.

Based on whether the phosphorylation of KIDINS220 by PKD is needed for the cancer-promoting effects of PKD, this could be used by

targeting, for example, the phosphorylation site of KIDINS220 with an antibody. This would then inhibit specifically the KIDINS220-related effect of PKD whereas other potentially interesting phosphorylated proteins would not be affected. For the specific case of ALL patients expressing the fusion protein PAX5-KIDINS220,⁵² a specific monoclonal antibody targeting the fusion protein could be developed and used for treatment purposes. This would however depend on the membrane expression of the fusion protein. Finally, targeting the extracellular domains of KIDINS220 with therapeutic monoclonal antibodies could be a possibility if this would be associated internalization of the protein, induction of cell death via the activation of the immune system (antibody-dependent cell cytotoxicity) or with structural modifications leading to decreased intracellular signalling or protein-protein interactions. This latter is, however, less likely as the extracellular domain of KIDINS220 is very small.

6 | CONCLUDING REMARKS

As described in this review, KIDINS220, a protein that was first studied at from a biochemical and a cellular biology point of view, has now clearly been associated with the development or the status of several diseases. Possible molecular explanations of these roles have sometimes been proposed, but much is still to be done both in order to understand the shown roles and to validate them in additional patient groups. As for cancer, KIDINS220 has a major role in cell proliferation in several cancer types, and the mechanism seems to be quite specific for each cancer and difficult to generalize. Since the inhibition of KIDINS220 expression is often associated with decreased cell proliferation, this intriguing protein could turn out to be an interesting drug target. Another remaining part in the field of cancer and KIDINS220 is to understand the expression of this protein in some cancers, and whether this could be related to re-expression of embryonic genes or not.

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General Discussion and perspectives

Our two papers containing the work of my PhD add important and quantitative information about what the 5'-nucleotidases cN-II and CD73 are doing in cancer cells. As presented both in the general introduction, in the introductions of the submitted manuscripts and in their discussions, several aspects were already known as to their involvement in responses to cancer drugs, in regulations of nucleotide pools in cancer cells, and in some metabolic pathways and intracellular regulatory machineries. However, the question is vast and every work performed in appropriate conditions gives valuable information about these two intriguing enzymes. This is particularly evident when we consider cell-specificity and tissue-specificity as experiments then needs to be confirmed in various cell or animal models. Some of the results described in the manuscripts will be discussed again here, within an effort to take into account data from both papers.

There is not much data available related to changes in intracellular and extracellular nucleotide pools in different cell models prepared so far with modulated expression of cN-II or CD73. Several studies have reported a slight decrease in NTP pools in cN-II overexpressing non-cancerous cells (Federico Cividini, Filoni, et al. 2015; Gazzola et al. 1999) and no effect in shRNA-mediated stable downregulation of cN-II (Bricard et al. 2016). The impact of CD73 expression on intracellular nucleotide pools, is however not known to our knowledge. We showed some modifications in intracellular nucleotides in our MDA-MB-231 models, underlining the implication of these enzymes in the regulation of these pools and potentially in thereby regulated cellular processes. However, whether these modifications are important enough to have phenotypic implications is not yet clear. What can be noted, is the clear involvement of cN-II in the regulation of both adenosine and inosine derivatives upon cellular exposure to adenosine or AMP (figure 3 in paper 1). In addition, nucleotide pools in our CRISPR/Cas9 models for NCI-H292 were not determined, and conclusions on these can therefore not be done. Some data are available for NCI-H292 cells with downregulated (but not abolished) cN-II expression (Bricard et al. 2016), but they can most probably not be extrapolated to our current models.

Further experiments are thus warranted in order for example to link changes in nucleotide pools to modified sensitivity to vincristine and mitomycin C in these cells. However, it needs further studies to know if these changes have any role to play in the differential phenotypic behavior shown by our cell models.

The involvement of CD73 in cell migration, both through the production of adenosine and directly through the protein, has already been suggested, whereas nothing was known for cN-II before our study (R. Sadej, Sychala, and Skladanowski 2006; Bavaresco et al. 2008; Rafal Sadej and Skladanowski 2012). cN-II deficiency increased migration in MDA-MB-231 cells, whereas CD73 deficiency decreased migration in NCI-H292 cells. As for the stress conditions, AMP caused a surprising modulation in cell migration even in CD73 deficient cell lines. We further managed to decipher the molecular mechanism (COX-2, AKT pathway) involved in the modification in migration in MDA-MB-231 models, that was not found to be actively implicated in NCI-H292 or 4T1 cells, thus being cell specific. This finding showing a link between cN-II expression and COX-2 expression etc, is of major importance for our work. It shows indeed that the modulation of an enzyme in nucleotide metabolism can modify cellular processes and pathways that seem to be independent from purines and pyrimidines. Again, the involvement of nucleotides remains uncertain. In view of this, we have performed RNAseq analysis and targeted metabolomics analysis (Collaboration with C Machon and J Guitton at HCL, Lyon) on our MDA-MB-231 cell models, and will continue the work on these to identify other major modifications induced by cN-II and/or CD73 deficiency. New phenotypes will be studied as well based on the results we will get and potentially highlight even more new roles for these two enzymes.

If we take into account the minor differences observed between NCI-H292 cell models in our first study (almost no modification in gene expression, no modification in cell proliferation, slight modification in migration, increase in COX-2 expression), it is not evident to explain at a molecular level the differences observed and presented in the second study (HIF1- α induction, drug sensitivity). We therefore suggest that the observed phenotypes here are due to modifications in cellular parameters that have not yet

been studied, and can include the cytoskeleton, DNA repair mechanisms, microtubules, intracellular second messenger etc.

If we look at all the presented work globally, we can identify some limits of our work. These include:

- We transfected cancer cells with plasmids and worked on batches and not on isolated clones. This has the advantage of leaving some heterogeneity within the cell model. However, we do not know if the observed phenotypes were due to modifications in 5'-nucleotidases or rather to other modifications induced during selection or already present in some cells. We planned to re-express cN-II and CD73 using mutated and CRISPR/Cas9-insensitive constructs in order to have clear positive controls for this, but these are still unavailable.
- Not all of our results were obtained or even studied in all cell models. It is therefore difficult to make a general conclusion each time since some of the effects are clearly cell-line dependent.
- Not all results were confirmed by using different techniques (migration, drug sensitivity). This can be due to the involvement of different cellular processes when changing the method, or the fact that the effect is not robust enough to be observed every time.
- Indeed, nucleotide metabolism is dependent on a large number of proteins whereas we only studied thoroughly the 5'-nucleotidases (cN-II and CD73). However, it is evident that transporters, kinases, ADO receptors and other proteins are involved in the cellular response to stress, in particular to adenosine and to AMP. Thus, a larger study on the protein expression of these enzymes, transporters and receptors would be of interest in order to better conclude on our work.
- Finally, as these models are the first described models with CRISPR/Cas9-mediated modulation of cN-II and CD73 expression, it is somehow difficult to compare our results with those presented in the literature and obtained with shRNA, siRNA or inhibitor-mediated inhibition

of the studied enzymes. Also, we are the first to describe the concomitant inhibition of the two 5'-nucleotidases in cell models.

The study of the biological role of cN-II and CD73 in cancer cells will continue in our laboratory, both with the described models and with the development of new ones. Short term and long-term perspectives include:

- The analysis of RNAseq and metabolomics data from MDA-MB-231 cells: this will allow us to identify other modifications dependent on cN-II and/or CD73 expression in these cells, and the potential link between gene expressions and metabolite abundance. Once identified, we will develop new cell models with over or underexpression of given genes and see if the observed phenotypes (migration, COX-2 expression...) are similar in these models.
- Additional studies including stress response in MDA-MB-231 cells (similar to what was done on NCI-H292 cells) and nucleotide pools in NCI-H292 cells. This will complete the already performed work and help making conclusions on more than one single model each time.
- As mentioned, the reintroduction of wild-type cN-II and CD73 in our models will allow us to conclude on the functional role of these enzymes if phenotypes are reverted. We will also introduce mutant enzymes in order to study these in a cellular environment without any wild type protein.
- The study of cN-II deficient mice. These mice are available, and we plan to study the growth of tumors in these mice, using both cN-II expressing and deficient cell models such as the already obtained 4T1 model. Such experiments will help understanding the role of cN-II both on tumor cell and on stromal cells in the growth of such tumors. We will be particularly interested in the immune infiltration in the tumors of these mice.
- The study of cN-II and CD73 expression on human tumor samples. As we were initially interested in the interplay between intracellular and extracellular nucleotides and the role of cN-II and CD73 in this phenomenon, we would like to evaluate the expression of the two

proteins in lung cancer patients samples using immunohistochemistry. This will allow both to evaluate the co-expression of the enzymes in such clinical samples, and to see whether the two enzymes can predict clinical outcome and especially response to treatment.

- The study of cN-II and CD73 inhibitors, both developed within our group (see next paragraph) or by others, both alone and in combination with classical or immune-targeting cancer therapies.

Our laboratory is involved in the development of cN-II and CD73 inhibitors for several years. I was also involved in an ongoing INCa-funded study for the identification of new small molecule inhibitors for CD73 and for the development of *in vivo* suitable options for cN-II inhibitors. This project is done in collaborations with chemists (S Peyrottes, IBMM, Montpellier), enzymologists (L Chaloin, IRIM, Montpellier) and immunologists (C Caux, CRCL, Lyon). During my PhD thesis, I have participated in several meetings with our partner researchers related to this project and performed several experiments related to the validation of our lead compounds in our *in vitro* and *in vivo* models. The results we have obtained so far are confidential and will be published once all the related experiments are completed. More work is needed in order to understand clearly how such inhibitors should be used (alone, in combination with nucleoside analogues, in combination with immune-modulators..), in addition to obtaining highly efficient inhibitors. Once available, they would both represent interesting tools and be potential cancer drugs. Their clinical use will then be dependent on all findings and information obtained about cN-II and CD73, including their role in cancer cell biology.

In conclusion, this work is a part of the global project on 5'-nucleotidases, nucleotide metabolism, cancer biology, and cancer treatment. It can be seen as a drop in the ocean, but adds itself to a large amount of scientific data within these fields. Further work both in our laboratory as well as in other groups will in the upcoming years continue to make the knowledge on this topic grow. The actual

impact of this work in particular and of the nucleotide metabolism or 5'-nucleotidases in cancer will be clearer and clearer, and I truly hope that I have participated in making a difference.

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