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“Your funeral… my trial”: a review of the mechanisms leading to cancer cells death when targeting nicotinamide adenine dinucleotide

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Abstract

Le nicotinamide adénine dinucléotide (NAD) est un métabolite jouant un rôle crucial dans la vie cellulaire. Dans la mitochondrie, il est surtout utilisé comme co-enzyme d'oxydo-réduction afin de produire de l'énergie sous forme d'ATP (respiration cellulaire). Dans le noyau, il est utilisé en tant que co-substrat par plusieurs enzymes (sirtuines, PARP,...) impliquées dans diverses fonctions d'expression génique, de réparation cellulaire ou d'apoptose. Le NAD tend dès lors à devenir une cible clef dans le traitement de certaines maladies, et particulièrement des cancers, dans les cellules desquels ses taux semblent être augmentés. Certaines molécules visant directement ou indirectement son métabolisme ont montré leur efficacité dans l'inhibition de la croissance tumorale ou dans la potentialisation d'autres thérapies anti-cancéreuses. Le but de cette revue de littérature est de rassembler les mécanismes connus menant à la mort des cellules cancéreuses lors d'un traitement visant directement ou indirectement le NAD.

Mots-clés : NAD, cancer, nicotinamide, mort cellulaire, métabolisme

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Louis Morisod

Le titre « Your funeral… my trial », est emprunté à l'album du même nom, de Nick Cave, paru chez Mute Records en 1986.

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1. Introduction

Nicotinamide adenine dinucleotide (NAD) is a molecule made out of 2 pyridines nucleotides, a nicotinamide and an adenine connected to ribose units joined by two phosphate groups. Otto Warburg first identified it in the 1930's, and its functions were intensively investigated during the following years (1). It is a metabolite found in all living cells that plays crucial roles in many cellular processes. It functions either as co-enzyme or is used as substrate in several biochemical reactions, including mono- and poly-ADP-ribosylation, protein deacetylation, and ADP-ribose cyclization. As coenzymes, NAD and its phosphorylated form – nicotinamide adenine mononucleotide phosphate (NADP) – are electron acceptors and donors. Thus, they might turn from reduced forms (NADH, NADPH) into oxidized forms (NAD+, NADP+), and *vice versa*. This redox function is of particular importance in the metabolism of the cell, particularly in energy metabolism, reductive biosynthesis, and antioxidation. Lack of nicotinamide or nicotinic acid, the precursors of NAD, has been shown to induce pellagra (2). Since a number of metabolic pathways have been discovered to synthesize NAD, it was reasonable to suggest that NAD could intervene in several biological processes. Investigations since the 1960's highlighted a new role of NAD as a co-substrate in many reactions participating in aging, cell death, calcium homeostasis, gene expression, DNA repair, and circadian clock (3). These cellular reactions involve the consumption of the NAD/NADP molecules, with a necessity for the cell to re-synthesize NAD (4). The physiologic and pathologic importance of these reactions is becoming increasingly appreciated over the recent years. Of interest, cancer cells have an increased need of NAD compared to normal cells, since most cancer cells have continuous PARP activation through DNA damage and genomic instability and have higher energy consumption demands relative to other cells. Thus, enzymes involved in NAD+ metabolism are attractive targets for drug discovery against a variety of human diseases, including cancer (5).

The continuous discovery of NAD-metabolism targeting drugs enhances the comprehension of its roles in cancer cells and provides an exciting field for oncologic research. Stimulating results in xenograft tumors or *in vitro* models indicate potent effects that still need to be clarified (6)(7). Many studies focus on the mechanisms of cell death induced by NAD-depletion. It is likely that some have not been discovered yet and that cancer type specificities will still emerge. Moreover, interactions with other chemotherapeutic agents are emerging, maximizing their effects (8) (9).

This review will attempt to enumerate and present the already-known NAD-involving pathways, with specific focus on cancer conditions. The metabolism of NAD will be presented, from its production to its functions as a co-enzyme and a co-substrate. Then, mechanisms of cell death linked to NAD metabolism will be developed with an emphasis on the reactions that are impaired upon NAD depletion. Finally, the consequences of NAD-depletion in cancer cells will be reviewed and explained.

2. NAD biosynthesis

a. General information on NAD

Nicotinamide adenine dinucleotide (NAD) is a dinucleotide made out of an adenosine and a nicotinamide, joined by two phosphate groups (Figure1). Production of NAD is an essential issue for cell life because of the numerous functions this molecule fulfills. In bioenergetic pathways, NAD functions as an electron carrier coenzyme that is reversibly shuttled between its oxidized (NAD+) or reduced (NADH) forms, and does not require continuous production. Conversely, NAD is also used as substrate in numerous biochemical and biological processes, including those catalyzed by PARP1 (poly[ADP-ribose]polymerase 1), sirtuins, and ADP-ribosyl cyclase 1-6. In these reactions, NAD is split into two nucleotides and the cell needs to replenish its pools to maintain homeostasis. Due to the central role of NAD in cell metabolism, NAD is synthesized in humans from various sources (Figure 2) through *de novo* and/or salvage pathways.

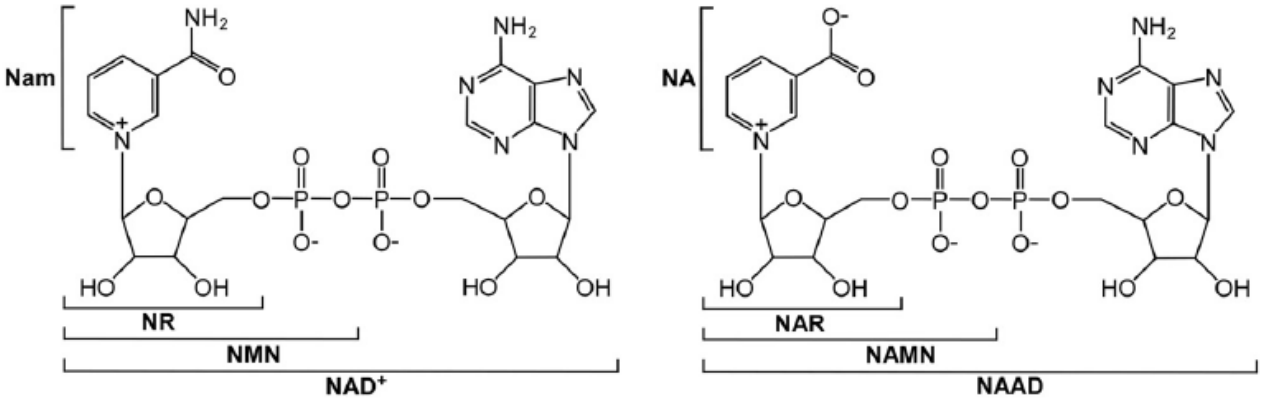

Figure 1. Chemical structures of oxidized nicotinamide adenine dinucleotide (NAD+) and nicotinic acid adenine dinucleotide (NAAD). Abbreviations : Nam : nicotinamide ; NR : nicotinamide riboside ; NMN : nicotinamide mononucleotide ; NA : nicotinic acid ; NAR : nicotinic acid riboside ; NAMN : nicotinic acid mononucleotide.

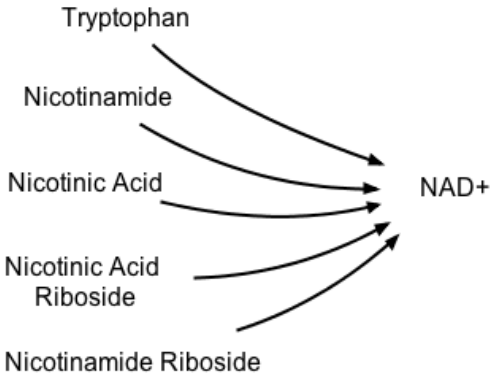

Figure 2. Precursors of nicotinamide adenine dinucleotide. Tryptophan, nicotinamide, nicotinic acid, nicotinic acid riboside and nicotinamide riboside are all precursors of NAD, which can further be turned into nicotinamide adenine dinucleotide phosphate.

b. *De novo* pathway

The *de novo* pathway of NAD synthesis starts from tryptophan, a rare and essential amino-acid (10). L-tryptophan is turned into N-formyl-kynurenine by either tryptophan 2,3-dioxygenase (TDO) in the liver, or indoleamine 2,3-dioxygenase (IDO) in extra-hepatic tissues, to enter the kynurenine pathway. In this pathway, several reactions lead to the formation of quinolinic acid (QA). QA is then transferred onto activated ribose to yield nicotinic acid mononucleotide (NAMN), one of the two pyridine mononucleotides, by quinolinic acid phosphoribosyl-transferase (QAPRT). NAMN is subsequently converted into nicotinic acid adenine dinucleotide (NAAD) by nicotinic acid mononucleotide adenylyltransferase (NMNAT). Finally, NAAD is amidated into NAD with NAD-synthase (NADS) (Figure 3).

c. Salvage pathways

NAD can be synthesized from other precursors. They are first converted into mononucleotides and then dinucleotides. These pathways are known as salvage pathways because preformed components are used. Nicotinic acid (NA) and nicotinamide (Nam) taken together are named vitamin B3 or niacin. Their related riboside forms, nicotinic acid riboside (NAR) and nicotinamide riboside (NamR), are found in food or body fluids. Niacins are converted into their respective mononucleotides by phosphoribosyltransferases, and then to dinucleotides to form NAD.

The Preiss-Handler pathway has been described in all organisms and uses NA to synthesize NAD, via nicotinic acid mononucleotide (NAMN) and nicotinic acid adenine dinucleotide (NAAD) (11)(12). Nicotinic acid phosphoribosyltransferase (NAPRT) converts NA into NAMN, which then follows the steps described in figure 3.

The salvage pathway using nicotinamide (Nam) is the most rapid and economical way to produce NAD in mammalian cells (13). Nam is the product of all NAD-consuming reactions and its levels in plasma are higher than those of nicotinic acid (10). NAMPT is the rate-limiting enzyme of the pathway and, thus, a key player to maintain NAD pools in cells (14).

Recently, riboside forms of NA and Nam (NAR and NamR, which is found in milk) were characterized as NAD precursors (15) (16). Nicotinic Riboside Kinase 1 and 2 (NRK1,2) are able to convert NAR/NamR into NAMN and NMN respectively, which can be subsequently transformed into NAD as shown in figure 3.

Most mammalian cells can used both NAD biosynthesis pathways depending on available precursors (17). In rats, it has been reported that tryptophan increases NAD levels, more specifically in the liver than in other organs (18). Another study performed in mice showed that nicotinamide was a more efficient precursor of NAD than nicotinic acid (19).

These pathways are summarized in figure 3 to provide a global vision of NAD synthesis. Of great interest NMNAT is involved in each of these pathways. It is thus an ideal target to disrupt NAD production.

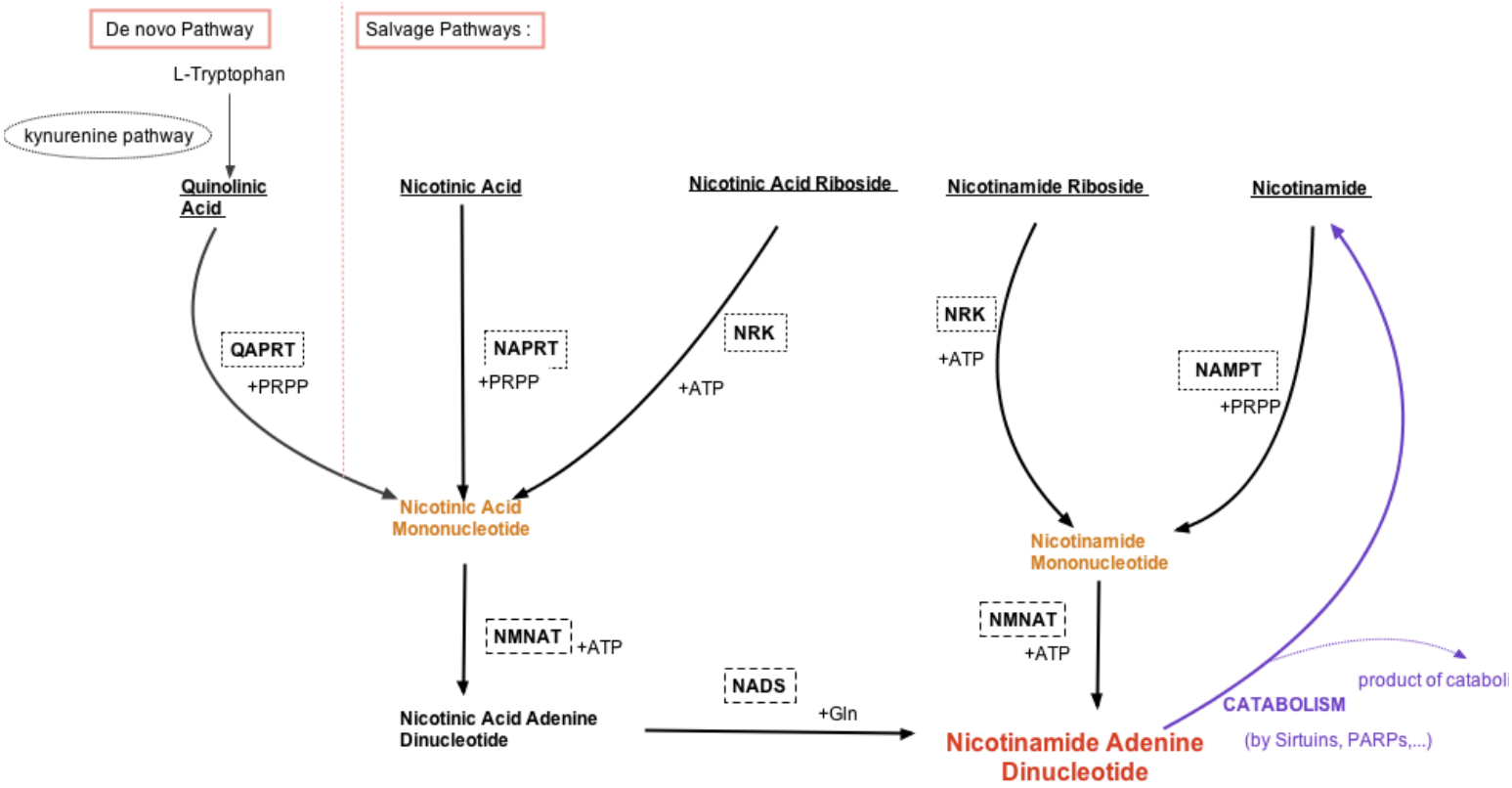

Figure 3, adapted from Reference (13). In *de novo* pathway, the kynurenine pathway, L-tryptophan is turned into quinolinic acid, which is then converted into nicotinic acid mononucleotide, that is changed to nicotinic acid adenine dinucleotide and then nicotinamide adenine dinucleotide, the final product of this synthesis. Salvage pathways produce NAD from different precursors: nicotinic acid, nicotinamide and their respective riboside-forms, nicotinic acid riboside and nicotinamide riboside. NAD may then be used for several reactions, including some that turn it into nicotinamide that can thus be recycled in a salvage pathway. QAPRT : quinolinic acid phosphoribosyltransferase; PRPP : phosphoribosylpyrophosphate; NAPRT : nicotinic acid phosphoribosyltransferase; NRK : nicotinamide (nicotinic acid) riboside kinase; NAMPT : nicotinamide phosphoribosyltransferase; ATP : adenosine triphosphate; NMNAT : nicotinamide mononucleotide adenylyltransferase; NADS : NAD-synthase; Gln : glutamine; PARPs : poly (ADP) ribose polymerases.

3. NAD functions and catabolism

a) NAD as a co-enzyme

NAD/NADH and their related phosphorylated forms, NADP/NADPH, are important co-enzyme of redox reactions: they carry hydrogen atoms from a molecule to another (20). These reactions are used for several functions such as energy production (especially NAD+/NADH) or prevention of oxydative stress and reductive systems (especially NADP+/NADPH).

NAD+/NADH cell contents play an important role in all oxydative reactions. Therefore, NAD+/NADH ratio in the cell is tightly controlled and kept very high (17). After its reduction (NADH), it is then reoxidized (NAD+) by lactate dehydrogenase in anaerobic conditions, or by the mitochondrial respiratory chain in aerobic conditions. Reoxydation of reduced NADH by oxygen in the electron transport system provides energy to create a proton gradient across the inner mitochondrial membrane, that is used to produce adenosine triphosphate (ATP), the main source of energy of all living cells.

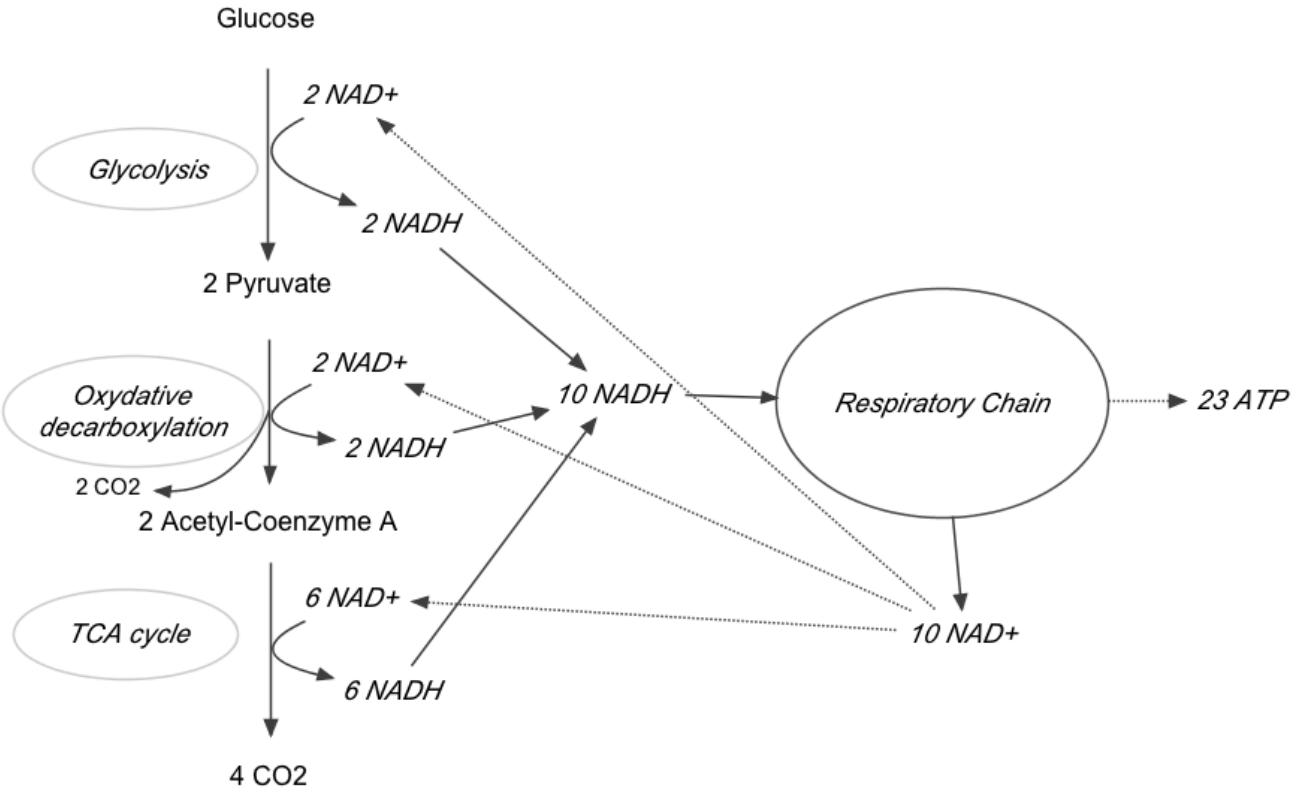

Figure 4. Adapted from reference (17). In aerobic conditions, NAD+ is reduced in several steps of metabolism, including glycolysis, oxidative decarboxylation and tricarboxylic cycle. NADH is then used in the respiratory chain in order to create a gradient of protons, used for the synthesis of adenosine triphosphate (ATP). In anaerobic conditions (not shown in the figure), NADH is reoxidized during the reduction of pyruvate into lactate.

NADP plays an important role in reductive reactions. Therefore, NADP+/NADPH ratio is kept very low in the cell (17). NADP+ is produced from NAD+ by a NAD

kinase. It may be reduced into NADPH by various enzymes that include glucose-6-phosphate-dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in the hexose monophosphate shunt, a cytoplasmic and mitochondrial isocitrate dehydrogenase (IDH) 1 and 2, malic enzyme (ME) or aldehyde dehydrogenase (ALDH) (21) (22).

Then, NADPH is used in several reactions including: (i) fatty acids and steroids synthesis, (ii) reduction of glutathion (via glutathion peroxydase) to protect against free radicals and peroxides and xenobiotics (by the family of cytochromes P450), and (iii) synthesis of free radicals in "respiratory burst" thanks to the NADPH-oxidase.

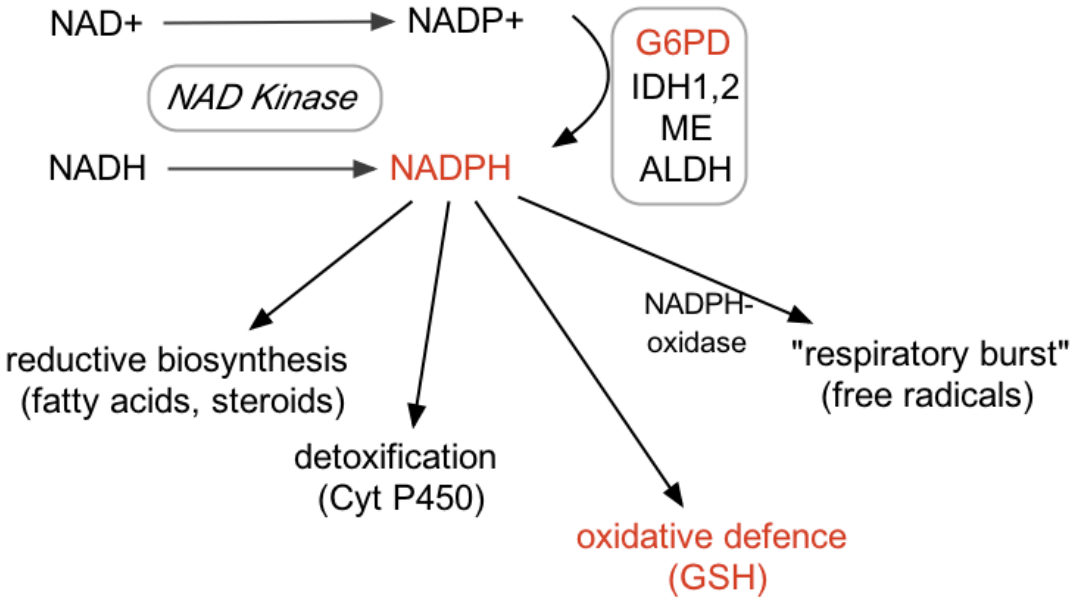

Figure 5. Adapted from (21): NAD+ and NADH may both be turned into their respective phosphorylated form by NAD kinase. Moreover, NADP+ might be turned into NADPH by other enzymes, such as glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), isocitrate dehydrogenase (IDH) 1 and 2, malic enzyme (ME) and aldehyde dehydrogenase (ALDH). NADPH may then enter various processes: reductive biosynthesis, detoxification, oxidative defense and oxidative burst. Cyt P450: cytochrome P450; GSH: reduced glutathion.

As they only carry electrons from a molecule to another one, dinucleotides in redox reactions are not consumed and the concentrations of NAD are kept constant.

b) NAD as a co-substrate

The role of NAD in redox reactions has been known for decades now. It is more recently that NAD functions as a co-substrate were identified and investigated. In these reactions, NAD+ is cleaved by three well known enzyme families: (i) ADP-ribose transferases, (ii) sirtuins and (iii) cyclic-ADP ribose synthases. These NAD-consuming proteins are thought to be sensors of the state of the cell allowing further reactions to happen or not ; nevertheless, this hypothesis is

hard to confirm due to the difficulty of measuring exactly NAD+ concentrations in different subcellular localizations (17).

Unlike, the metabolic redox reactions which do not affect the NAD cell content, the cleavage of NAD+ requires a constant re-synthesis of the molecule to maintain NAD+/NADH homeostasis.

i) ADP-ribosylation

ADP-ribosylation is a reaction using NAD+ as a cosubstrate to transfer an ADP-ribosyl group on an amino-acid or on another ADP-ribosyl residue and releases also a nicotinamide that can enter the salvage pathways. This reaction is made by an ADP-ribose transferase.

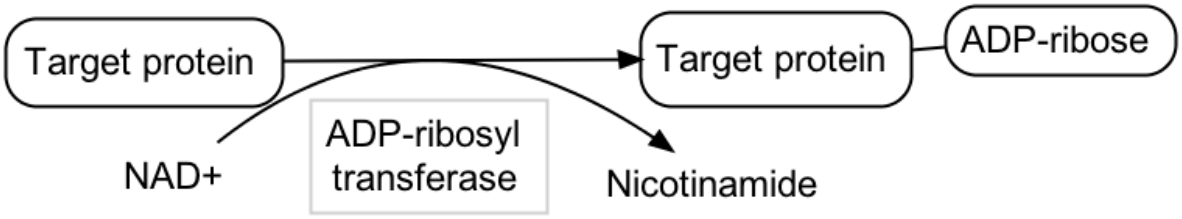

Figure 6. ADP-ribose transferases, such as the members of the PARP-family, transfer an ADP-ribose on a protein by cleaving a NAD+ molecule. This releases a molecule of ADP-ribose, which is put on the target protein, and a nicotinamide.

Addition of several ADP-riboses to a target protein is called poly-ADP-ribosylation, and is carried out by poly(ADP)-ribosyl transferases (PARPs), the best known ADP-ribosylation transferases (23). The amino-acid acceptor might be a glutamic acid, an aspartic acid or a lysine residue. 18 genes coding for PARPs are known (23).

ADP-ribosylation has three structural particularities. First, transfer of ADP-ribose on an amino-acid residue induces a covalent modification of the protein. Secondly, PARPs are able to elongate chains of ADP-ribose on a protein, with up to 200 units of ADP-riboses. Finally, some members of the PARP family may create branches on the chain of poly-ADP-riboses (23). These elongations and branches form a conserved motif, which interacts non-covalently with several DNA-stability proteins, such as p53 and p21 (24).

Consequences of ADP-ribosylation are many. PARP-1 was identified as a nuclear protein involved in the base excision repair (BER) pathway: it can recognize and bind DNA strand breaks. Then, via ADP-ribosylation, it recruits downstream proteins for DNA-repair and induces chromatin conformation changes (25). In genotoxic conditions, PARP-1 activity was shown to increase 500-fold (26). In this regard, PARP-1 is of great importance in DNA-damaging conditions such as alkalyting agents, oxidation or ionizing radiations.

With years, PARP-1 was also identified in other DNA-repair systems interacting with Werner syndrome protein or Cockayne syndrome B protein (27)(28). During apoptosis, PARPs are cleaved by caspases (29). PARP-1 may play a role in

genomic stability and prevention of cancer (30). Conversely, PARP-1 may also participate in caspase-independent cell death called chromatinolysis, via the apoptosis-induced factor (AIF) translocation from the mitochondria (31). PARPs were also reported to be involved in spermiogenesis (32). Finally, a group of PARPs named Tankyrase interact with telomeres (25)(23).

PARPs are thought to be one of the most important NAD-consuming proteins of the cell. They decreases NAD+ concentration and need continuous NAD-synthesis by *de novo* or salvage pathways to fulfill their functions (17).

ii) Sirtuins

Sirtuins are NAD-consuming enzymes with various activities. There are seven mammalian sirtuins (SIRT1 to SIRT7), with different cellular localization and variable functions. Most of sirtuins (SIRT1, SIRT2, SIRT3, SIRT5, SIRT6) have a lysine deacetylase activity (33). The reaction releases nicotinamide, O-acetyl ADP ribose and the deacetylated protein. The main target of these sirtuins is histones, giving them an important role in gene expression. The other activity of sirtuins is mono-ADP-ribosylation (SIRT4, SIRT6), with the release of nicotinamide. SIRT2 and SIRT3 are thought to combine both activities (Figure 7) (17).

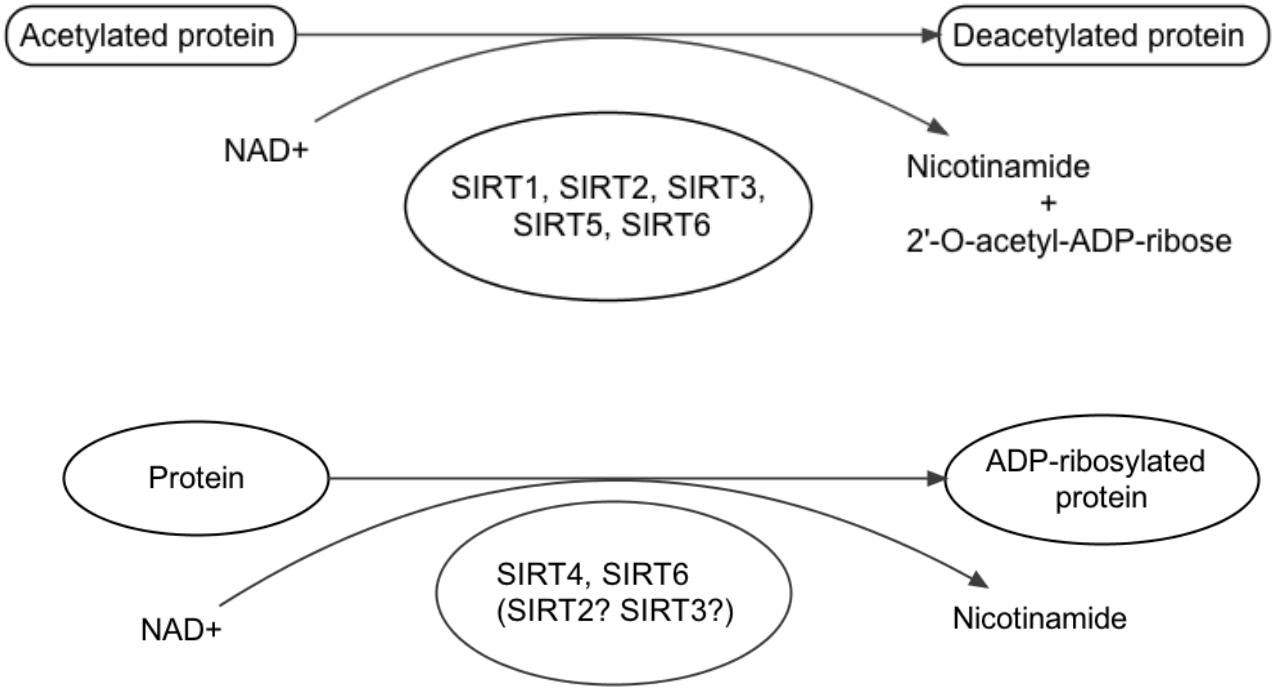

Figure 7. Adapted from reference (17). SIRT1,2,3,5,6 are able to deacetylate proteins using NAD+ and releasing nicotinamide and 2-O'-acetyl-ADP-ribose. SIRT4,6 and maybe SIRT2,3 are ADP-ribosyl transferases, with the classic use of NAD+ and the release of nicotinamide.

Sirtuins activity is thought to depend on NAD+ concentrations, but difficulties to precisely measure NAD+ subcellular concentration make it hard to prove (17).

Conversely, it has been clearly established that nicotinamide is a non-competitive inhibitor of sirtuins (34).

Like PARPs, actions of the sirtuins are numerous but specific to each isoform of the family. In general, as part of the DNA repair, chromatin structure changes and telomeres maintenance, sirtuins have an important role in genomic stability and lifespan regulation. Second, sirtuins, especially SIRT1, the best-studied member of the family, are implicated in several metabolic activity such as gluconeogenesis and fatty acid oxidation in the liver, insulin secretion in the pancreatic cells, metabolism of white adipose tissue and muscles or prevention of age-associated diseases via caloric restriction (35) (36).

In the past years, hypothesis of a correlation between metabolic state of the cell and DNA-repair emerged and recent studies showed a direct link between DNA-damage and metabolic response (37). Furthermore, SIRT1 and SIRT3 were shown to have tumor suppressive activities (38)(39). In other tumors, expression of SIRT1 was correlated with malignancy (36). It is thought that SIRT1 can prevent apparition of tumors but also maintain growth of already existing tumors (40).

Finally, SIRT3, SIRT4 and SIRT6 all regulate carbon uptake aimed at biomass formation from glycolysis (SIRT3, SIRT6) or from glutaminolysis (SIRT4). The Warburg effect, a mechanism used by tumoral cells to potentiate uptake of carbon for DNA elongation by increasing glycolysis and glutaminolysis, represses the activity of these three sirtuins (Figure 8, for SIRT3 and SIRT6). Therefore, glycolysis is upregulated and so able to provide more carbon substrates for DNA formation upon intense cell proliferation (41)(37)(42). Loss of SIRT3 was found in breast and ovarian cancers and loss of SIRT6 in 20% of all tumors (37)(42)(43).

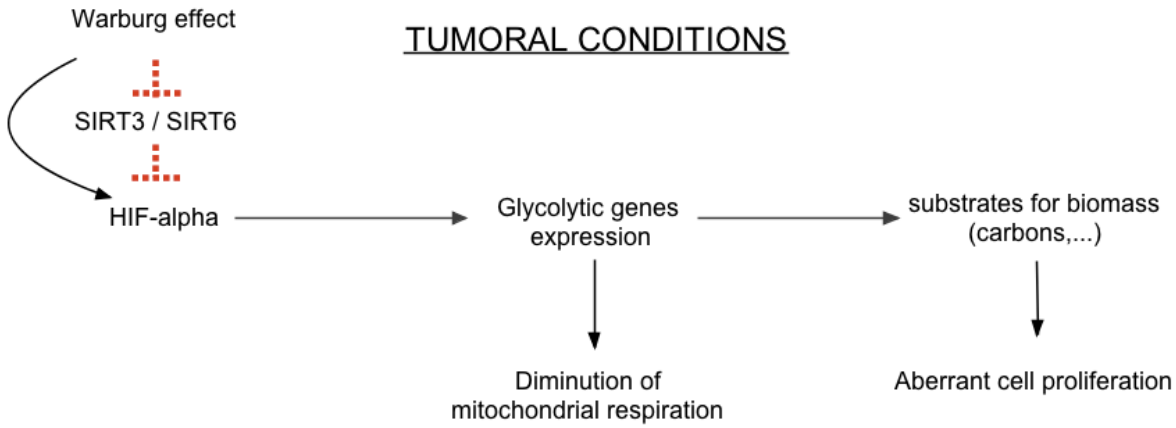

Figure 8. Consequences of Warburg effect on SIRT3 and SIRT6. In normal conditions, SIRT3 and SIRT6, two members of the sirtuins family, inhibit hypoxia-inducible factor α (HIF-α). This factor induces expression of glycolytic genes in order to produce more substrates for the cell proliferation, in case of hypoxic conditions. In tumoral conditions, SIRT3 and SIRT6 are inhibited, and thus more substrates used for the cell proliferation are obtained.

The discovery of sirtuins, 14 years ago, opened a large field of investigations that has to be explored to improve knowledge of several diseases, from obesity to cancer (40).

iii) c-ADP ribose transferases

CD38 and CD157 are cyclic-ADP-ribose (cADPR) synthases consuming NAD+ for the reaction. CD38 was first identified as a lymphocyte antigen, but then was found ubiquitously in all cells and many organelles (44)(45). It catalyzes the cyclization of NAD into cADPR but also the formation of nicotinic acid adenine dinucleotide phosphate (NAADP) from NADP and nicotinic acid, and the formation of ADP-ribose from cADPR (46) (Figure 9). cADPR is a second messenger of calcium signaling pathway that binds the ryanodine receptors. It is used by several signaling pathways, including fertilization, insulin release and muscle contraction (47).

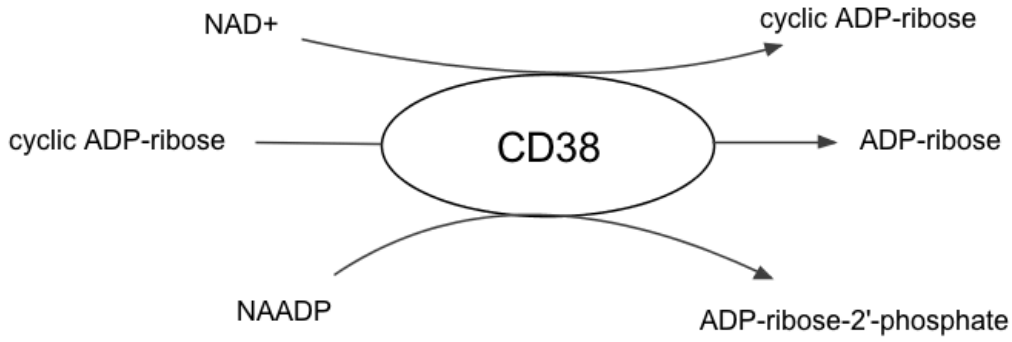

Figure 9. Adapted from reference (46). CD38 i) turns approximately one out of hundred NAD+ molecules into one cyclic ADP-ribose (cADPR), an important second messenger for calcium signaling in the cell, ii) turns cADPR into ADP-ribose and iii) turns nicotinic acid adenine dinucleotide phosphate (NAADP) into ADP-ribose-2'-phosphate.

CD38 is thought to be an important NAD-consuming enzyme. In steady state conditions, about 1% of NAD is turned into its cyclic form (48) (49).

4. Targeting NAD in cancer therapy and mechanisms of cell death associated with NAD metabolism

In this section, some particularities of cancer cells will be briefly presented in order to show specific mechanisms of the cancer cells in relation with NAD metabolism that could be potential target for drugs. Then, the two most important drugs leading to NAD+ depletion will be described in details with focus on their activity, effects and resistance. Finally, all the mechanisms known to rely on NAD+ and to be potential target to counteract the effects of cancer will be reviewed, and the consequences of their inhibition suggested, if not already tested.

a) NAD and energy metabolism in cancer cells

Tumor cells undergo metabolic changes in order to cope with their high-energy dependent proliferation. One of the most important metabolic change, the Warburg effect, is an important shift of metabolism that leads to an increased rate of glycolysis partially due to hypoxia inducible factor 1α (HIF-1α), whereas the rate of oxidative phosphorylation is reduced (50) (13) (Figure 10). This seems to be a paradox in aerobic conditions because glycolysis furnishes less ATP than oxidative phosphorylation. Nevertheless, this shift provides other advantages, such as providing more glucose in the pentose phosphate pathway for the synthesis of 5-phosphate ribose, a precursor of nucleotides for DNA duplication, and to generate NADPH, an indispensable antioxidant for tumor cells (51). Even if the mechanisms of the Warburg effect remain poorly understood, it is widely admitted that it is a crucial event in the development of tumors, and therefore a good target for chemotherapeutic drugs that could be achieved through NAD depletion (52). Indeed, NAD is involved in glycolysis by serving as the co-factors for the glycolytic enzyme GAPDH. Its depletion in tumor cells using NAD biosynthesis inhibitor, results in a blockade of glycolysis at the step of GAPDH. This leads to depletion of pyruvate, an important substrate for pyruvate dehydrogenase that produces acetyl coenzyme A into TCA cycle. Because depletion of acetyl coenzyme A starves the TCA cycle from its substrates, it also decreases production of ATP, which will ultimately result in ATP depletion and necrotic cell death (53) (54) (Figure 14).

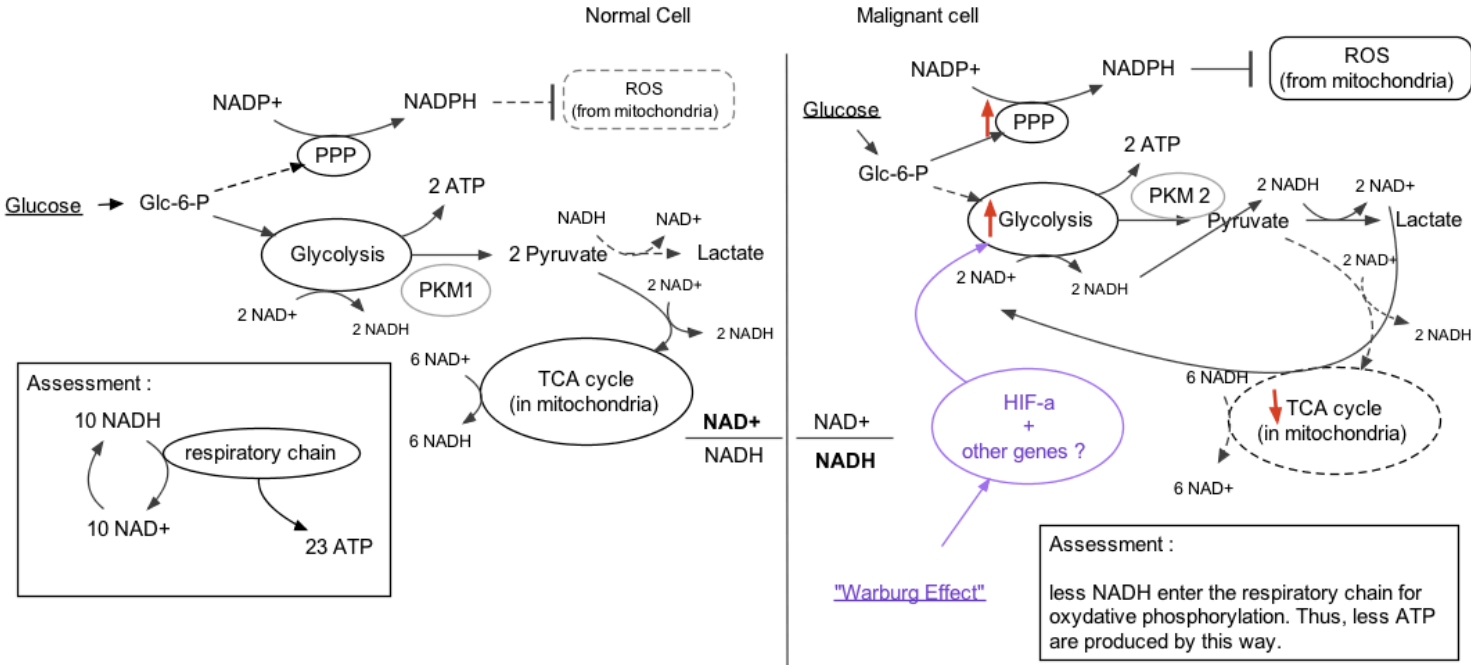

Figure 10. The Warburg effect. Adapted from reference (13). In normal cells, NAD+ reduction occurs in the glycolysis, with production of pyruvate, in oxidative decarboxylation of pyruvate into acetyl-coenzyme A and in tricarboxylic (TCA) cycle. Pyruvate mainly enters the TCA cycle in order to reduce more NAD+, which will increase ATP production by the respiratory chain. The NAD+/NADH ratio is kept high. Given that ROS are relatively low in healthy cells, a low reduction of NADP+ in the pentose phosphate pathway (PPP) is sufficient to maintain an optimal redox state in the cell. In malignant cells, the Warburg effect increases glycolysis and reduces the oxidative phosphorylation, probably via HIF-a and other genes. This induce more glycolytic substrate to enter the PPP pathway, in order to reduce more NADP+ and thus face elevated ROS, a specificity of cancer cells. Pyruvate is mainly reduced into lactate via lactate dehydrogenase, in order to re-oxidize NADH. This leads to a reduced NAD+/NADH ratio. Pyruvate kinases M (PKM1 and PKM2) may also play a role in the future of the metabolites of the glycolysis and will be discussed below. Glc-6-P : glucose-6-phosphate.

In addition, NADH creates reducing conditions that are favorable for many biological functions including electron transport chain, TCA cycle, beta-oxidation of fatty acids and conversion of pyruvate to acetyl coenzyme A. All these reactions would be perturbed by NAD depletion, leading to an inefficient performance of oxidative phosphorylation. This contributes to undesirable effects of NAD depletion, because healthy cells rely relatively more on these reactions than cancer cells. These, which are more dependent on glycolysis, will particularly suffer from ATP depletion by inhibition of glycolysis.

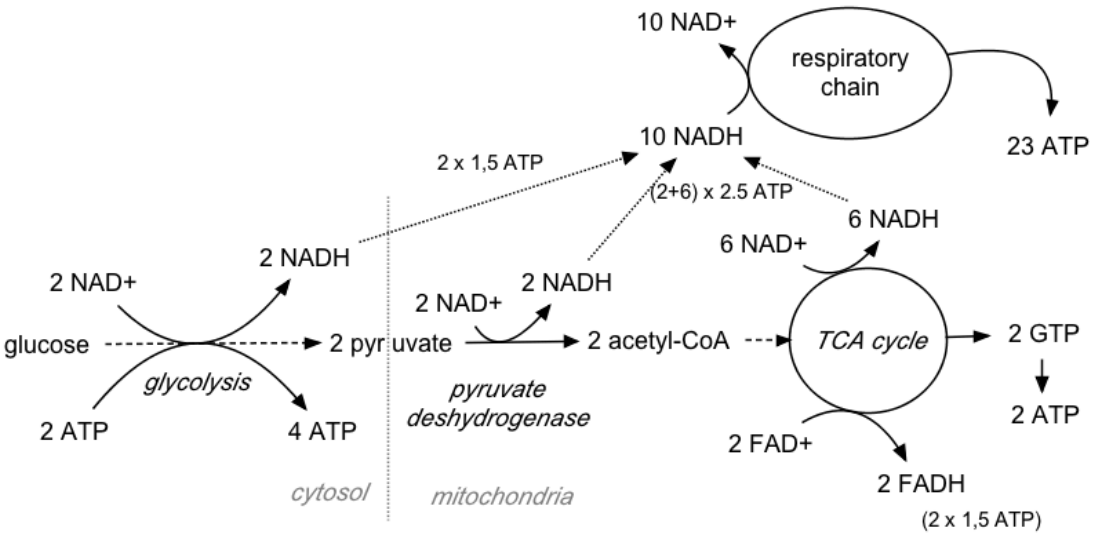

Figure 11. Reduction of one cytosolic NAD leads to 1.5 ATP, although reduction of one mitochondrial NAD leads to 2.5 ATP. Depletion of NAD blocks glycolysis, pyruvate dehydrogenase, the TCA cycle and the electron transport system, thus inhibiting both sources of ATP production: glycolysis and oxidative phosphorylation.

NAD is required for energy production in all cells, and particularly in tumor cells. This justifies research for drugs interacting with its metabolism.

b) NAD and oxidative stress cell defense systems

NAD is a key player in antioxidation and generation of oxidative stress through several pathways. NAD(P)H/NAD(P)+ cell contents are indispensable in numerous redox reactions. Cellular redox status is one of the main mechanisms involved in control and regulation of cell death pathways, including apoptotic, autophagic and necrotic processes (55). NAD+ is converted by NADK into NADP+, the precursor for NADPH formation (Figure 11). NADPH is one of the most important factors in cellular antioxidation through various processes and is generated from either NADP+ or NADH through different reactions that involve many cytosolic/mitochondrial enzymes (Figures 5 and 12) (56). In healthy cells, pyruvate-kinase M1 is mainly expressed. It has a high affinity for its substrate, phosphoenolpyruvate (PEP), and turns it into pyruvate, the final product of glycolysis. In cancer cells, pyruvate-kinase M2 (PKM2) is overexpressed, and PKM1 less expressed. PKM2 has a lower affinity to PEP, which results in lower levels of reactions. This leads to the accumulation of preceding metabolites of the glycolysis, which are driven into others metabolic pathways such as the PPP (57). Thus, by the expression of PKM2, more NADP are reduced in the PPP (Figure 12) (58). NADPH plays a central role in oxidative cell defense (Figure 14). It is indispensable for production of GSH from GSSG by glutathione reductase. GSH is crucial for activity of several essential anti-oxidant enzymes that comprise glutathione S-transferases and glutathione peroxidase. NADPH has an important role in antioxidant activity of the thioredoxin systems (59). NADPH has been reported to reactivate catalase when it is inactivated by excess of H2O2 (60). Paradoxically, NADPH can also generate ROS when serving as a substrate for NADPH oxidases, processes that play important roles in many biological

pathway and pathological situations (56). Targeting NADPH depletion trough inhibition of PPP or NAD biosynthesis has dramatic consequences for tumor cells since it results in excessive ROS production that is generally harmful because they react with cell components such as DNA, proteins and lipids ending up with cell death (61).

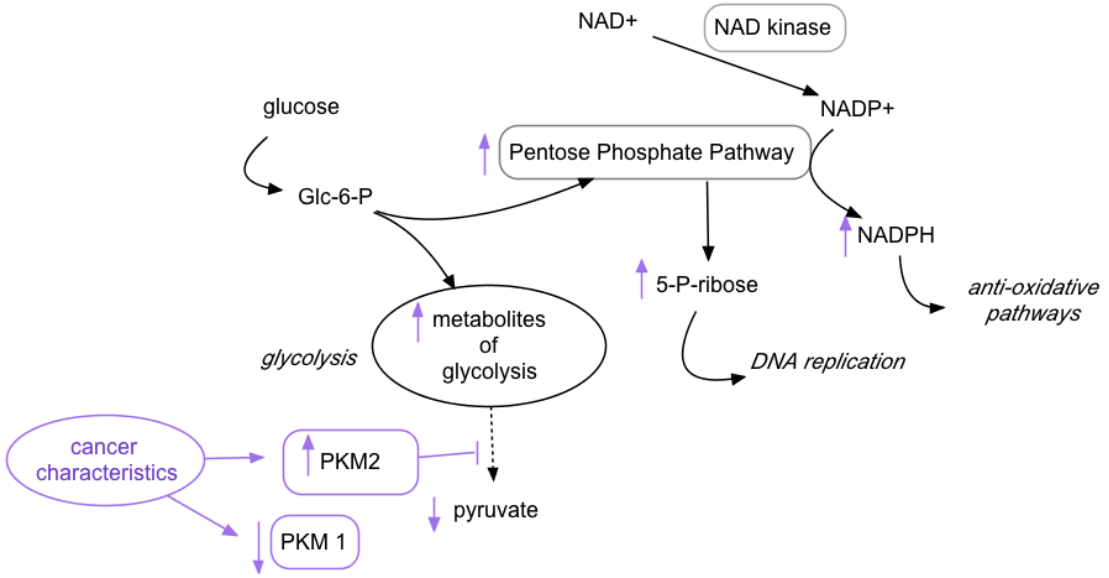

Figure 12. PKM2 is often increased in tumor cells, in order to bring more substrates of the glycolysis into the pentose phosphate pathway. This is due to a lower affinity of PKM2 for its substrate, PEP, than PKM1, which is expressed in healthy cells. Indeed, PKM2 has a higher K_M than PKM1. The benefits are production of 5-phosphoribose, a precursor of nucleotides, and reduction of NADP+. NAD depletion would lower the activity of NAD kinase and therefore counteract the efforts of the tumor cells to increase NADPH. Glc-6-P : glucose-6-phosphate.

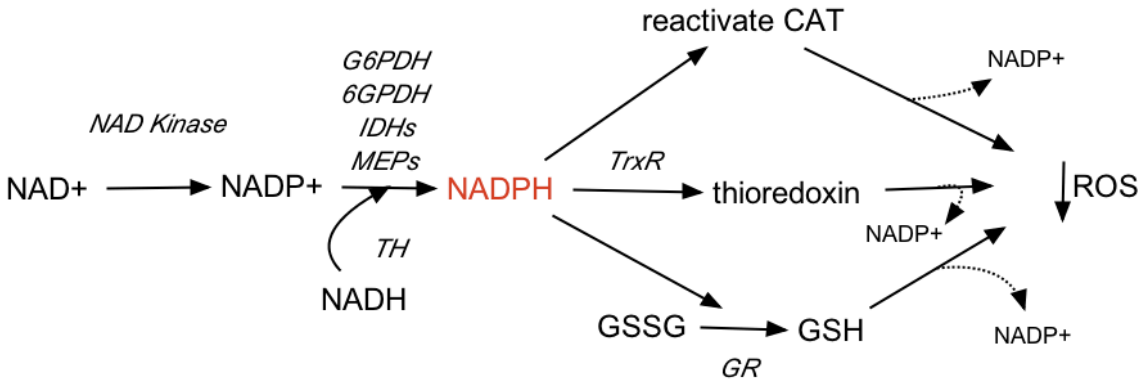

Figure 13. Adapted from reference (56). NAD kinases are essentials to produce NADP+, which is reduced by the following enzymes : glucose-6-phosphate dehydrogenase (G6PDH), 6-gluconate phosphate dehydrogenase (6GPDH), NADP-dependent isocitrate dehydrogenases (IDHs), NADP-dependent malic enzymes (MEPs), and transhydrogenase (TH). It enters one of the three anti-oxidative pathways by 1) reducing oxidized glutathione (GSSG) into reduced glutathione (GSH) thanks to glutathione reductase (GR), 2) reactivating H_2O_2-inactivated catalases and 3) regenerating thioredoxin via thioredoxin reductase (TrxR).

c) Inhibitors of NAD biosynthesis as potential antitumor drugs

During recent years, targeting cancer metabolism has emerged as a hot topic for drug discovery. Most cancers have a high demand for metabolic inputs (i.e. glucose/glutamine/NAD), which aid proliferation and survival. In this context, cancer cells have an increased need of NAD compared to normal cells, since most cancer cells have continuous PARP activation through DNA damage and genomic instability and have higher energy consumption demands (53). Thus tumor cells are expected to be more_vulnerable to NAD depletion than normal cells. To achieve this goal, enzymes involved in NAD synthesis are the best drug targets.

i. Inhibitors of nicotinamide mononucleotide adenylyltransferases

Inhibitors of the nicotinamide mononucleotide adenylyltransferases (NMNATs) are attractive drugs because there are three isoforms of NMNAT in humans, with designated nuclear, cytoplasmic or mitochondrial localizations. Specific inhibitors could therefore lead to reduced side effects compared to a pan-inhibiting molecule. Surprisingly, NMNAT have been shown to be depleted in tumor cells, compared to healthy cells, despite the increased need of NAD in tumor cells (62). Although NMNAT is an ideal chemotherapeutic target, there are few molecules developed and furthermore most of them such as gallotannin are only efficient in vitro (63). NMNATs are also known to activate NAD-depleting drugs such as tiazofurin, but research on this latter molecule and others related molecules were abandoned because of their high toxicity (64) (65) (13). Although they are promising agents with an interesting cell-localized action, efficient NMNAT inhibitors are still to be developed.

ii. Inhibitors of nicotinamide-adenine phosphoribosyl-transferase

In mammals, Nam is the most widely used precursor for NAD biosynthesis (13). NAMPT is the rate limiting enzyme that catalyzes the phosphoribosylation of Nam to produce NAD. Furthermore, the expression of NAMPT is upregulated in several cancers (66). Two forms of NAMPT have been evidenced: the intracellular form (iNAMPT) and the extracellular form (eNAMPT) (67). Whereas the role of iNAMPT has been well identified in the salvage pathway of NAD, the functions of eNAMPT are not clearly understood. The renewed interest in the potential anticancer benefit of targeting metabolism has led to development of several NAMPT inhibitors: APO866 (also known as FK866, or WK175), GMX1777 (prodrug of GMX1778, also known as CHS-828), CB30865, AS1604498, AS2292427, AS2334990, GNE-618, STF-118804 (68) (69) (70). Although these compounds have unrelated chemical structures, they are all specific and competitive inhibitors of NAMPT. Of note, the inhibitors of NAMPT have effects on inflammatory, angiogenic or hormonal functions, but these aspects will not be addressed in this review.

Data derived from the in-vitro and in-vivo studies indicate that NAMPT inhibitors exhibit mechanism-based efficacy against a wide range of human solid tumors and blood cancers (71) (72) (73). Preclinically, NAMPT inhibitors were tested on a variety of human tumor xenografts (7). Most xenografts showed

either tumor regression or stabilization after treatment. The mode of action of NAMPT inhibitors is under intense investigation. The molecular basis for the inhibitory activity of some of the compounds has been revealed by the crystal structures of its complex with human or rat NAMPT (67). These studies showed that NAMPT inhibitors are competitive inhibitors versus the nicotinamide substrate. Treatment of cancer cells with NAMPT inhibitors strongly decreases NAD cell content, which is followed by ATP decline, cytochrome c release, and ultimately cell death (73) (74). Cell death induced by these novel antitumor drugs occurs either in caspase-dependent or –independent manner, and involves ROS production, mitochondrial dysfunction, and autophagy (74) (75) (76) (77). The kinetic of these events and type of programmed cell death may dependent on tumor cell types. Evidence that cell death induced by NAMPT inhibitors relies only on NAD depletion is provided by the protective effects of nicotinamide in presence of these compounds (78) (79). Additional point of interest concerning the NAD depleting mode of action of NAMPT inhibitors is that infusion of nicotinamide or nicotinic acid within 72 hours of a lethal dose prevents death in all animals tested, indicating their potential as antidotes to protect patients from the drug side effects. Evidence of autophagic cell death induced by NAMPT inhibitors has been recently reported (80) (77). The regulation of autophagy-mediated cell death by NAMPT inhibitors (FK866) was evidenced and includes transcriptional-dependent (TFEB) and independent (PI3K/mTORC1) activations (Figure 14) (77). Although NAMPT inhibitors display tremendous antitumor activities on various malignant cells, resistance to these novel drugs has been reported recently. These are in-vitro induced resistance by culturing malignant cells with increasing concentrations of NAMPT inhibitors (GMX1778 and FK866) and they are associated with mutations which decrease the drug binding affinity without affecting that of Nam (79) (81). NAMPT inhibitors (FK866/GMX1778) are currently in phase I/II studies to assess their safety and tolerability for the treatment of various cancers including refractory B-CLL, metastatic melanoma, and refractory solid tumors or lymphoma (82) (70) (83)(84).

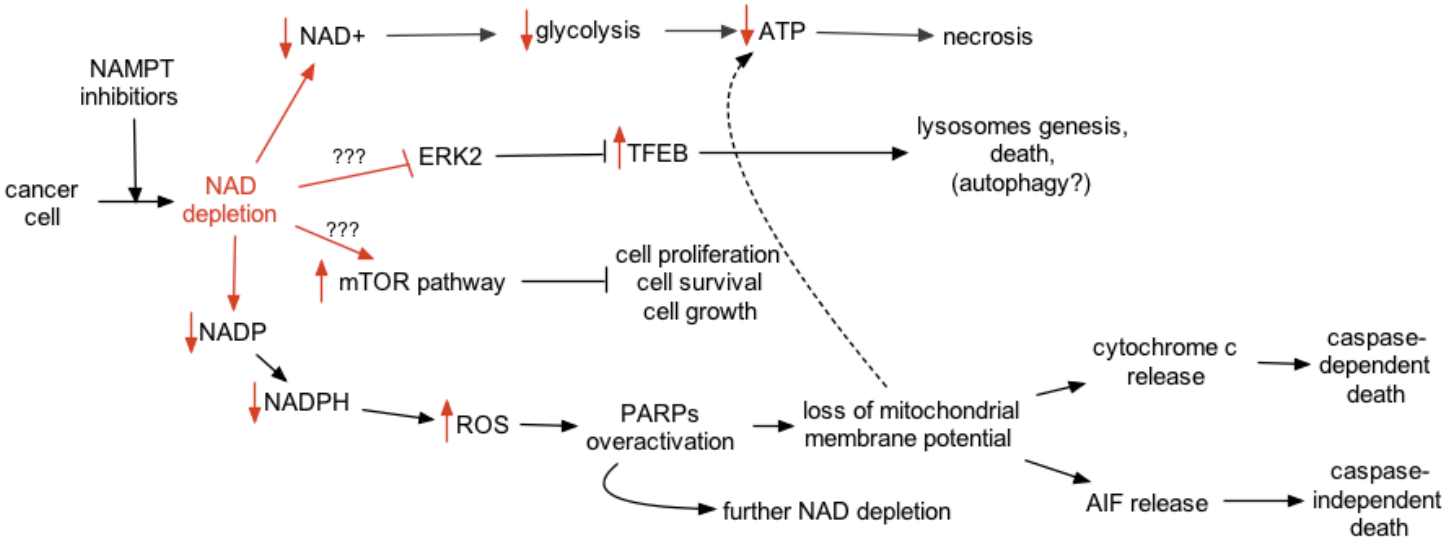

Figure 14. NAMPT inhibitors induce NAD depletion, with several consequences. First, depletion of NAD+ impairs TCA cycle and glycolysis, resulting in ATP depletion that might lead to necrosis. Second, NAD depletion seems to inhibit ERK2 and its inhibition on TFEB. TFEB can thus promote the genesis of lysosomes that might play a role in autophagic death. Third, NAD depletion induces

the mTOR pathway, which blocks cell proliferation, survival and growth. Finally, the loss of oxidative protection by NADPH results in increased concentrations of reactive oxygen species (ROS). As described below, in the PARP section, accumulation of ROS leads to DNA damage and subsequent PARPs overactivation. This overactivation disrupts the mitochondrial membrane potential, with two possible routes : a) cytochrome c release leading to caspase-dependent death or b) the release of the apoptosis-inducing factor (AIF) that leads to a caspase-independent death.

d) NAD, classical chemotherapeutic drugs and PARP-1 inhibitors

Classical chemotherapeutic drugs, such as alkylating agents (chlorambucil, cyclophosphamide, temozolomide, cisplatin…) have been reported to induce DNA damage and are efficient and widely used to treat various cancer types. DNA repair pathways following such injury induce hyper-activation of PARP-1. PARP-1 is known to be a NAD-dependent enzyme that intervenes in DNA-repair. It is often increased in cancer cells, because of the genomic activity and their high DNA-replication profile (53). Depending on the type or the severity of DNA damages, PARPs response might be reasonable or excessive. If the DNA-damage is mild, PARP-activation might lead to DNA repair and survival of the cell. If the DNA-damage is more important, PARPs-activation might be insufficient to cope with the breadth of the work: the cell reasonably activates the apoptosis pathway, with cleavage of the PARPs. Finally, in the case of immoderate DNA-damage, PARPs response might be excessive, with exaggerated activity, resulting in NAD+ depletion, followed by ATP depletion and necrotic cell death, because cells cannot undergo apoptosis in ATP depletion conditions (8). In addition, PARP1 hyperactivation produces poly-ADP ribose moieties that disrupts mitochondrial membrane potential and provokes release of apoptosis-inducing factor and cytochrome c, thus leading to caspase-dependent and independent apoptosis (figure 14) (85). Mechanism of AIF induction by PARP-1 remains unclear but was recently reproduced by use of alpha-eleostearic acid (86) (87).

On the other hand, inhibition of PARPs activity has two further consequences (Figure 15). In the case of mild DNA-damage, it drives the cell into the apoptotic route instead of survival, because of the inefficiency of PARPs activity. In the case of excessive DNA-damage, PARPs inhibition prevents the immoderate consumption of NAD+ that leads to ATP drop and necrosis. It also drives the cell into the apoptosis route (8). Since PARP inhibitors have been discovered, there have been more investigations on them than on PARP inhibition via NAD+ depletion. PARP-1 inhibitors are used in combination with DNA-damaging agents such as temozolomide, cisplatin or ionizing radiations to potentiate their effects (88). They have entered clinical trials. In tumors that miss other DNA-repair functions, such as BRCA-1 and BRCA-2 tumors, monotherapy of PARP-1 inhibitors seems to be an efficient and promising cancer treatment (Figure 16) (89) (90). PARPs are promising drug targets with various effects in several diseases. PARP-1 inhibitors have shown efficacy in tumor cells, either with DNA-damaging agent or in monotherapy, according to the kind of tumor. These two subjects warrant further investigations in order to completely use the resources of this target and require also a better understanding of the mechanisms leading to cancer to select patients who would benefit most of these drugs.

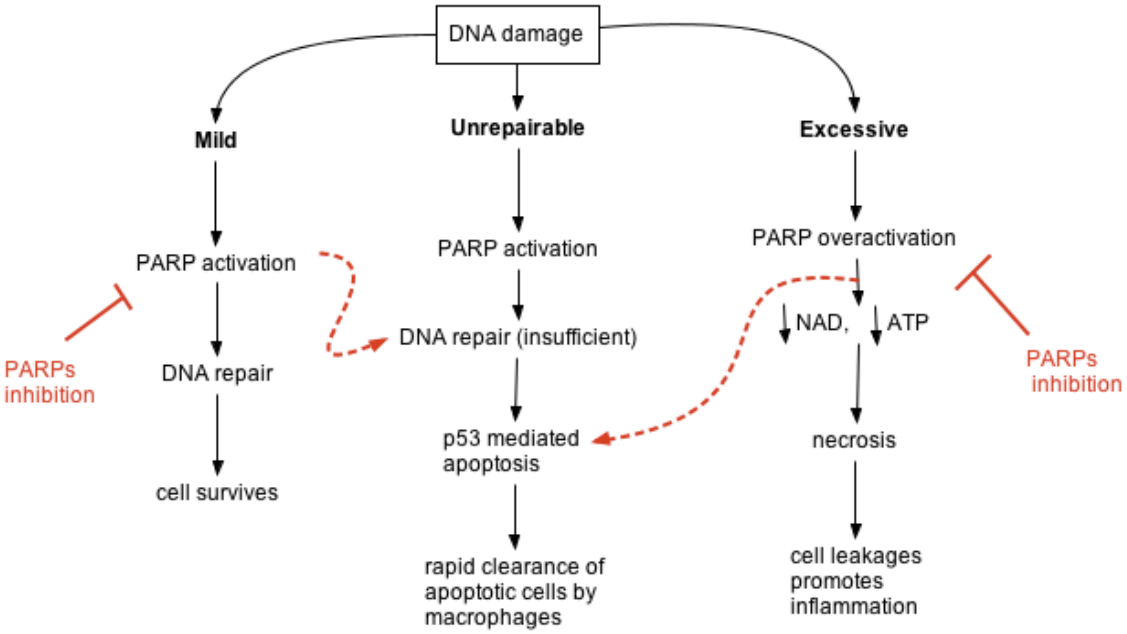

Figure 15. DNA damage may lead to three different outcomes, according to the severity of the insult. If it is mild, PARP activation leads to a normal function with DNA repair and survival of the cell. If the damage is too big for the PARP to cope with it, the insufficient repair of DNA is recognized and the p53-mediated apoptosis activated, leading to elimination of the cell, with no perturbation for the environment. If the DNA-damage is excessive, an overactivation of PARP may deplete NAD, and thus ATP stores, with an ineluctable drive into necrosis, including promotion of inflammation. PARPs inhibitors may drive both the mild and the excessive DNA damage into the same final outcome as unrepairable DNA-damage, leading to a "clean" death by apoptosis. This is an interesting outcome to help mild DNA-damaging agents to induce cell death in tumors.

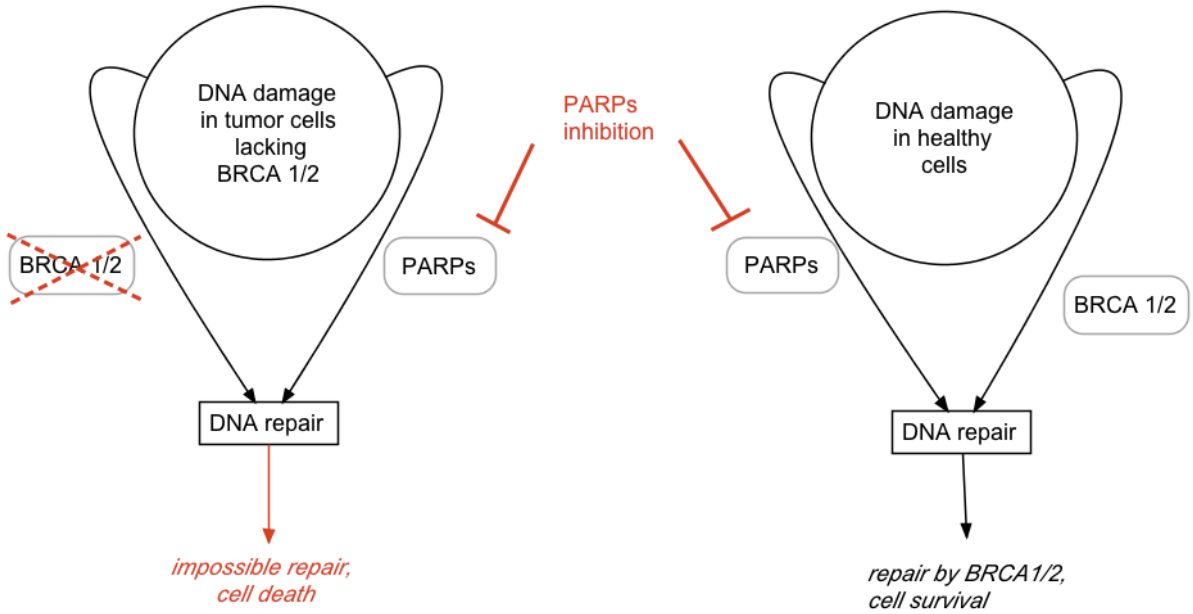

Figure 16. PARPs inhibition would impair PARP-DNA-repair function in every cell of the organism. Nevertheless, BRCA1/2 is another way to repair DNA damage, and can then rescue healthy cells from DNA-damage. In BRCA ½ deficient cancer cells, this pathway is inefficient, leading to no ability for these cells to be rescued from DNA insults.

e) NAD and sirtuins

The mammalian sirtuins (SIRT1–7) are a family of enzymes with several functions (such as NAD+-dependent lysine deacetylase, desuccinylase, deacylases, demalonylases and ADP-ribosyl transferase) that play several and sometime opposite roles in cancer cells and tumorigenesis (see references (91) and (92)). Sirt1 is the most studied member of sirtuins. Beside the role of SIRT1 in circadian clock and lifespan, it has DNA-repair, transcription and genomic stability functions. In human cancers, no deletion neither mutation of SIRT-1 have been found, but rather an overexpression. In neoplastic conditions, SIRT-1 is enhanced together with NAMPT expression to maintain its efficacy (93) (94). In contrast, in mice, overexpression of SIRT-1 is able to reduce cancer formation (38) (95). Increased expression of SIRT-1 may confer a better DNA-repair function that is required due to the high ROS concentrations that can damage DNA in tumor cells (96). Furthermore, this DNA repair mechanism is thought to be less efficient than in normal conditions, leading to the accumulation of new mutations. It has been proposed that this genomic instability is beneficial for the cell in two ways: (i), it drives the cell into a more malignant profile and (ii), it may confer new resistance to the drugs (97). SIRT-1 acts in tumorigenesis through different ways (shown in Figure 17):

(a) Sirt1 deacetylates and therefore inactivates p53, a tumor-suppressor gene that induces apoptosis in the case of DNA lesions. Loss of this function confers a power to the cancer cells to proliferate without being arrested by DNA-lesions (98) (99). Abrogation of this mechanism in SIRT-1-/- mice was able to sensitize cancer cells to imatinib (100).

(b) SIRT-1 deacetylation is also responsible for the activation of Ku70, which subsequently sequestrates the Bcl-2 pro-apoptotic factor Bax into the mitochondria, leading to inhibition of the apoptosis (101). In leukemia cells, inhibition of SIRT-1 results in apoptosis *in vitro* and suppression of tumor growth in xenograft tumors *in vivo* (102).

(c) BCL6 is a transcriptional regulator, which inhibits apoptosis and differentiation of cells in lymph nodes (103). It is activated by the SIRT-1 deacetylation (104). Inhibition of SIRT-1 counteracts lymphoma attributes (105).

(d) SIRT-1 can increase the expression of multidrug resistance gene *mdr1*, coding for a P-glycoprotein, a pump that plays an important role in multidrug resistance, which confers resistance to chemotherapy, including doxorubicin. (106)

(e) SIRT-1 can function as an activator of hypoxia-induced factor 1 (HIF1), which is expressed in hypoxic conditions, to increase angiogenesis and glucose uptake (92). In this regard, inhibition of SIRT-1 prevents this metabolic shift that takes part in the Warburg effect (107) (108).

(f) and finally, Acetyl-CoA synthetases is activated by SIRT-1. It converts free acetate into acetyl-CoA that may enter the TCA cycle to improve metabolism (109) (110).

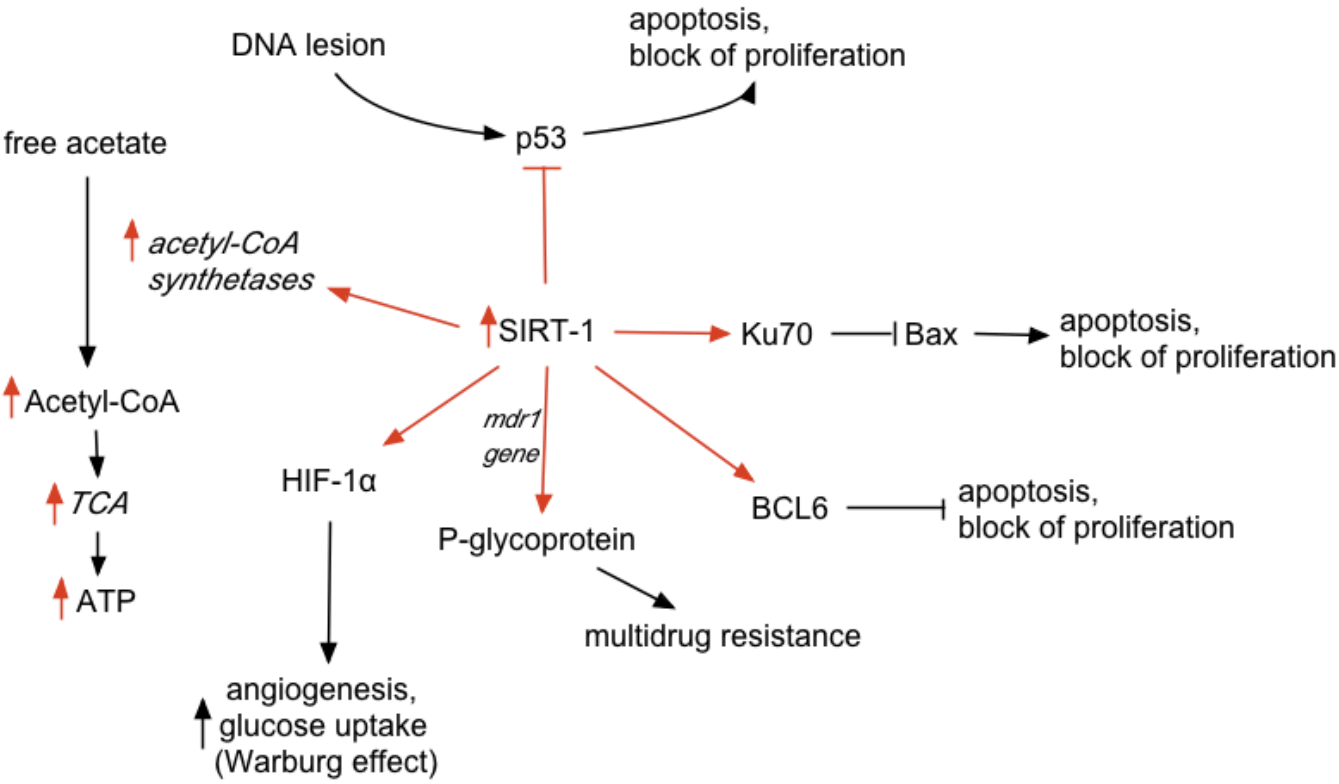

Figure 17. a) SIRT-1, which is increased in some types of cancer cells, inhibits p53 in order to enhance proliferation even in the case of DNA lesions. b) SIRT-1 may increase the activity of Ku70 in order to block Bax activity and thus avoid apoptosis of the cell. c) SIRT-1 activates BCL6 and thus inhibits apoptosis, in order to permit cell proliferation. d) SIRT-1 activates P-glycoprotein via expression of the gene mdr1, which provokes an efflux of the some drugs out of the cell. e) SIRT-1 activates hypoxia-induced factor 1 (HIF1), a transcription factor leading to increased angiogenesis and increased glucose uptake for the cell supply. HIF-1 might be one of the important gene of the Warburg effect. f) Free acetate may be turned into acetyl-coenzyme A (Acetyl-CoA) by Acetyl-CoA synthetases, in order to furnish more substrates to the TCA cycle, which leads to production of energy (ATP) for the cell. SIRT-1 activates acetyl-CoA synthetases.

Interestingly, it has been shown that depletion of SIRT-1 could increase PARP-1 activity after DNA-damage, leading to an AIF-mediated cell death (111). This observation supports the importance of the interactions between NAD-dependent enzymes.

Emerging pathways of angiogenesis and metastasis may also include sirtuins in their cascade, and some of them are presented in reference (92). Furthermore, investigations on others sirtuins (SIRT-2 to SIRT-7) may bring interesting clarification of their roles and the consequences of their inhibition. The many roles of SIRT-1 in cancer make it a good drug target. Although sirtuins-inhibitors are only few (tenovin-6 and cambinol are the most important, both acting on SIRT-1 and SIRT-2), it is believed that the understanding of sirtuins and the

research of their inhibitors will be of particular interest in the future (105) (112).

f) NAD and CD38

CD38 is an important NAD-ases in cells, and might be potential targets in cancer cells. Although it has been revealed that NAD concentrations depend more on PARP in stressed or tumor cells than on CD38, it is thought that CD38 could change the activity of sirtuins by modulating NAD concentrations (113). Two CD38-inhibitors have been discovered (quercetin and apigenin) and benefit of treatment with these molecules could be found for metabolic diseases or parasitic infections, but no profit has been found in cancer treatment yet (113) (114).

Conclusion

NAD is an indispensable small molecule that plays many important cellular functions. Targeting this sole element impairs many biological reactions, as they are many to use it as fuel. In addition, given its important roles, its expression is increased in cancer cells, which require many adaptations in order to face intense proliferation. The increased expression of enzymes involved in NAD biosynthesis reveals the importance for these cells to rely on NAD and suggests that NAD depletion could harm them. The goal of this work was to review the known processes in which NAD participates and that could impair development of cancer cells. NAD depletion affects variably many cellular processes: NAD depletion grossly disturbs the energy metabolism of the cell, that require the redox function of NAD. More specific are the effects of NAD depletion on NAD-consuming enzymes, whose functions are indispensable in cancer cells. PARPs and sirtuins functions are beneficial for the cell survival, and targeting their actions by direct inhibition or through NAD-depletion would be detrimental to tumors.

Inhibitors of NAMPT are efficient at inducing an important NAD depletion, and are currently under intense development. They seem to be promising agents for several reasons : (i) malignant cells have an increased metabolism, partially relying on NAD and NADP; (ii) they show a higher level of NAMPT, which is a rate-limiting enzyme of NAD salvage and thus a regulator of important enzymes such as PARPs and sirtuins ; (iii) this augmentation is typical of proliferating cells and do not rely on specific mutations, allowing an efficacy on a wide range of tumors; (iv) it is easy to evaluate, via a needle-based biopsy, markers of efficacy of the drug in the target cells, such as the metabolites of glycolysis (53); (v) nicotinic acid or nicotinamide may be potent antidotes. As every rose has its thorn, these inhibitors show also undesirable effects. For instance, targeting an enzyme present in healthy cells has adverse effects on normal proliferating cells, such as hematopoietic cells. Thrombocytopenia and lymphopenia are unwelcome events in cancer patients and limit the recommended dose. However, these effects can be sought in inflammatory diseases, such as experimental autoimmune encephalomyelitis (115). Pharmacokinetics is another issue in the utilization of these drugs. Each inhibitor holds a pyridine ring to resemble NAD, and this ring is thought to be the cause of the rapid clearance of these drugs. Research presently seeks for novel inhibitors, in which the pyridine ring is converted into another heterocyclic ring in order to diminish the clearance of NAMPT inhibitors (70). Studies on the effects of these drugs have to be multiplied.

Whilst the effects of NAMPT inhibitors on xenograft tumors or *in vitro* studies are promising, the results in clinical trials were not as convincing as expected. Just a few patients got a stabilization of their tumor, and toxic effects were numerous. Still, the combination with DNA-damaging agents seems and is thought to provide synergistic effects, namely more than the addition of the effect of each drug. It seems that their effects would likely potentiate the ones of others cancer treatments, such as chemotherapy or radiotherapy. Few studies have focused on combinative therapies at the moment, and clinical trials with such combinations are now undertaken.

The potential of NAD-depleting agents is still growing, as new implications in cell metabolism and novel inhibitors are continuously discovered. The comprehension of these numerous implications warrant further studies, and probably years, if not decade, to realize and take advantage of the promising potential of this domain.