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List of non-standard abbreviations:

2-DG-6-P: 2-deoxyglucose-6-phosphate
2-DG: 2-deoxy-D-glucose
ADP: Adenosine 5'-diphosphate
AMP: Adenosine 5'-monophosphate
AMPK: 5'-AMP activated protein kinase
ATP: Adenosine 5'-triphosphate
ER: Endoplasmic reticulum
FDG: 2-fluoro-2-deoxy-D-glucose
GDP: Guanosine 5'-diphosphate
GLUT: Glucose transporter
HAD: haloacid dehalogenase
HXT: hexose transporter
MAP kinase: Mitogen-activated protein kinase
mTORC1: Mammalian target-of-rapamycin complex 1
PET: Positron emission tomography
PP1: Protein phosphatase 1
PPP: Pentose phosphate pathway
UDP: Uridine 5'-diphosphate
UPR: Unfolded protein response

Abstract.

Most malignant cells display increased glucose absorption and metabolism compared to surrounding tissues. This well-described phenomenon results from a metabolic reprogramming occurring during transformation, that provides the building blocks and supports the high energetic cost of proliferation by increasing glycolysis. These features led to the idea that drugs targeting glycolysis might prove efficient in the context of cancer treatment. One of these drugs, 2-deoxyglucose (2-DG), is a synthetic glucose analog that can be imported into cells and interfere with glycolysis and ATP generation. Its preferential targeting to sites of cell proliferation is supported by the observation that a derived molecule, 2-fluoro-2-deoxyglucose (FDG) accumulates in tumors and is used for cancer imaging. Here, we review the toxicity mechanisms of this drug, from the early-described effects on glycolysis to its other cellular consequences, including inhibition of protein glycosylation and endoplasmic reticulum stress, and its interference with signaling pathways. Then, we summarize the current data on the use of 2-DG as an anti-cancer agent, especially in the context of combination therapies, as novel 2-DG-derived drugs are being developed. We also show how the use of 2-DG helped to decipher glucose-signaling pathways in yeast and favored their engineering for biotechnologies. Finally, we discuss the resistance strategies to this inhibitor that have been identified in the course of these studies and which may have important implications regarding a medical use of this drug.

1. A short history of the use of 2-deoxyglucose

Oncogene activation, tumor suppressor loss, and other stresses are believed to shift cancer cells towards an increased glycolytic metabolism, with a decreased contribution of respiration and oxidative phosphorylation [1]. These metabolic features may allow the development of anticancer treatment and glycolytic inhibitors have been, and are still, investigated for this purpose [2]. One of the most widely studied glycolytic inhibitors is 2-deoxyglucose (2-DG), a synthetic glucose analog in which the hydroxyl group at the second carbon atom is replaced by hydrogen (**Figure 1**).

This drug has been studied for almost 70 years, and in this review, we will detail how it has contributed to many aspects of biomedical research, being used as a surrogate for glucose in uptake assays, as an enzyme inhibitor, a metabolic blocker and ATP-depleting agent, and much more. We will see that 2-DG has contributed to a better understanding of metabolism in a variety of organisms, and we will particularly emphasize its importance in the delineation of glucose-signaling pathways in yeast and fungi and its use in strain improvement for biotechnologies. However, contrasting with its simple structure, we will see that 2-DG has many effects in the cell that go beyond a simple block of glycolysis. This is because 2-DG displays some structural similarities with mannose (**Figure 1**), and as such, also competes with its metabolism and affects the corresponding cellular processes such as protein and lipid glycosylation.

We will summarize the potential of 2-DG as an anti-cancer agent as concluded from past and recent studies, as well as the use of other 2-deoxy derivatives of glucose, such as 2-fluoro-2-deoxy-fluoroglucose (FDG, **Figure 1**) which is used for cancer imaging and diagnosis. Excellent reviews on the toxicity of 2-DG in the context of mammalian cell homeostasis and cancer were previously published [3,4]. Finally, we will discuss the various resistance mechanisms that have been described in the literature over the years, highlight what these tell us about the cellular toxicity of 2-DG, with important implications to its potential use as an anticancer agent.

1.1 2-DG as a tool to interfere with glucose metabolism and cell proliferation

Because of the structural similarities between 2-DG and glucose (**Figure 1**), it was expected that 2-DG would act as a competitive inhibitor of glucose metabolism and thus might strongly affect the growth of cells heavily dependent on glucose, such as cancer cells running aerobic glycolysis. As such, it was one of the first compounds and probably the most widely studied for its interference with tumor metabolism.

As early as 1952, Woodward and Cramer reported an effect of 2-DG on the proliferation of rat Walker 256 mammary carcinoma cells [5]. An inhibitory effect on the growth of embryonic chicken heart fibroblasts was also noted [6]. In parallel, the effect of 2-DG on yeast growth, metabolism [7], and glucose fermentation [8] were also investigated, leading to the conclusion that 2-DG competes with glucose for its metabolism. Other carbohydrate analogs were tested for their effect on fermentation [9] but only those with a substitution at their C2 position inhibited anaerobic fermentation, with 2-deoxyglucose being

the most potent. Further work confirmed the competitive nature of 2-DG over glucose utilization, both in normal tissues and tumors [10] and Krebs ascites carcinoma cells [11]. The fact that 2-DG blocks glucose absorption in response to insulin in extrahepatic tissues without evidence of subsequent metabolism, and the competing nature of its effect over glucose further contributed to establishing 2-DG as a blocker of glucose metabolism [12]. This was confirmed by observations made in animals, in which the administration of 2-DG led to an increased blood glucose concentration, suggesting competition with endogenous glucose distribution and metabolism [13]. These studies were soon translated to cancer patients, as early as 1958, with similar observations [14]. An inhibitory effect of 2-DG on tumor growth was noted *in vitro* [15] and in a transplantable tumor in mice [16]. This has generated a lot of interest regarding the possible use of this molecule as an anti-tumor agent.

1.2 Use of 2-DG to decipher how glucose regulates gene expression in yeast

The yeast *Saccharomyces cerevisiae*, also named budding yeast or baker's yeast, has been exploited for thousands of years by humans for their ability to ferment beverages and their action on the leavening of dough. Nowadays, it is also an important tool for the generation of biofuels using byproducts from various industries. Budding yeast and several other yeast species have a peculiar metabolism that favors the use of glucose (or fructose) over alternate carbon sources, likely resulting from their evolution in plant-based ecological niches [reviewed in 17]. Even in the presence of oxygen, yeasts mostly get their energy from glycolysis with only a limited contribution of respiration (Crabtree effect). This is because glucose represses the expression of genes involved in respiration and oxidative phosphorylation at the transcriptional level [for review, see 18]. As described for cancer cells, the strategy of glycolysis-based proliferation relies on a fast glucose uptake from the medium, and this could provide yeast a competitive advantage relative to other species that consume glucose less aggressively.

The generated by-product of glycolysis, pyruvate, is then converted to ethanol by fermentation. This apparent waste of energy, since more ATP would be generated per molecule of glucose if it were to be respired rather than being fermented, is believed to provide several ecological advantages [17]. First, it is not a waste of energy, but merely a temporal separation of glycolysis from respiration, since once glucose is exhausted from the medium, genes involved in respiration are expressed and cells can then start using ethanol for growth (diauxic shift). Ethanol degradation begins with the oxidation of ethanol to acetaldehyde (by an alcohol dehydrogenase), which is then converted into acetate (by an aldehyde dehydrogenase) and then acetyl-CoA (by acetyl-CoA synthetase) to feed the Krebs cycle [17]. Second, the ability to convert the energy contained in pyruvate into a chemical (ethanol) that is poorly metabolized by other species provides an opportunity to restrict access to these stores. Finally, ethanol accumulation prevents the thriving of other organisms that are sensitive to this compound, thus limiting competition in the wild.

A better understanding of how glucose regulates gene expression in yeast was driven both by a need to understand the mechanism of gene regulation at a fundamental level [19], but also because of the need to ameliorate carbon source usage of various yeasts (eg. starch, xylose) [20–22], for industrial needs such as brewing [23,24] or dough leavening [25], or to ameliorate the expression of enzymes of industrial interest that are sometimes glucose-repressed [26,27].

2-DG has been used as a tool to identify genes involved in the glucose repression pathway because 2-DG activates this pathway and thus prevents the use of alternative carbon sources [28–30]. The characterization of mutants able to use raffinose or sucrose as a carbon source despite the presence of 2-DG, which would normally prevent their utilization, led to the identification of several actors involved in glucose repression [19,31,32] that will be described later in this review. In addition to revealing critical actors of the glucose-repression pathway, these experiments and others pointed out to the importance of the yeast hexokinase in mediating 2-DG toxicity [33] (see also part 5. on the mechanisms of resistance).

2. Direct metabolic effects of 2-DG and consequences

2-DG, by affecting hexose metabolism, inhibits cell growth by multiple mechanisms, and work over the last 60 years delineated its various impacts on cellular physiology, although additional aspects likely remain to be discovered. Particularly, 2-DG has different effects depending on the metabolic context, and seminal work from the Lampidis lab demonstrated that 2-DG has various toxicity mechanisms depending on the metabolic state of tumor cells and their accessibility to oxygen [34], as detailed later in this review. This has important consequences in tumor biology since tumors are heterogenous populations made of oxygenated, proliferating cells as well as hypoxic, slow-growing cells, the latter being more difficult to target by conventional therapies. Similarly, in yeast, 2-DG seems to have differential effects depending on whether cells are growing in the presence of abundant or limiting amounts of glucose (or alternative carbon sources) [35]. The effects of 2-DG on early steps of glucose metabolism are presented in **Figure 2**, and an overview of the cellular consequences of these inhibitions is depicted in **Figure 3.** Individual aspects of these inhibitions will be discussed in the following sections.

2.1 Phosphorylation of 2-DG and its impact on glycolysis and the pentose phosphate pathway, and the generation of ATP

Like glucose, 2-DG uses hexose transporters to be transported across the cell membrane. In yeast, glucose competed with 2-DG for import, suggesting they use similar transport systems [36]. In mammalian cells, both sugars also compete for their uptake and their import responds to insulin, suggesting that they use the same transport systems [13]. The properties of 2-DG uptake, as determined in intestinal epithelial cells or Caco-2 cells, indicate transport by glucose transporters of the GLUT/SLC2 family [37–39] and 2-DG transport correlate with GLUT expression in various cell lines [40]. For this reason and the fact that it

shows very limited metabolism (see below), radio-labeled 2-DG is often used as a surrogate substrate in glucose uptake assays.

Both glucose and 2-DG accumulate intracellularly by a concentrative uptake system depending on their phosphorylation [41]. After incubation of muscle cells from rat diaphragm with 2-DG, there was no evidence for free 2-DG accumulating inside the cells, suggesting that the 2-DG phosphorylation rate is higher than that of its transport [37,42]. Free 2-DG was not detected in adipocytes shortly after treatment, but became detectable after 1h, probably due to the depletion of ATP caused by 2-DG phosphorylation (see next paragraph) which limits the hexokinase reaction [43]. In this case, 2-DG reached an intracellular concentration similar to the concentration of the medium, in agreement with the hypothesis of a transport by passive diffusion which should lead to an equal concentration on both sides of the membrane at the equilibrium.

Effect of 2-DG on glycolysis. 2-deoxyglucose can be phosphorylated by yeast, muscle, and brain hexokinase [8,44–46] into 2-deoxyglucose-6-phosphate (2-DG-6-P) (**Figure 2**). This species is the main 2-DG-derived species accumulating in yeast cells [47], and similarly, in adipocytes, 2-DG-6-P accounts for most if not all of the 2-DG-derived species shortly after import [43]. There is no evidence for further metabolism of 2-DG-6-P in glycolysis (**Figure 2**), as it is neither a substrate of phosphoglucose isomerase (which would require a hydroxyl group in position C2 of glucose) nor of glucose-6-phosphate dehydrogenase [42,44] although the existence of small amounts of 2-DG-1-phosphate and 2-DG-1,6-bisphosphate species was reported in yeast [48] and rat brain [49], suggesting that 2-DG-6-P may be a substrate of phosphoglucomutase. Therefore, 2-DG phosphorylation consumes ATP to generate 2-DG-6-P but cannot continue to the energy payoff phase of glycolysis.

Moreover, 2-DG prevents the phosphorylation of other available sugars such as glucose [50]. Early work in rat tumors suggested that 2-DG treatment blocks glycolysis at the hexokinase step, supported by the fact that 2-DG inhibits glycolysis more than fructolysis (given that hexokinase activity in the brain and in yeast has a better affinity towards glucose than towards fructose) [10]. This was further confirmed by studies showing that 2-DG acts as a non-competitive inhibitor of hexokinase [51] and as a competitive inhibitor of phosphoglucose isomerase [45] (**Figure 2**). These data are in agreement with a more recent study showing that fructose-6-phosphate was almost not detected in a 2-DG-treated cell line, accompanied by a sharp decrease in all downstream glycolytic intermediates [52]. Moreover, its lack of metabolism leads to the accumulation of 2-DG-6-P in cells, and initial measurements proposed that the amount of 2-DG-6-P reaches up to 80-fold that of glucose-6-phosphate [47], likely bolstering its inhibitory effect on glycolysis. Note that 2-DG phosphorylation was also reported to induce a depletion in the free phosphate pool [36], further impairing ADP-rephosphorylation and ATP-dependent processes [53,54]

All of these features have led to the widespread use of 2-DG to simulate nutrient or energy (ATP) depletion in cell culture, as well as its use as an ATP "trap" *in vitro* (in combination with hexokinase) [55]. This was also observed in the context of tumors [56]. However, the next sections will show that ATP depletion is only one of the cellular effects of 2-DG. The addition of pyruvate in the medium was shown to attenuate the 2-DG-induced decrease in cellular ATP and was used to discriminate between the effects of 2G on cellular energetics vs. other effects [57,58]. This result further demonstrates that mitochondria are functioning and are not necessarily defective in tumor cells.

Interactions of 2-DG with the pentose phosphate pathway (PPP).

Evidence supports the idea that 2-DG can partially enter the PPP by which 2-DG-6-P is converted to 6-phospho-2-deoxygluconate by glucose-6-phosphate dehydrogenase, thus providing an important source of NADPH [59,60] (**Figure 2**) which is a source of reducing equivalents for the glutathione system [61]. In this respect, 2-DG treatment was shown to rescue cells from glucose starvation-induced cell death by restoring tolerable levels of reactive oxygen species [59]. However, because 2-DG-6-P also inhibits glucose-6-phosphate dehydrogenase itself, this may only occur under a certain 2-DG-6-P concentration [62]. The 6-phospho-2-deoxygluconate generated can probably not be further metabolized in this pathway as it is a poor substrate of 6-phosphogluconate dehydrogenase [63].

Extrapolating on the inhibition of glycolytic enzymes by 2-DG-6-P, one could expect that 2-DG interferes with the PPP. Inhibition of glycolysis after the hexokinase step can lead to an increased flux of glucose-6-phosphate into the pentose phosphate pathway (PPP). Indeed, it was reported in both yeast or fibroblasts that 2-DG treatment led to the accumulation of erythrose-4-phosphate, an intermediate of the nonoxidative branch of the PPP [64] which is also a competitive inhibitor of phosphoglucose isomerase [65]. A metabolomics study examining the effects of 2-DG on primary metabolism in a human endometrial carcinoma cell line revealed that 2-DG indeed led to an increased concentration of PPP metabolites [52] (**Figure 3**). Importantly, in the course of this study, the authors identified 20 peaks that were specific of 2-DG-treated cells and differed from untreated cell metabolites by the mass of an oxygen atom, suggesting that many other 2-DG-derived metabolites remain to be characterized.

2.2 UDP-2-DG and the interference of 2-DG with structural and storage carbohydrates as well as glycosphingolipids

Initial observations reported that the inhibitory effect of 2-DG on yeast growth was much higher than its effect on glycolysis and fermentation [9], suggesting an additional growth inhibitory mechanism which still required phosphorylation of 2-DG [66] (**Figure 3**). Interestingly, this did not occur in a medium devoid of an additional carbon source, and stationary phase cells were less affected than exponentially growing cells suggesting a structural defect occurring during cell division [35,66].

The cell wall of yeasts and other fungal cells are composed of carbohydrate-containing layers providing rigidity and resistance towards many stresses [67]. Exposure of growing fission yeast *Schizosaccharomyces pombe* to 2-DG led to more fragile cell walls, leading to their lysis at their zones of growth [68], and this was also observed in two species of budding yeasts [69]. In all three species, lysis happened in the main regions of β-glucan synthesis, suggesting interference of 2-DG with this process. In the pathogenic yeast *Candida albicans*, 2-DG was found to incorporate into β-glucan polymers of the cell wall and alters their stability [70]. Accordingly, a yeast-specific MAP kinase-based signaling pathway that responds to cell wall damage is activated in response to 2-DG treatment, suggesting that 2-DG triggers perceivable damage to the cell wall structure [71]. Proof of the existence of UDP-2-DG [72] and GDP-2-DG in 2-DG-treated yeast were later provided, suggesting interference with cell wall synthesis either through trapping of cellular uridine- or guanosine-nucleotide pools or by competitive inhibition with endogenous precursors [48,73]. Incorporation of 2-DG in storage carbohydrates, such as glycogen (both *in vitro* and *in vivo*) [74,75] or trehalose were also reported [41,76]. UDP-2-DG was found to be a substrate for purified yeast, calf liver, and mung bean UDP-glucose 4-epimerase, raising the possibility that UDP-2-deoxygalactose could be generated *in vivo* [77]. UDP-2-DG and GDP-2-DG were also detected in chick embryo fibroblasts incubated with 2-DG [60]. This suggests that 2-DG might alter other biosynthetic pathways in animals involving UDP-Glc and GDP-Glc, such as protein glycosylation (see next section) but also glycogen metabolism [78] (**Figure 3**).

Another such pathway is the biosynthesis of glycosylated lipids. Early studies in yeast [79] and hamster embryo fibroblasts [80] revealed that 2-DG can incorporate into glycolipids. The addition of glucose to a ceramide molecule is catalyzed by a Golgi-associated cytosolic enzyme named glucosylceramide synthase, which also uses UDP-glucose as a glucose donor. Glucosylceramide can be used as a precursor for further glycosylation events, leading to a variety of glycosphingolipids [for review, see 81]. Indeed, 2-DG incorporates into the carbohydrate moiety of newly synthesized glycosphingolipids [57,82] (**Figure 3**), but it is not yet clear whether the 2-DG-modified versions of these lipids have the same properties as the endogenous ones. Yet, these lipids participate in many cellular pathways, from cell proliferation, senescence and apoptosis to cell migration, endocytosis, intracellular trafficking, autophagy, and even inflammation [81] so altering their biosynthesis should lead to a series of consequences which, up to now, have been poorly documented.

Similarly, data is scarce on the effect of 2-DG on inositol metabolism. Glucose-6-phosphate is used as a precursor for inositol synthesis by inositol-3-phosphate synthase, but an *in vitro* study using a plant enzyme reported that 2DG-6-P actually inhibits this activity [83], suggesting that it may not incorporate into this pathway but rather be inhibitory to inositol synthesis. This could explain another observation made in mice indicating that 2-DG reduces inositol content in liver and testis [84]. Overall, a careful evaluation of the impact of 2-DG exposure on inositol metabolism awaits further investigations.

2.3 GDP-2-DG and inhibition of protein N-glycosylation, causing ER stress and activation of the Unfolded Protein Response

Mannose is the C2 epimer of glucose (**Figure 1**), so 2-deoxyglucose is also equivalent to 2-deoxymannose. Consequently, 2-DG can also hijack mannose metabolism in cells, particularly regarding protein glycosylation (**Figure 3**). In particular, 2-DG incorporation into GDP-2-DG competes for GDP-mannose for incorporation by mannosyltransferases into N-linked glycans on lipid-linked oligosaccharides, resulted in the synthesis of partially glycosylated or unglycosylated proteins, as initially demonstrated on for viral glycoproteins [85–90] and *in vitro* [91]. Interestingly, the multiplication of various enveloped viruses occurred at concentrations below those required to block energy supply, arguing in favor of a direct effect of 2-DG on this pathway, and could be counteracted by exogenous mannose through the restoration of normal glycosylation [87]. This effect of 2-DG is not limited to viral proteins, and 2-DG treatment can, for example, lead to a lack of expression of glycoproteins at the cell surface, as demonstrated in the case of ligands of the activating receptor NKG2D (Natural killer group 2D) [92] – a defect compensated by the addition of exogenous mannose. Work initiated by the Lampidis lab showed that this effect on protein glycosylation is the main mechanism by which 2-DG is toxic to oxygenated cells, which are less sensitive to the metabolic effects of 2-DG because of their ability to respire and thus cope with a decreased glycolytic efficiency [3,34].

This additional effect of 2-DG on protein glycosylation also likely explains the strong effect of 2-DG on yeast cell lysis, which in addition to the perturbation of cell wall structural carbohydrates exerts an additional inhibition on mannan biosynthesis, a key component of the outer layer of the cell wall [93] (**Figure 3**). Mannans can sometimes be attached to other cell wall components, but they are often made of highly glycosylated proteins first synthesized in the endoplasmic reticulum (ER) or the Golgi, with a similar topology as mammalian high-mannose N-glycans, which can be further decorated with up to 50-200 mannosyl units [67]. Accordingly, 2-DG inhibited cell wall regeneration on yeast with a degraded cell wall (protoplasts), mostly by preventing the normal synthesis of mannans and with evidence for the incorporation of 2-DG into these macromolecules [94–96].

But more generally, because of the general interference with N-glycosylation, 2-DG causes protein misfolding, endoplasmic reticulum stress and the onset of the unfolded protein response (UPR) [34,97–100] (**Figure 3**). Interestingly, in normoxic tumor cells, this seems to be the primary mechanism of its toxicity, rather than blockade of glycolysis, since the observed 2-DG-mediated cell death can be compensated to a large extent by exogenous mannose [34,100]. Accordingly, 2-DG causes glycosylation defects in yeast and induces the UPR response, and yeast mutants defective for UPR are hypersensitive to 2-DG, except when supplied with exogenous mannose [71]. Moreover, 2-DG-mediated N-glycosylation inhibition was reported to trigger autophagy [100], AMPK (5'-AMP activated protein kinase) activation

[101] but also to downregulate ERK (extracellular signal-regulated kinase) and protein kinase B/AKT signaling [102], adding to the other effects that 2-DG has on signaling pathways, as described in the next section.

3. Other indirect effects of 2-DG on signaling and cellular pathways

3.1 Effect of 2-DG on AMPK signaling in mammalian cells

Through its many ways of interfering with carbohydrate metabolism, 2-DG triggers many signaling events affecting normal cell function. A first, well-known effect of 2-DG is the activation of the nutrient sensor 5'-AMP-activated kinase, AMPK (**Figure 3**). AMPK is key for the metabolic adaptation of cells during nutrient stress, through the inhibition of ATP-consuming processes and the simultaneous activation of alternative ATP-generating pathways [103]. In cells that primarily use glycolysis as a source of ATP, 2-DG likely activates AMPK through ATP depletion and the subsequent increase in AMP or ADP [104]. The mechanistic details of AMPK activation by 2-DG have not been fully addressed and they may depend on the cell lines considered and their metabolism [eg. regarding the involvement of the upstream kinase LKB1, 105,59]. Interestingly, AMPK activation participates in the survival of cancer cells to 2-DG, suggestive of an adaptation to this metabolic stress [59,106].

Additionally, it was shown that AMPK can also be activated by 2-DG-induced ER stress in a pancreatic tumor cell line, particularly in aerobic conditions [101], whereas reactive oxygen species generated in response to 2-DG treatment participated in AMPK activation in bovine aortic endothelial cells [107] suggesting that several 2-DG-induced stresses may contribute to AMPK activation.

A careful study comparing various AMPK agonists demonstrated that 2-DG treatment of mouse embryonic fibroblasts leads to the activation of canonical AMPK signaling, including phosphorylation of AMPK itself (Thr172) and of its model substrate acetyl-CoA carboxylase (Ser79) [108]. In breast cancer cells, 2-DG treatment triggers the phosphorylation of CREB (cAMP response element-binding protein) on Ser133 and activates downstream signaling [106]. However, contrary to the synthetic compound A-769662 which activates AMPK allosterically [109], all commonly used AMPK activators including 2-DG have additional, AMPK-independent effects on cell growth [108]. Accordingly, some effects of 2-DG are not recapitulated by other AMPK activators [110], which is not surprising because of its other cellular effects detailed earlier in this review.

3.2 Other signaling pathways affected by 2-DG: mTORC1, tyrosine kinases and beyond

In itself, AMPK activation already has a plethora of consequences [111] and this likely participates in the complexity of 2-DG-mediated toxicity. A first obvious consequence of AMPK activation is the inhibition of mTORC1 (mammalian target of rapamycin complex 1) activity, another central regulator of metabolism that functions opposite to AMPK. TOR favors biosynthetic metabolism, protein translation,

and proliferation while it inhibits autophagy [112]. Accordingly, 2-DG, like most other AMPK agonists, led to increased phosphorylation (inhibition) of the mTORC1 component and AMPK substrate, Raptor (Ser792); however, 2-DG did not prevent the phosphorylation of the mTORC1 substrate S6K1 (Thr389) contrary to other AMPK agonists, indicating only partial inhibition of mTORC1 by 2-DG [108], although others reported a stronger inhibition [113]. This inhibition may account for the known effect of 2-DG on triggering autophagy in various cell lines [114–117], and indeed the effect of 2-DG on mTORC1 activation was attenuated in breast carcinoma cells with impaired AMPK activity [118]. Besides, inhibition of N-glycosylation by 2-DG and the ensuing ER stress were found to be the trigger of autophagy, since 2-DG-induced autophagy was abrogated by the addition of exogenous mannose which should only alleviate the glycosylation defects [100]. This suggests again that several 2-DG-induced stresses may synergize to inhibit mTORC1.

In many cancer cell lines, 2-DG treatment was shown to rapidly and sustainably induce the activation of the growth-promoting kinase Akt through its phosphorylation at Thr308 and Ser473, but this was independent of glycolysis inhibition or AMPK activation and required PI3K signaling [119,120]. Interestingly, the activation of Akt dampened the growth-inhibitory effects of 2-DG, resulting in partial tolerance to 2-DG. This occurred through IGF-1 (insulin-like growth factor 1) receptor signaling, a key regulator cell growth, and cell cycle progression [119]. Further demonstrating the pleiotropic effects of 2-DG on cellular signaling, the use of a phospho-specific antibody microarray on 2-DG-treated non-small human lung carcinoma cells (H460) revealed an increase in the phosphorylation of 7 tyrosine kinases and 64 sites on target proteins including Raf-MEK-ERK kinases targets, cell cycle or DNA damage checkpoint proteins (p53, Chk1/2, RB, BRCA1) or JAK/STAT proteins [119], some of which depended on LKB1/AMPK signaling [121].

2-DG induced apoptosis *in vitro* in various cancer cell models [122–124], and although this could be due to the impairment of glycolysis, there are instances where it has also been linked to ER stress [125]. It should also be noted that environmental conditions and drug dosage are likely to influence the mechanism by which this occurs [reviewed in 4].

Similar to other ER-stressing agents such as tunicamycin and thapsigargin, 2-DG induces calcium release from the ER to the cytosol, contributing to AMPK activation through the calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) in human pancreatic tumor cells [101], and likely to many other pathways. A depletion of ER Ca^{2+} stores was also observed after 2-DG treatment in Hep2 cells [57], and it was suggested that this mediates protection towards Shiga toxins. These are bacterial toxins that bind glycosphingolipids of the cell surface and are endocytosed and retrograde-trafficked to the ER, where their enzymatically active moiety (Stx-A1) is released into the ER lumen and then translocated to the cytosol to inhibit protein synthesis. 2-DG treatment specifically impaired Stx-A1 release in the ER, possibly through its effect on Ca^{2+} homeostasis and independently of glycosylation inhibition [57].

3.3 Effect of 2-DG on AMPK signaling in yeast

The effects of 2-DG on the *Saccharomyces cerevisiae* AMPK orthologue, Snf1, are much less clear. In budding yeast, the presence of non-fermentable carbon substrates as a sole energy source or the depletion of glucose leads to the activation of Snf1, which plays an essential role in the adaptation to non-glucose growth conditions [for review, see 18]. This includes the derepression of otherwise glucose-repressed genes involved in alternative metabolism. Snf1 activity requires the phosphorylation of a threonine residue in its activation loop [126]. The signal which regulates Snf1 phosphorylation as a function of glucose availability remains unknown. This phosphorylation is controlled by three protein kinases ["upstream kinases", 127–129] whose activities on Snf1 are counteracted by phosphatases, the main one being the Protein Phosphatase 1 (PP1) complex Glc7/Reg1 (Glc7 being the catalytic subunit and Reg1 a glucose-specific regulatory subunit) [130–132]. Therefore, the activation state of Snf1 is the resulting balance of these two opposite reactions. Since there is no evidence of a regulation of upstream kinases by glucose, Snf1 dephosphorylation is likely the main mechanism for the regulation of its activity by glucose availability [133]. This would imply that glucose metabolism regulates the Snf1-inactivating phosphatase Glc7/Reg1, and/or that the accessibility of the activation loop's threonine varies with the energy status of the cell. In agreement with the latter hypothesis, Snf1 phosphorylation was found to be protected against dephosphorylation by ADP [134], as demonstrated for AMPK [135,136].

When cells are grown in the presence of glucose (ie. low Snf1 activity), the effect of 2-DG on Snf1 activation is rather modest, as measured by its phosphorylation of the activation loop [35]. Moreover, this does not seem to affect the phosphorylation of a Snf1 model substrate, the transcriptional repressor Mig1 [35], although other Snf1 substrates may, of course, behave differently. Interestingly, lack of Snf1 renders glucose-grown cells hypersensitive to 2-DG, suggesting that (a) Snf1-dependent process(es) participates in the relative 2-DG tolerance of wild-type cells [35].

However, the situation seems quite different when 2-DG is added to yeast grown in the absence, or in limiting amounts, of glucose. In this case, it seems that yeast cannot discriminate between glucose and 2-DG, because both sugars trigger the rapid inhibition of Snf1 phosphorylation [28,35]. Therefore, regarding Snf1 signaling, 2-DG is perceived as glucose by yeast cells, at least when cultivated in a less-preferred carbon source, and this actually founded the basis for the use of 2-DG to identify glucose-insensitive mutants (see part 1) [19,31,32]. Of note, Snf1 targets are also dephosphorylated in response to 2-DG [35,137], and the effect is almost immediate, suggesting it could be attributed to their 2-DG-induced dephosphorylation by the Glc7/Reg1 phosphatase. Whether 2-DG or a derived metabolite regulates Glc7/Reg1 remains to be established. Intriguingly, the effects of 2-DG on glucose-starved cells are annihilated by the overexpression of a 2-DG-6-P phosphatase, suggesting that 2-DG-6-P accumulation

is required for this regulation [29]. In any case, decades after its first use for yeast research, 2-DG still appears as a crucial tool to decipher open questions on AMPK signaling in yeast.

4. Use of 2-DG in the context of cancer treatment or cancer imaging

4.1 Evaluation of the use of 2-DG for cancer treatment

Because cancer cells generally display an altered metabolism with increased glucose uptake and utilization by glycolysis, there is a large potential of therapeutic interventions by targeting the early steps of glucose uptake and metabolism, more specifically glycolysis [138].

The first report of the use of 2-DG in patients dates from 1958 [14]. In this study, 2-DG was infused intravenously in 8 patients affected by various cancers. 2-DG induced a rise in blood glucose, concomitantly to hypoglycemia-like symptoms such as flushing, sweating, drowsiness, and hypothermia, which disappeared after 90 min. This is in agreement with our current knowledge that 2-DG both competes with glucose uptake and its metabolism. A single infusion did not impact the progression of the disease, suggesting that multiple treatments would be required, and in light of its toxicity which prevented the use of higher doses, further attempts at using 2-DG for cancer therapies were abandoned for several decades. It is also interesting to note that in early trials, only 30% of the dose of 2-DG administered to patients was excreted in the urine, and the fate of the 70% left in the body was unknown [14]. A phase I dose-escalation trial aimed at establishing the recommended doses of 2-DG which, when used as a single agent, would allow targeting hypoxic cells with little toxicity on normoxic tumor cells was published in 2010 [139]. 2-DG treatment competed with FDG uptake as determined by PET-scan, and expression of the autophagy protein p62 in peripheral blood mononuclear cell decreased in 5 out of 6 patients tested, although the rationale for the latter is not clear. Out of 7 castration-resistant prostate cancer patients who completed the study, none showed a decrease in prostate-specific antigen.

Several studies of the impact of 2-DG on the growth *in vitro* of human leukemia cells isolated from patients [15] and in various transplantable tumors in mice [16] as well as the *in vivo* effects of 2-DG administration on tumor growth in rats were very encouraging [140,141]. However, later investigations using nude mouse xenograft models of human osteosarcoma and non-small cell lung cancer showed that 2-DG treatment, when provided as a single agent, does not inhibit tumor growth [4,142]. Altogether, given its obvious toxic effects on the proliferation of various cancer cell lines *in vitro* [143,144] these results were disappointing and suggested that the use of 2-DG as a single therapeutic molecule is unlikely. Moreover, 2-DG activates pro-survival pathways in several cancer cell lines that could attenuate its anticancer effect [119], justifying the use of an additional agent when using 2-DG for therapy.

4.2 2-DG in the context of combination therapies to target slow-growing, hypoxic cells

The fact that cancer cells are still able to use other carbon sources through oxidative phosphorylation when oxygen is present as well as the existing metabolic cooperation between tumor cell populations [145] render tumors less sensitive to glycolysis inhibition than anticipated. Similarly, 2-DG is efficient at inhibiting the growth of most carcinoma cells lines in (normoxic) culture but does not cause their death [146]. Moreover, when it does, it is rather through the inhibition of N-glycosylation than a metabolic blockade [34].

Instead, studies revealed that cancer cells in hypoxia, which rely more heavily on anaerobic glycolysis for their survival, are more susceptible to the 2-DG-mediated arrest of proliferation and death [146,147]. This implied that the hypoxic subpopulation of tumor cells, that are slow-growing and generally considered as less sensitive to chemotherapeutic agents and radiotherapy, may be selectively targeted by 2-DG and possibly other glycolytic inhibitors [148]. Indeed, similar observations were made for the hexokinase inhibitor 3-bromopyruvate [149].

These data suggested that 2-DG should rather be used in the context of combination therapies, in addition to drugs targeting oxygenated, proliferating cells. Indeed, when 2-DG combined with other, more systemic chemotherapeutic agents targeting proliferating cells such as adriamycin (DNA topoisomerase II inhibitor) or paclitaxel (another microtubule depolymerization inhibitor), it was found to potentiate their effect on tumor growth inhibition and on promoting animal survival in lung cancer and osteosarcoma mouse xenograft models [142]. In mouse models of retinoblastoma, 2-DG was found to enhance the effects of a commonly used antineoplastic agent for this cancer, the platinum compound carboplatin, specifically by promoting the death of hypoxic tumor cells [150], confirming that 2-DG offers a way to target this population. 2-DG also sensitized cells to the ABT class of apoptosis inducers and Bcl-2 antagonists in a mice tumor xenograft setting of hormone-independent, chemo-resistant human prostate cancer [151]. An interesting synergy was also discovered between 2-DG and the DNA-damaging agent etoposide (topoisomerase II inhibitor), whereby it enhances the antitumor T-cell response in the tumor microenvironment in a mouse model of lymphoma [152]. Thus, traditional chemotherapies might benefit from a combination with 2-DG to increase their clinical effectiveness.

In another phase I dose-escalation trial, 2-DG was tested alone or in combination with a microtubule-stabilizing drug, docetaxel, in patients with advanced solid tumors [153]. Side-effects of 2-DG treatment again included increased blood glucose and hypoglycemia-like symptoms, as well as some gastric ulcers (probably caused by increase gastric acid production) and prolonged but asymptomatic QTc intervals. The combination was clinically tolerable with no pharmacokinetic interaction. Overall, one-third of the patients showed stable disease for the duration of the treatment (8 weeks), suggesting a clinical benefit. Additional optimizations will be required to enhance this treatment in the future.

Many other studies combining 2-DG with either another metabolic perturbator, chemotherapeutic drugs, or even radiation therapy suggest it is a promising approach for improving therapies [reviewed in 143]. Among the metabolic perturbators, an *in vitro* study showed that 2-DG potentiates the cytotoxic effects of oligomycin, a mitochondrial respiration inhibitor, in glioblastoma multiforme cells [154]. Particularly, because of the known effects of 2-DG on AMPK activation, its action was also evaluated in the context of AMPK inhibition to antagonize this metabolic adaptation, for example by Compound C in breast cancer cell lines [106]. Alternatively, several studies aimed at reinforcing the ATP-depleting properties of 2-DG, for example with the use of metformin which is probably the most studied drug in combination with 2-DG. Metformin is a compound widely used for type 2 diabetes treatment which lowers hyperglycemia by inhibiting hepatic lipogenesis and gluconeogenesis and promotes insulin sensitivity [reviewed in 155]. At the cellular level, metformin is a mild mitochondrial complex I inhibitor, leading to decreased ATP levels and AMPK activation, although akin to 2-DG, some consequences of metformin are independent of AMPK [156]. The combination of 2-DG and metformin synergistically depleted cellular ATP, and sustained activation of AMPK and autophagy in several cancer cell types [157]. Interestingly, the presence of metformin shifted the 2-DG response of prostate cancer cell lines from autophagy to apoptosis [114], and also triggered apoptosis in ovarian cancer cells and esophageal squamous cell carcinoma [158,159]. This combination also caused a decrease in angiogenesis in endothelial cells more efficiently than the drugs alone [160], in line with other investigations supporting the use of 2-DG to inhibit angiogenic potential [102,161–163]. However, recent results showed that this combination induced a detachment of breast cancer cells *in vitro* that remain viable [110]. Whether this is significant for the metastasis potential *in vivo* remains to be evaluated, as it could be counteracted by the inhibition of cell migration by 2-DG [163].

Finally, because 2-DG triggers ER stress and this appears to be its most toxic action in normoxic cells, preventing the onset of a proper unfolded protein response by targeting components of this signaling pathways shifts the cell growth-inhibitory effect of 2-DG to cell death, which could be exploited in the future to potentiate 2-DG toxicity [164].

4.3 2-DG as a sensitizer for radiation therapies

Work initially performed in yeast demonstrated that 2-DG inhibits DNA repair in respiratory-deficient cells [165]. This inspired further work aimed at determining the potential effect of 2-DG in the context of radiation-induced therapies. Indeed, subsequent findings revealed that 2-DG potentiated the radio-sensitization effect on cancer cells [166,167]. These observations and others also led to clinical trials aimed at evaluating the effect of 2-DG treatment in the context of radiation therapy [168], but only indicating a mild benefit – the amount of 2-DG being limited by its side-effects and toxicity [reviewed in 169]. Another clinical trial suggests that 2-DG can be used in combination with radiation therapy in

patients with glioblastoma multiforme with enhanced survival when 2-DG is provided at high doses, when tolerated [170].

4.4 Use of 2-DG derivatives for cancer imaging by positron emission tomography

The principle of using 2-deoxyglucose-derivatives for cancer imaging is based on the premise that increased glucose utilization is a nearly universal feature of cancers, as described by Warburg nearly a century ago. The rationale is that poorly metabolizable glucose analogs such as 2-deoxyglucose should be preferentially transported across the membrane of highly glycolytic cells, and thus should concentrate in regions of increased glycolysis. Consequently, the use of a traceable version of this drug would provide a whole-body readout of regions with strong glucose import.

Developed in the 1970s to study glucose metabolism [171], the localization of the glucose analog ^{18}-Fluoro-2-deoxyglucose (^{18}FDG) can be followed by positron emission tomography-based imaging (PET) and its accumulation in cells depends on glucose transporters and hexokinase [172]. ^{18}FDG is now commonly used to localize metabolically active tumors for diagnosis as well as to evaluate cancer evolution or response to treatment in various types of cancer (lymphomas, head and neck, lung, or gynecologic cancers), but also in the context of Alzheimer's disease and other disorders [for review, see 173].

4.5 Novel 2-deoxyglucose-derived drugs on the rise

Other 2-deoxy-substituted glucose-derived drugs have been developed and compared to 2-DG [for a recent review, see 143]. For example, Lampidis et al. [174] tested the efficacy of glucose analogs that are halogen-substituted in position 2 (Fluoro/Chloro/Bromo-2-deoxyglucose) for their ability to block glycolysis and kill hypoxic tumor cells. They showed that FDG inhibited glycolysis more strongly than 2-DG and was more cytotoxic than 2-DG, in agreement with previous findings whereby FDG appeared more toxic than 2-DG to glucose-grown yeast cells and imposed a stronger block on glycolysis [175]. The authors also noted a relationship between the halogen size and its inhibitory potential on glycolysis [174].

On the contrary, FDG is less efficient at inhibiting glycosylation than 2-DG [176], probably because the extra fluoride atom makes it less of a mannose mimic. Thus, the comparison between the effects of 2-DG and FDG allows one to discriminate between the effects of 2-DG on glycosylation and glycolysis [3], although other pathways might also be differentially affected. For example, contrary to 2-DG, FDG did not incorporate into glycosphingolipids but blocks the biosynthesis of the glucosylceramide precursor [177]. Still, because of this preferential interference with glycolysis, FDG might be more efficient in killing hypoxic cells *in vivo*, as demonstrated in a transgenic mouse model of retinoblastoma [178].

More 2-DG analogs are also being developed and assessed for possible medical use. WP1122 is a 2-deoxyglucose-derived molecule with additional acetyl substitutions at positions 3 and 6 (3,6-di-O-acetyl-

2-deoxy-d-glucose) [179]. Interestingly, its chemical structure allows it to cross the blood-brain barrier by passive diffusion rather than through glucose transporters, suggesting potential applications to target brain tumors. Its intracellular deacetylation by esterases allows trapping of the molecule, leaving a 2-deoxyglucose remnant that can be phosphorylated and inhibitory just like 2-deoxyglucose. This new way of delivery seems to achieve a greater accumulation of 2-DG in plasma but also a longer half-life of the molecule, and a higher efficiency than 2-DG on a glioblastoma cell line *in vitro* [143,179].

5. 2-DG resistance strategies in yeasts and filamentous fungi

The ability to generate mutants able to tolerate 2-DG has been observed very early on in mammalian cell culture [180] and in microorganisms, both in budding yeast [181] and *E. coli* [182]. To better understand how glucose is sensed and regulates the expression of specific enzymes such as invertase [a secreted enzyme which hydrolyzes sucrose into glucose and fructose, with important industrial uses, 183], but also to study the process of glucose phosphorylation by hexokinases, several groups performed genetic screens to isolate 2-DG-resistant mutants of budding yeast. These studies were facilitated by the fact that 2-DG resistance occurs at high frequency [184]. **Table 1** summarizes the mutants obtained in the course of these studies and they will be discussed in greater detail in the next sections. A model diagramming the affected pathways is presented in **Figure 4**.

At first, it seemed unclear how cells could become resistant to a drug like 2-DG, which competes with a nutrient as essential as glucose and alters many aspects of cellular metabolism. In principle, resistance could result from a decreased import of 2-DG into cells, a decrease in 2-DG phosphorylation, or a detoxification of 2-DG-6-P. The latter hypothesis was supported by the early discovery of an endogenous phosphatase with activity towards 2-DG-6-P [181].

5.1 2-DG import

In principle, since 2-deoxyglucose enters the cells by glucose transporters, mutation of these transporters should abolish 2-DG toxicity. However, to our knowledge, none of the resistant mutants identified to date are mutated in a glucose transporter. This is likely because *S. cerevisiae* has an unusually large and redundant set of glucose transporters [185]. Among the family of 20 putative *HXT* (hexose transporter) genes, 7 are known to participate in glucose uptake, with various affinities and thus acting at various concentrations, and that are differentially regulated. Consequently, it is unlikely that a screen would lead to mutations in all 7 genes known to import glucose. Additionally, more recent screens were performed in the context of using glucose as a carbon source [71,186], and given the structural similarities between glucose and 2-DG, mutation of glucose transporters that would affect 2-DG transport might equally impair glucose transport and thus prevent growth on glucose medium.

However, in yeasts with less redundant transport systems, such as *Kluyveromyces lactis*, mutants of the low-affinity glucose transporter Rag1 were identified [187], and indeed, deletion of glucose transporters in another strain renders it resistant to 2-DG [188]. Similarly, deletion of the gene encoding the hexose transporter HxtB in the filamentous fungus *Aspergillus nidulans* causes higher 2-DG tolerance [189].

The kinetic characterization of glucose uptake in several 2-DG resistant mutants sometimes led to the observation that glucose transport is affected in these mutants, however, these could be correlations and may be a consequence of general deregulation of glucose signaling or metabolism caused by the (generally uncharacterized) mutations carried by these strains, and there is sometimes little proof of causality between the decreased glucose uptake and increased 2-DG tolerance. For example, 2-DG-resistant mutants of budding yeast and the filamentous fungus *Neurospora crassa* were shown to lack low-affinity transport systems and instead express the high-affinity counterparts [190,191], but this would be expected from mutations affecting glucose repression, which provides additional means of resistance (discussed below). Similarly, a 2-DG-resistant mutant of the fission yeast *Schizosaccharomyces pombe* was defective in glucose transport but upon its genetic characterization, its 2-DG resistance did not segregate with the lack of transport [192], and additional mutants obtained by mutagenesis display either glucose transport defects or glucose-repression defects but the corresponding genes were not identified [193,194]. Mutation of components of the SWI/SNF (SWItch/Sucrose Non-Fermentable) chromatin remodeling complex in fission yeast causes resistance to 2-DG, maybe through their action on hexose uptake transport systems [195]. Altogether, whereas inhibition of 2-DG import should give rise to resistance, it is rather poorly documented in genetic screens.

5.2 2-DG phosphorylation

As would be expected from our current knowledge of 2-DG toxicity, in which 2-DG-6-P plays a pivotal role, the isolation of spontaneous 2-DG-resistant budding yeast strains consistently retrieved hexokinase mutants (**Table 1**) [196,32,19,64,71,186] and indeed, deletion of the main hexokinase gene *HXK2* results in 2-DG resistance [35,64]. This is also the case in many other species, such as in the thermotolerant yeast *Kluyveromyces marxianus* [197], *Kluyveromyces lactis* [187], *Schizosaccharomyces pombe* [68] and others.

Whereas the mechanism by which hexokinase mutations lead to 2-DG resistance seems quite straightforward, it is actually more difficult to understand than anticipated. Indeed, budding yeast contains two genes encoding hexokinases and one encoding a glucokinase. *HXK2* is the main hexokinase gene expressed in glucose-grown cells, but the expression of its paralog, *HXK1*, is increased when *HXK2* is absent [198] so that Hxk1 should compensate for the loss of Hxk2 regarding 2-DG phosphorylation. Previous work showed that the affinity of yeast hexokinase preparations towards 2-DG is in the same

range as that for glucose [41,199,200] and recent work further showed that both hexokinases had a similar activity toward 2-DG *in vitro* [186]. This goes against the idea that 2-DG is not phosphorylated in *hxk2Δ* cells. Accordingly, the deletion of phosphatases detoxicating 2-DG-6-P (discussed in the next paragraph) in a *hxk2Δ* mutant partially sensitizes this strain to 2-DG, suggesting that there is (at least) some phosphorylation of 2-DG in this strain and thus that (an)other sugar kinase(s) can perform this function [71,186]. Consequently, it remains unclear how the loss of *HXK2*, in the context of other enzymes competent for 2-DG phosphorylation, might lead to 2-DG resistance.

However, a difference between Hxk2 and other glucose-phosphorylating enzymes is that Hxk2 has long been known to participate in the glucose-mediated repression of genes [201,202], and as such, may contribute to 2-DG detoxification (see paragraph 5.1.4, below). Indeed, in a screen using a genomic library to identify genes restoring glucose repression in a hexokinase-deficient mutant, only plasmids encoding *HXK2* were identified, suggesting that other isoforms could not take over this function [203]. This led to the hypothesis that Hxk2 carries a signaling function in addition to its enzyme activity [reviewed in 204]. Indeed, a fraction of the Hxk2 pool localizes to the nucleus [205,206]. The N-terminus of Hxk2 is required for this localization and the transcriptional regulation of several genes [207], and is regulated by phosphorylation of a serine residue within this domain [208]. These analyses have allowed the functional dissection of this enzyme and the generation of separation-of-function alleles [209]. However, using these alleles, recent work suggests that the gene-regulating function of Hxk2 was not involved in 2-DG resistance [35]. Moreover, the analysis of a series of point mutants and truncated proteins had revealed a strong correlation between catalytic activity and activity on glucose repression [210–215]. Altogether, it seems that further work will be needed to unambiguously address the mechanism by which the loss of Hxk2 leads to 2-DG resistance.

5.3 2-DG-6-P dephosphorylation/detoxification

A third possible mechanism of resistance is through the dephosphorylation of the generated 2-DG-6-P species, which would allow for both the detoxification of the toxic 2-DG-6-P species and the liberation of 2-DG back into the medium. The ability of yeast cells to rapidly phosphorylate 2-DG into 2-DG-6-P after import, and then rapidly dephosphorylate it back to 2-DG was indeed observed in early studies using radioactive-labeled 2-DG [33,36,41].

The characterization of this phosphatase activity showed a relative specificity for 2-DG-6-P [216] and several 2-DG-resistant mutants exhibited an increase in this 2-DG-6-P phosphatase activity, which could detoxify the cells of this metabolite and dampen its negative effects on cellular physiology [181,216]. Later, by screening a genomic library and identifying genes whose overexpression allows 2-DG resistance, two 2-DG-6-P phosphatases were subsequently cloned and named *DOG1* and *DOG2* [217,218]. These belong to a superfamily of phosphohydrolases named haloacid dehalogenase (HAD)-like

phosphatases that are encountered in all living organisms and perform a wide range of functions, some being very specific to a single substrate and others being more promiscuous [219]. Since there is no evidence that 2-DG exists in nature, there is probably an endogenous substrate for these enzymes, but so far, the physiological functions of these 2-DG-6-P phosphatases are unknown. Dog1 and Dog2 preferentially hydrolyze phosphorylated carbohydrates, and their affinities towards various substrates have been documented [219]. However, it remains difficult to firmly conclude on their physiological functions based on these *in vitro* data, since post-translational modifications, protein compartmentation or expression levels might modulate their function. For instance, Dog1 has a higher activity on 2-DG-6-P than Dog2 [219], but yeast lacking *DOG1* are less sensitive to 2-DG than those lacking *DOG2*, presumably because Dog2 is much more expressed that Dog1 [71]. However, an analysis of the proteomic response of yeast to 2-DG revealed that they are induced upon 2-DG treatment, specifically through UPR signaling and the cell-wall integrity MAPK cascade [71]. Moreover, Dog2 was found to be glucose-repressed, which likely contributes to the resistance of de-repressed mutants as discussed in the next paragraph. Metabolomics studies of a yeast strain lacking *DOG1* and *DOG2* may help identify their endogenous substrates.

Intriguingly, Dog1/Dog2 overexpression should only prevent 2-DG-6-P accumulation, and presumably not increase the ATP levels (since ATP is still consumed for the futile phosphorylation of 2-DG, counteracted by the DOG enzymes). So, the fact that the simple overexpression of Dog1/Dog2 can override the growth inhibitory effect of 2-DG suggests that cells can accommodate the energy cost of 2-DG phosphorylation (and the expected drop in ATP), provided that 2-DG-6-P does not accumulate and inhibit glycolysis, glycosylation, or maybe alter phosphate pools.

5.4 Snf1/AMPK-dependent resistance mechanisms

As previously mentioned when discussing the resistance mechanism at stake in *hxk2* (hexokinase) mutants (part 5.1.2), a recurrent mechanism allowing 2-DG resistance is the loss of the glucose-repression pathway (**Table 1**). This explains why the isolation of 2-DG-resistant mutants was such a successful strategy to isolate strains with constitutive expression of normally glucose-repressed enzymes (see part 1.2).

Glucose derepression is positively regulated by yeast AMPK Snf1 such that mutants carrying a constitutively active Snf1 kinase have limited glucose repression and are strongly resistant to 2-DG. This is the case of mutants of the PP1 phosphatase (Glc7/Reg1), which negatively regulates Snf1. As a matter of fact, *reg1* and *glc7* mutants were found in the early-characterized 2-DG resistant mutants [19,32] and were found since then in other screens [35,64,71,186,220]. These mutations, impacting on PP1 activity, lead to higher Snf1 kinase activity, and indeed, the 2-DG resistance depends on Snf1 integrity and its phosphorylation on the activation loop [35,186]. Moreover, this is also the case of mutations in *HXK2*, as

discussed previously (see part 5.1.2), as well as of gain-of-function mutations of *SNF1* (Snf1-L183I, Snf1-G53R) [35,186,221]. Even though all of these data are fully consistent with the role of Snf1 in promoting 2-DG resistance, it is not fully clear how this is achieved.

One proposed mechanism is that by lifting the inhibition of glucose-repressed genes, the resistance gene *DOG2* is overexpressed, contributing to the observed resistance. In agreement with this, other glucose-repressed mutants such as *mig1Δmig2Δ* and *cyc8* are resistant to 2-DG and involved in glucose repression [32,71,220]. Yet, deleting *DOG2* (and its paralogue *DOG1*) in strains with hyperactive Snf1, such as *reg1Δ*, does not make them as sensitive as a WT strain [71,186], suggesting additional roles of Snf1 in 2-DG resistance. This is supported by the fact that an isolated point mutation in *REG1* (*reg1*-P231L) causing 2-DG resistance has no impact on the glucose-mediated repression of genes [186] and therefore is not expected to lead to an increase in *DOG2* expression.

A second Snf1-dependent mechanism relates to the effect that 2-DG exerts on nutrient transporter homeostasis. Indeed, cells are very sensitive to changes in nutrient sources and concentration and have evolved mechanisms so that the nutrient transporter repertoire at the plasma membrane matches the available nutrients at any given time [222]. This is particularly true in response to carbon sources perturbations, and Snf1/AMPK acts as a master regulator of transporter endocytosis events triggered by perturbation of glucose metabolism [137,223–225]. Particularly, 2-DG treatment was shown to induce the endocytosis of two low-affinity glucose transporters, Hxt1 and Hxt3 [226]. This process requires two proteins of the alpha-arrestin family, Rod1 and Rog3, that are themselves regulated by Glc7/Reg1 and Snf1 [137,224,226,227]. An interesting parallel can be made between the regulation of the arrestin-related protein TXNIP in mammalian cells, which is a substrate of AMPK (particularly upon 2-DG treatment, but also during glucose starvation) and regulates the endocytosis of the glucose transporter Glut1 [228]. Intriguingly, the arrestin mutant *rod1Δ* is somewhat resistant to 2-DG [35,64] and the additional deletion of its paralog, *ROG3*, further increases this phenotype [226]. Point mutations aimed at abolishing their function in endocytosis also led to increased resistance to 2-DG [226] suggesting that both proteins mediate 2-DG toxicity through their function in endocytosis. Moreover, overexpression of the Hxt1 and Hxt3 transporters restore *snf1*Δ resistance to 2-DG, highlighting the links between glucose transporter homeostasis and 2-DG resistance [226]. It was proposed that 2-DG toxicity is mediated through increased endocytosis and degradation of transporters that would lead to glucose deprivation, and that the loss of both arrestins would suppress this effect by stabilizing transporters at the cell surface. However, this strategy appears only valid in the context of the *snf1Δ* mutant, since an increased Hxt1/Hxt3 expression does not confer 2-DG resistance in WT cells [226]. Altogether, it seems that our understanding of how Snf1/AMPK contributes to 2-DG resistance is currently only partial and merits further investigation.

5.5 Other glucose-repression-dependent and -independent mechanisms

In addition to the abovementioned mutations in Hxk2 (hexokinase) and Reg1/Glc7 (PP1), early screens also identified mutations in the *GRR1* gene (Glucose-Repression Resistant) as causing 2-DG resistance (**Table 1**) [19,229,220]. The isolated *grr1* mutants are defective in the repression of several enzymes by glucose [229,230] and thus it was expected that Grr1 is another actor of the glucose repression pathway. Yet, the resistance mechanism caused by this mutation, although it may be partially overlapping with that observed in other glucose-repression mutants, appears slightly different. In particular, the *grr1*Δ mutant exhibited significant resistance only on sucrose medium [35] whereas the other glucose-repression mutants discussed up to now are also resistant to 2-DG when cells are grown on glucose medium. This difference might be explained by the fact that depending on the medium or the genetic alterations, yeast are facing different physiologies, with a different pattern of expression of hexose transporters at the membrane with various affinities and/or a different hexokinase isoform expressed, and as in the case of mammalian cells [146,147], they might be differentially susceptible to the inhibitory effects of 2-DG.

We know now that Grr1 is an F-box containing subunit of a ubiquitin ligase complex that regulates glucose transporter genes by triggering the proteasomal degradation of a transcriptional repressor, Mth1 [231,232]. However, Grr1 also plays other important roles in the glycolytic/gluconeogenic switch, as well as the response to extracellular amino acids, and even cell cycle and meiosis, indicating that Grr1 links cell cycle progression to nutrient availability [233,234]. Because all of these attributed functions, how the mutation of *GRR1* confers resistance to 2-DG remains unclear.

Several other mutants were identified as resistant to 2-DG with no obvious relationships to the glucose-repression pathway [64] but not all of them have been confirmed [35]. This might be due to the appearance of additional mutations in the course of the experiments since spontaneous mutations causing 2-DG resistance are easily obtained [184]. However, the deletion of *LSM6*, which encodes a member of a cytoplasmic complex involved in mRNA decay, does confer 2-DG resistance [35,64], although it is surprising that the screen did not uncover other mutants of this complex [64]. The 2-DG resistance observed in the *lsm6Δ* mutant depends on the integrity of Snf1/AMPK, but whether this is linked to the known effect of Snf1 in the regulation of mRNA stability by glucose availability [235] remains to be established.

Other resistance strategies may include the bypass of limiting metabolic reactions, the characterization of which may inform us of the cellular toxicity of 2-DG. For example, overexpression of a phosphorylating ($NADP^+$-dependent) glyceraldehyde-3-phosphate dehydrogenase from the yeast *Kluyveromyces lactis* named Gdp1 rendered *S. cerevisiae* resistant to 2-DG, suggesting that the NADPH pool, or more globally an altered cellular redox state participates in 2-DG toxicity [64].

Within the currently described mechanisms of 2DG resistance, no mutation has ever been isolated in pathways known to be inhibited by 2DG treatment (eg. glycosylation or cell wall synthesis). This may indicate that mutations in only one of the multiple pathways targeted by 2DG may not be sufficient to attain global resistance, as opposed to other mutations which act more systemically such as those leading to the overexpression of a 2DG6P phosphatase. It is also likely that novel resistance mechanisms remain to be discovered.

Altogether, despite decades of research, it seems that we still do not have a full understanding of the mechanisms allowing 2-DG resistance. The ease of yeast genetic manipulations coupled to advances in whole-genome sequencing techniques and their affordability have already been fruitful at identifying mutations conferring 2-DG resistance [71,186] which will probably be an asset for the isolation and characterization of additional mechanisms.

6. 2-DG resistance in mammalian cells

Compared to our knowledge in yeast, and in sharp contrast with the numerous studies using 2-DG in mammalian cells, very little is known about the resistance mechanism to 2-DG.

In a landmark study from 1962, S. Barban obtained a 2-DG-resistant HeLa cell line after prolonged incubation (2-3 weeks) in 2-DG-containing medium followed by further selection at higher 2-DG concentrations, resulting in a cell line able to grow in presence of a 10-fold excess of 2-DG over glucose [236]. These cells showed an increased dependence on pyruvate for growth and grew at about half the rate of the original, 2-DG-sensitive cell line. This is consistent with a decreased level of glycolysis and increased contribution of respiration. Accordingly, the resistant cells displayed lower incorporation of 2-DG, a lower hexokinase activity towards 2-DG but also other sugars, and a lower accumulation of 2-DG-6-P which reached a plateau within 30 min treatment [236]. However, Barban also made the surprising observation that an extract of 2-DG-resistant cells inhibited the hexokinase activity of an extract prepared from the original cell line. This “inhibitor” of hexokinase activity, which also acted on purified yeast hexokinase, was found to be heat-labile and non-dialyzable, arguing in favor of a large macromolecule, possibly a protein. The 2-DG-resistant cells also exhibited a higher phosphatase activity, with an alkaline pH optimum, which reacted on various phosphorylated sugars, especially on 2-DG-6-P. Whereas the main hypothesis proposed at the time was that 2-DG resistance in this cell line was due to a decreased transport and phosphorylation of 2-DG, the possibility that the observed phosphatase might counteract and detoxify 2-DG-6-P was also considered. Based on previous work showing that prednisolone, a synthetic corticosteroid, induces an alkaline phosphatase activity in cell cultures, Barban investigated the effect of this drug on 2-DG resistance and indeed demonstrated that prednisolone treatment results in 2-DG resistance of HeLa cells [237]. Finally, 2-DG resistance also correlated with an alkaline phosphatase activity in various cell lines or conditions [238,239]. Altogether, it seems that as previously mentioned in the yeast

S. cerevisiae, detoxification of 2-DG-6-P by dephosphorylation is likely to be an important part of 2-DG resistance. However, the phosphatase was never identified in these studies. More recently, it was shown that a human HAD-like phosphatase with sequence similarities to yeast Dog1/Dog2, named HDHD1 (Haloacid Dehalogenase-like Hydrolase Domain containing protein 1), also confers resistance to 2-DG when overexpressed in HeLa cells [71]. HDHD1 belongs to the beta-phosphoglucomutase (BPGM)-like family of HAD hydrolases, like the yeast Dog1/Dog2 and the *E.* coli enzyme YniC [which can also dephosphorylate 2-DG-6-P, 71,240]. HDHD1 had previously been described as a pseudouridine-5'-phosphatase [241], but as mentioned previously, most of the enzymes of this family can act with a low catalytic efficiency on a variety of phosphate esters, as shown with the yeast and bacterial enzymes [219,240]. Whether HDHD1 upregulation is responsible for the spontaneous resistance observed in earlier studies remains to be determined.

Another study used pig kidney cells that were made resistant to 2-DG upon serial cultivation in the presence of the inhibitor [242]. Interestingly, the results indicate a different mechanism to achieve 2-DG resistance, since no increased phosphatase activity was observed, despite a decrease in the accumulated 2-DG-6-P in resistant cells. The 2-DG resistant cells were able to grow at a higher rate than sensitive cells when cultivated at low glucose concentrations, suggestive of a metabolic alteration in this cell line that may facilitate adaptation to these conditions and explain their ability to tolerate 2-DG. Therefore, it is likely that several and distinct resistance mechanisms also exist in mammalian cells.

Moreover, there are several described contexts in which 2-DG toxicity can be attenuated. As alluded to earlier in this review, oxygenated cells have a decreased dependency on glycolysis and are therefore less susceptible to 2-DG [146]. Additionally, it has been shown that even in hypoxic conditions, which should potentiate 2-DG toxicity, a HIF-1 (Hypoxia-Induced Factor 1)-dependent increase in glycolytic enzymes (including hexokinase) may interfere with 2-DG toxicity [243]. Also, and as previously discussed, the fact that 2-DG activates pro-survival pathways in several cancer cell lines [119] could also provide a way to survive to toxic 2-DG concentrations.

The study of the 2-DG resistance mechanisms will be important to further investigate in the context of using this drug for cancer treatment, but also because detoxification mechanisms such as the one based on 2-DG-6-P dephosphorylation and efflux may alter ^{18}FDG accumulation in tumors and consequently, cancer imaging.

7. Concluding remarks and perspectives

In this review, we summarized decades of research on the cellular effects of 2-DG, its potential use in a medical setting, and the potential resistance mechanisms to this drug. Whereas it is usually recognized that early studies focusing on 2-DG were motivated by its effect on cancer cell growth *in vitro*, we also highlighted a large body of work performed in other model organisms, particularly in baker's

yeast, aimed at better understanding and controlling biotechnology-oriented processes such as fermentation. All of these studies contributed to a better understanding of the mechanism of action of 2-DG. They also revealed the complexity of this drug which, by mimicking glucose but also mannose, alters not only glycolysis but also many other processes such as glycosylation, causing both energy depletion and cellular stress. Efforts should be pursued in order to obtain a complete picture of the effects of 2DG on cellular metabolism and signaling.

Despite the specific targeting of 2-DG to highly glycolytic cells, its relative toxicity has made it a poor candidate for cancer treatment as a single agent. However, the fact that 2-DG toxicity depends on the physiological setting of the cells (normal or cancer cells, oxygenated or hypoxic, nature of the competing carbon source) provided new opportunities to target specific cell subpopulations in the context of combined therapies. Particularly, many synergies between 2-DG and other anti-cancer agents have been described and represent exciting avenues that should be further investigated. The precise profiling of the effects of 2DG on metabolism by global approaches could also help determine the susceptibility of specific tumors or specific cancer cell subpopulations within a tumor [244]. Other 2DG-derived inhibitors are being studied and may provide additional opportunities. Finally, the resistance of various cell types to 2DG have been observed but the strategies are poorly understood. Work in yeast has started to unravel these mechanisms and this is currently an active area of research, which should be pursued in human cells, for example by integration of genomic, proteomic, and metabolomic data on resistant cells. This will be useful for the further application of this remarkable sugar analog for cancer treatment.

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Figure legends.

Figure 1. Molecular structures of the sugars discussed in this review. The side chains of the C2 are indicated in red, showing the similarities between 2-deoxyglucose and mannose (circled) and the differences between 2-fluorodeoxyglucose and 2-deoxyglucose.

Figure 2. Metabolism of 2-deoxyglucose in the early steps of glycolysis and pentose phosphate pathway, and described inhibitory effects.

Figure 3. Examples of cellular and metabolic effects of 2DG and resistance mechanisms as determined in various model organisms, cells lines or animal models. Note that in cancer cells, the relative effect of 2DG on glycolysis or glycosylation inhibition can be modulated depending on whether cells are in normoxia or hypoxia. See text for details.

Figure 4. Overview of the identified mutations causing 2DG resistance in yeast place in the context of in the glucose sensing and signaling pathways. See text for details.

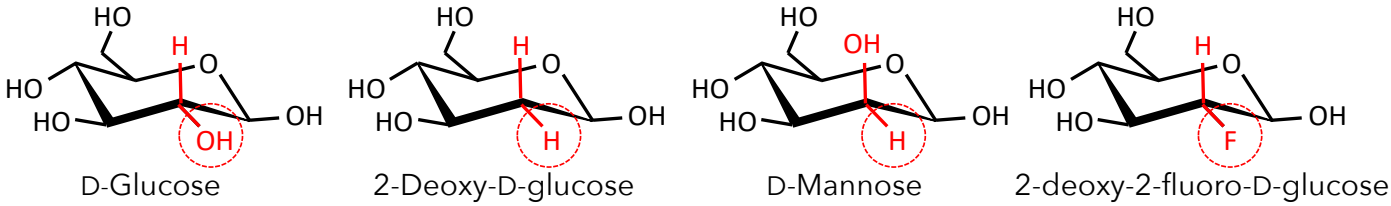
Table and Table legend.

Mutated gene(s):	***HXK2***	***REG1***	***GLC7***	***SNF1***	***CYC8***	***TUP1***	***MIG1 MIG2***	***GRR1***	***ROD1 ROG3***	***LSM6***
(note)	*(a,b,d)*	*(b,c,d)*	*(b,c,d)*	*(d,e)*	*(f)*	*(f)*	*(g)*	*(b,h)*	*(i)*	*(j)*
Reference (C source, if screen):										
Zimmerman and Scheel, 1977 [19] (Raffinose, galactose)	X	X						X		
Bailey and Woodward, 1984 [227] (Galactose)								X		
Neigeborn and Carlson, 1987 [32] (Sucrose)	X	X	X							
Schuller and Entian, 1991 [218]	X	X	X		X	X	X	X		
Ralser et al. 2008 [64] (Glucose)	X	X							X	X
McCartney et al., 2014 [35]				X		X		X		X
O'Donnell et al., 2015 [224]									X	
Soncini et al. 2020 [184] (Glucose)	X	X	X	X						
Defenouillère et al. 2019 [71] (Glucose)	X	X			X		X			

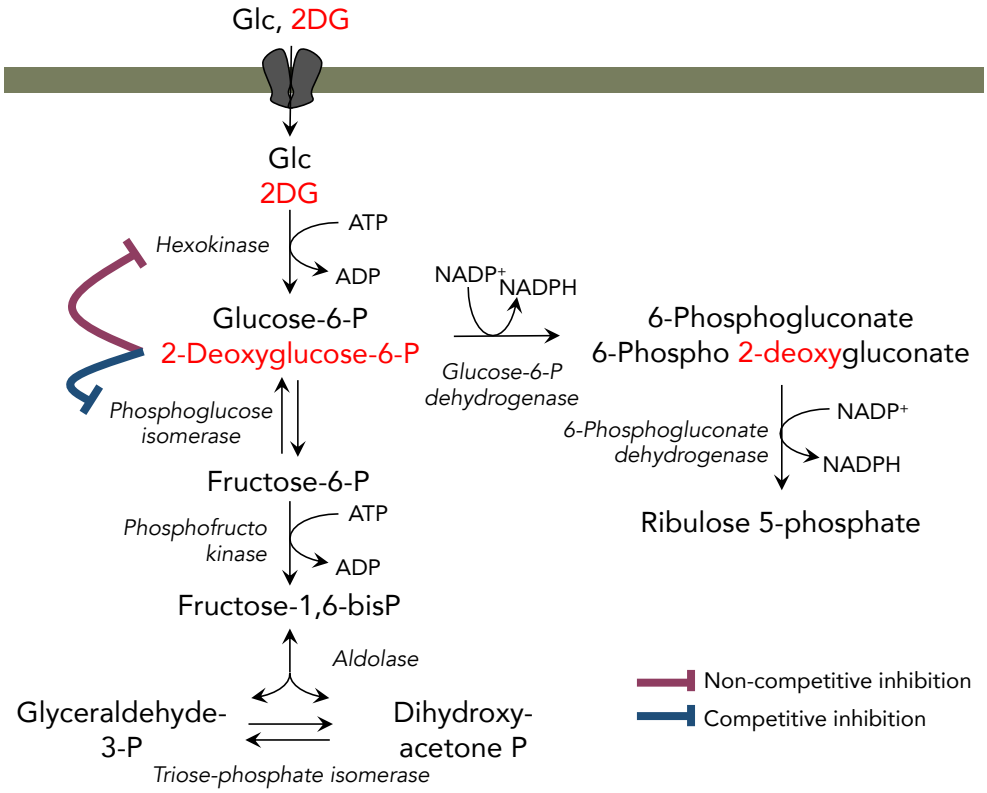
Notes: (a): Hexokinase; (b): Glucose repression mutant; (c): PP1 complex; (d): Activation of Snf1/AMPK activity; (e): Gain of function mutation; (f): Transcriptional repressor, general; (g): Transcriptional repressor, glucose-specific; (h): Glucose and amino acid signaling, cell cycle; (i): Endocytosis; (j): mRNA decay.

Table 1. Known 2-DG resistant mutants isolated by screening in the yeast *Saccharomyces cerevisiae*. The notes indicate the function of the corresponding genes. The references are indicated; when the results are obtained by screening, the carbon source used for the screen is indicated.

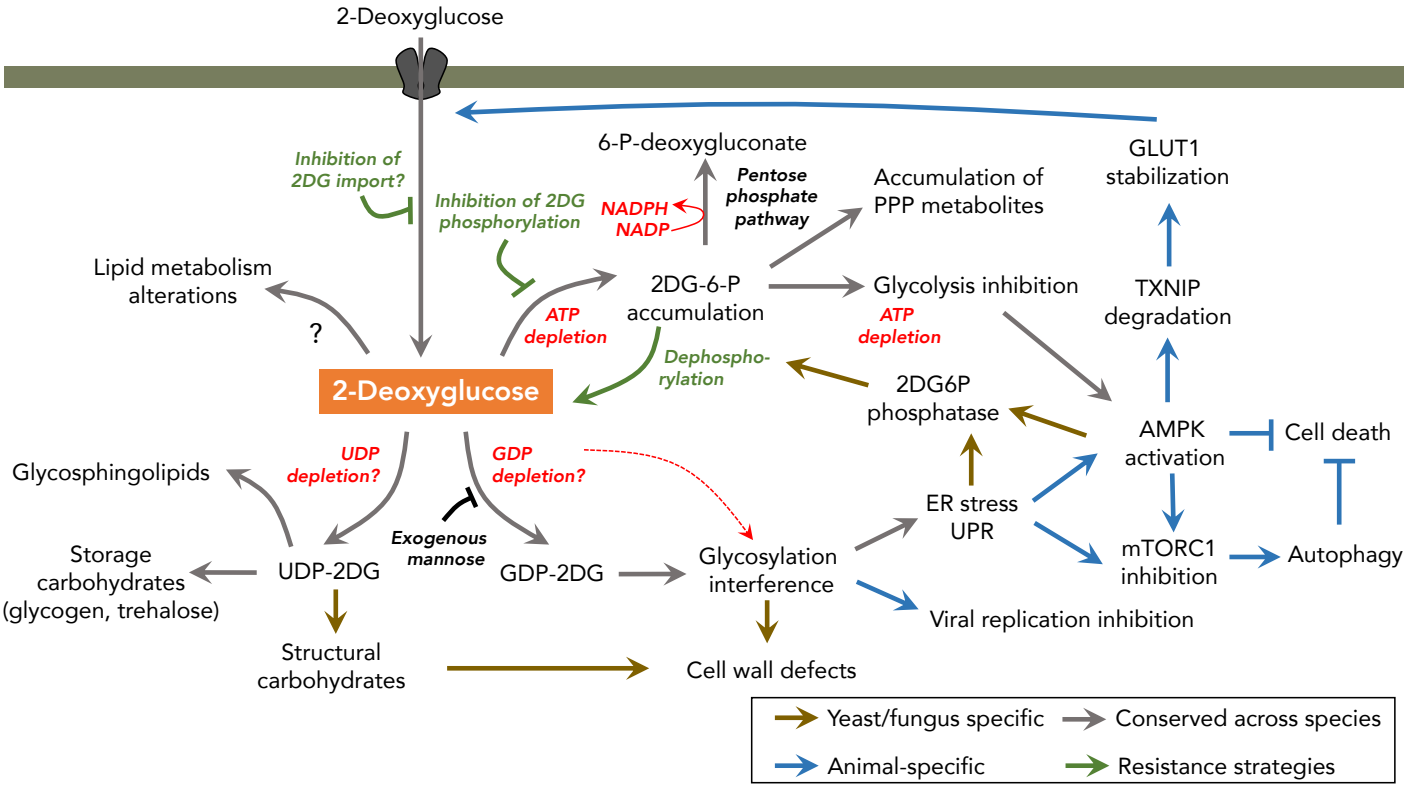
Figures.



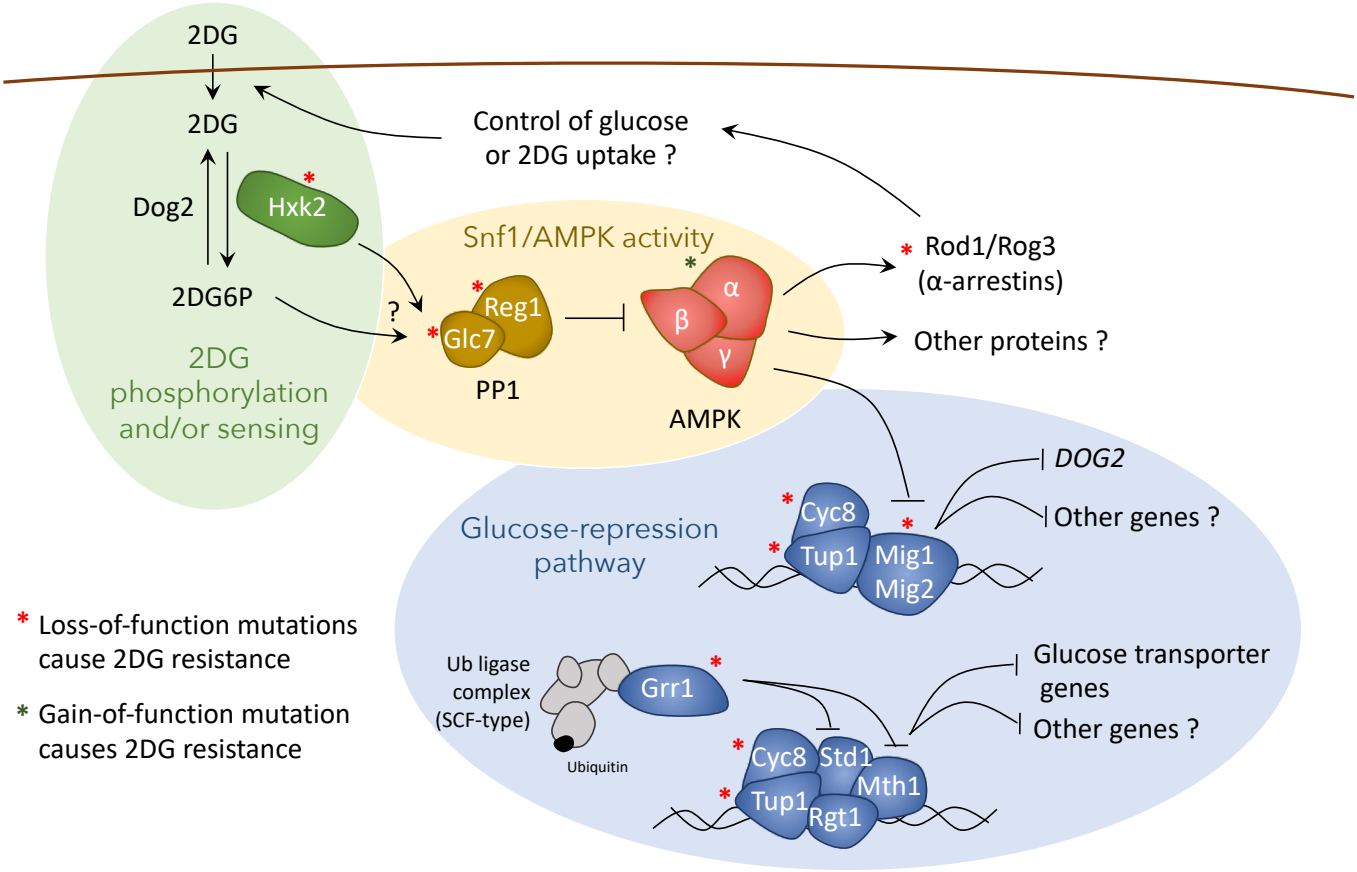
Laussel and Léon - **Figure 1.**



Laussel and Léon - **Figure 2.**



Laussel and Léon - **Figure 3.**



Laussel and Léon - **Figure 4.**