

Cellular and molecular mechanisms of metformin: an overview

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Abstract

Considerable efforts have been made since the 1950s to better understand the cellular and molecular mechanisms of action of metformin, a potent antihyperglycemic agent now recommended as the first line oral therapy for type 2 diabetes (T2D). The main effect of this drug from the biguanide family is to acutely decrease hepatic glucose production, mostly through a mild and transient inhibition of the mitochondrial respiratory-chain complex 1. In addition, the resulting decrease in hepatic energy status activates the AMP-activated protein kinase (AMPK), a cellular metabolic sensor, providing a generally accepted mechanism for metformin action on hepatic gluconeogenic program. The demonstration that the respiratory-chain complex 1, but not AMPK, is the primary target of metformin was recently strengthened by showing that the metabolic effect of the drug is preserved in liver-specific AMPK-deficient mice. Beyond its effect on glucose metabolism, metformin was reported to restore ovarian function in polycystic ovary syndrome, reduce fatty liver and to lower microvascular and macrovascular complications associated with T2D. Its use was also recently suggested as an adjuvant treatment for cancer or gestational diabetes, and for the prevention in pre-diabetic populations. These emerging new therapeutic areas for metformin will be reviewed together with recent data from pharmacogenetic studies linking genetic variations to drug response, a promising new step towards personalized medicine in the treatment of T2D.

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Introduction

Prevalence of type 2 diabetes (T2D) has reached epidemic proportions worldwide and promotes the risk for cardiovascular diseases and early mortality. Prevention and management of T2D has become a major public health challenge around the world. Metformin (1,1-dimethylbiguanide), a biguanide derivate, is the most widely prescribed drug to treat hyperglycemia in individuals with T2D and is recommended, in conjunction with lifestyle modification (diet, weight control and physical activity), as a first line oral therapy along in the recent guidelines of the American Diabetes Association and European Association of the Study of Diabetes [1, 2]. This recommendation was based on clinical studies as the UK Prospective Diabetes Study (UKPDS), a multi-centre randomized controlled trail of different therapies for T2D [3]. This landmark study reported that intensive glucose control with metformin appears to decrease the risk of diabetes-related endpoints and death in overweight diabetic patients, and is associated with less weight gain and fewer hypoglycaemic attacks when compared to insulin and sulphonylureas. The reduction of cardiovascular mortality by metformin compared with any other oral diabetes agent or placebo was confirmed by recent meta-analysis including more than 30 clinical trials [4, 5].

Despite being introduced clinically in the 1950s (although it was only available in the United States from 1995), the exact mechanism of action of metformin has not been fully elucidated. Recent clinical trials suggest that metformin, in addition to its efficacy in treating T2D, may also have therapeutic potential in other conditions including diabetic nephropathy, cardiovascular diseases, polycystic ovary disease and the prevention or treatment of cancer. This review will document the different mechanisms of metformin's action described to reverse these disturbances both in diabetic and non-diabetic patients.

A- Anti-hyperglycemic action of metformin

Metformin is currently the drug of first choice for the treatment of T2D, being prescribed to at least 120 million people worldwide. Metformin is regarded as an antihyperglycemic agent because it lowers blood glucose concentrations in T2D without causing overt hypoglycemia. Metformin is also frequently described as an insulin sensitizer leading to reduction in insulin resistance and significant reduction of plasma fasting insulin level. The improvement in insulin sensitivity by metformin could be ascribed to its positive effects on insulin receptor expression and tyrosine kinase activity [6]. Metformin may also exert its beneficial metabolic actions in part through the modulation of multiple components

of the incretin axis. Maida *et al.* have indeed recently reported that metformin acutely increases plasma levels of glucagon-like peptide 1 (GLP-1) and induces islet incretin receptor gene expression through a mechanism that is dependent on peroxisome proliferator-activated receptor (PPAR)- α [7]. However, a growing body of evidence from clinical studies and animal models suggests that the primary function of metformin is to decrease hepatic glucose production [8], mainly by inhibiting gluconeogenesis [9, 10]. Several mechanisms have been proposed to explain this inhibitory action on hepatic gluconeogenesis, including changes in enzyme activities [11-13] or reduction in hepatic uptake of gluconeogenic substrates [14]. The preferential action of metformin in hepatocytes is due to the predominant expression of the organic cation transporter 1 (OCT1), which has been shown to facilitate cellular uptake of metformin [15]. Consistent with this, accumulation of metformin in the liver has been shown to be higher than in other tissues, reaching hundreds of μM in the periportal area [16]. Furthermore, deletion of the OCT1 gene in mouse dramatically reduces metformin uptake in hepatocytes and human individuals carrying polymorphisms of the gene (*SLC22A1*) display an impaired effect of metformin in lowering blood glucose levels [15].

Although the molecular target of metformin was elusive for several years, Zhou *et al.* reported that the activation of AMP-activated protein kinase (AMPK) was intimately associated with the pleiotropic actions of metformin [17]. AMPK is a phylogenetically conserved serine/threonine protein kinase viewed as a fuel gauge monitoring systemic and cellular energy status and which plays a crucial role in protecting cellular functions under energy-restricted conditions. AMPK is a heterotrimeric protein consisting of a catalytic α -subunit and two regulatory subunits β and γ and each subunit has at least two isoforms. AMPK is activated by increase in the intracellular AMP-on-ATP ratio resulting from imbalance between ATP production and consumption. Activation of AMPK involves AMP binding to regulatory sites on the γ subunits. This causes conformational changes that allosterically activate the enzyme and inhibit dephosphorylation of Thr172 within the activation loop of the catalytic α subunit. AMPK activation requires phosphorylation on Thr172 by upstream kinases, identified as the tumor suppressor serine/threonine kinase 11 (STK11/LKB1) and CaMKK β , which is further stimulated by the allosteric activator AMP [18]. Moreover, it has been recently shown that ADP, and therefore the ADP-on-ATP ratio, could also play a regulatory role on AMPK by binding to specific domains on the γ subunit [19, 20]. Activated AMPK switches cells from an anabolic to a catabolic state, shutting down the ATP-consuming synthetic pathways and restoring energy balance. This regulation involves phosphorylation by AMPK of key metabolic enzymes and transcription factors/co-

activators modulating gene expression [18]. As a result, glucose, lipid and protein synthesis as well as cell growth are inhibited whereas fatty acid oxidation and glucose uptake are stimulated.

Metformin most likely does not directly activate either LKB1 or AMPK as the drug does not influence the phosphorylation of AMPK by LKB1 in cell-free assay [21]. Rather, there is evidence that AMPK activation by metformin is secondary to its effect on the mitochondria, the primary target of the drug. One of the most significant breakthroughs in the understanding of the cellular mechanism of metformin was indeed made in the early 2000s by two independent research groups reporting for the first time that this member of the biguanides family induced mild and specific inhibition of the mitochondrial respiratory-chain complex 1 (Figure 1). The initial observation was made in both perfused livers and isolated hepatocytes from rodents [22, 23] but later expanded to other tissues, including skeletal muscle [24], endothelial cells [25], pancreatic beta cells [26], and neurons [27]. Although the exact mechanism(s) by which metformin inhibits the respiratory-chain complex 1 remains unknown, we have recently shown that this unique drug effect does not necessitate AMPK and is also found in human primary hepatocytes [28]. In addition, it seems that mitochondrial action of metformin requires intact cells [22, 29, 30] and is prevented, at least in hepatocytes, neither by inhibition of nitric oxide synthase nor by various reactive oxygen species (ROS) scavengers [22]. In addition, the maximal inhibitory effect of metformin on complex 1 activity is also lower than the reference inhibitor rotenone (~40% with metformin compared with 80% with rotenone), suggesting that, owing to different physico-chemical properties, their respective site of action differ on one or several of the subunits of the respiratory-chain complex 1. For instance, the positive charge of metformin was proposed to account for its accumulation within the matrix of energized mitochondria, driven by the membrane potential ($\Delta\psi$) [23]. Furthermore, the apolar hydrocarbon side-chain of the drug could also promote its binding to hydrophobic structures, especially the phospholipids of mitochondrial membranes [31]. Interestingly, it has been recently shown that metformin, by contrast to rotenone, also exerts an inhibitory effect on mitochondrial ROS production by selectively blocking the reverse electron flow through the respiratory-chain complex 1 [32, 33]. Further investigations are still required to clarify the mechanism(s) by which metformin modulates the respiratory-chain complex 1 by such a unique way. It is also worth mentioning that metformin probably also exerts some non-mitochondrial effects since it has been shown to affect erythrocyte metabolism, a cell-lacking mitochondria, by modulating membrane fluidity [34, 35].

Taken together, the activation of AMPK by metformin in the liver, and probably in other tissues, is the direct consequence of a transient reduction in cellular energy status induced by the mild and specific inhibition of the respiratory-chain complex 1 by the drug [28] (Figure 1). In line with this, methyl succinate, a substrate of the respiratory-chain complex 2 bypassing the inhibition of complex 1, has been shown to antagonize the metformin-induced AMPK activation in pancreatic beta cell line [26]. Furthermore, Hawley *et al.* have recently reported that AMPK activation by metformin is abolished in cell line stably expressing AMPK complexes containing AMP-insensitive $\gamma 2$ mutant, demonstrating that increased cytosolic AMP indeed triggers the activation of the kinase by the drug [36].

AMPK involvement in the antidiabetic effect of metformin was initially supported by a study showing that the glucose-lowering effect of the drug is greatly decreased in mice lacking hepatic LKB1 [37]. LKB1/AMPK signaling has been reported to regulate the phosphorylation and nuclear exclusion of CREB-regulated transcription coactivator 2 (CRTC2, also referred to as TORC2) [37]. CRTC2 has been identified as a pivotal regulator of hepatic glucose output in response to fasting by directing transcriptional activation of the gluconeogenic program [38]. Non-phosphorylated CRTC2 translocates to the nucleus, where it associates with phosphorylated CREB to drive the expression of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) and its subsequent gluconeogenic target genes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Phosphorylation on the Ser 171 residue of CRTC2 by AMPK and/or AMPK-related kinases, including the salt-inducible kinases (SIK), is critical for determining the activity, cellular localization and degradation of CRTC2, thereby inhibiting the gluconeogenic program [37, 38]. However, since CRTC2 is O-glycosylated at Ser171 in insulin resistance state, making phosphorylation impossible [39], it is unlikely that metformin regulates gluconeogenesis through CTRC2 phosphorylation. A possible alternative mechanism for metformin inhibitory action on TORC2-mediated gluconeogenesis has been recently proposed (Figure 2), involving increase of hepatic SIRT1 activity, an NAD⁺-dependent protein deacetylase, through AMPK-mediated induction of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme for NAD biosynthesis [40]. SIRT1 has been reported to deacetylate CRTC2, resulting in the loss of protection from COP1-mediated ubiquitination and subsequent degradation [41]. This likely occurs in parallel with another mechanism for metformin action which involves the disassembly of the CREB-CBP (CREB binding protein)-CRTC2 complex at PGC-1 α and PEPCK promoters [42]. The regulation of gluconeogenic gene expression by metformin appears to be dependent on the phosphorylation of CBP at Ser436, but not CRTC2, through

AMPK-induced atypical PKC ι/λ activation [42]. In addition, a variety of transcription factors have been shown to participate in the metformin-induced inhibition of gluconeogenic genes in the liver (Figure 2). Kim *et al.* demonstrated that metformin regulates hepatic gluconeogenesis through an AMPK-mediated upregulation of the orphan nuclear receptor small heterodimer partner (SHP), which operates as a transcriptional repressor [43]. SHP inhibits CREB-dependent hepatic gluconeogenic gene expression via direct interaction with CREB and competition with CRTC2 binding in the CREB-CBP complex [44]. Takashima *et al.* have proposed a role for Krüppel-like factor 15 (KLF15) in the metformin-induced inhibition of genes coding for both gluconeogenic and amino acid catabolic enzymes, these later being potentially implicated in the regulation of gluconeogenesis through the control of gluconeogenic substrate availability [45]. Metformin suppressed KLF15 gene expression and promoted its ubiquitination for proteasomal degradation. Restoration of KLF15 expression only partially rescued the inhibitory effect of metformin on hepatic glucose production, indicating that other factors also contribute to metformin action [45].

Understanding the mechanism of action of metformin is further complicated by our recent study establishing that both LKB1 and AMPK activities are dispensable for metformin-induced inhibition of glucose output or gluconeogenesis [46]. We indeed reported that a reduction in hepatic energy status, but not AMPK activation, constitutes the critical factor underlying the effects of metformin on the regulation of hepatic glucose output [46]. Since the rate of hepatic glucose production is closely linked to hepatic energy metabolism (6 ATP equivalents are required per molecule of glucose synthesized), disruption of the main energy supply in hepatocytes (mitochondrial oxidative phosphorylation) through inhibition of the respiratory-chain complex 1 would have a profound effect on the flux through gluconeogenesis (Figure 2). In addition, as AMP tends to rise whenever ATP falls, this could also provide an alternative explanation for the acute inhibition of gluconeogenesis by metformin *via* allosteric regulation of key enzymes in this pathway, such as fructose-1,6-bisphosphatase [47]. Of particular note is the metformin-induced inhibition of glucose production independently of transcriptional repression of gluconeogenic genes. Interestingly, forced expression of key gluconeogenic genes through PGC-1 α overexpression did not reduced metformin-induced reduction of glucose output, but was again associated with a significant depletion of energy stores [46]. These results indicate that metformin could acutely suppress gluconeogenesis *via* a transcription-independent process and that changes in gene expression are therefore not the exclusive determinant in the regulation of glucose output (Figure 2). Interestingly, suppression of hepatic glucose production by metformin in insulin-

resistant high-fat fed rats is dependent on an inhibition of the substrate flux through G6Pase, and not of a decrease in the amount of the protein [13], supporting the notion that metformin action is related to a reduction of the gluconeogenic flux rather than a direct inhibition of gluconeogenic gene expression.

In addition to the suppression of endogenous glucose production, metformin has been shown to be beneficial in improving lipid metabolism. Evidence that metformin improved fatty liver disease by reversing hepatic steatosis in *ob/ob* mice [48, 49] and in rodents fed a high-fat diet [50] has been demonstrated but also reported in some clinical studies [51, 52]. Metformin-induced reduction in hepatic lipid content is consistent with an increase in both fatty acid oxidation and inhibition of lipogenesis, presumably mediated by AMPK activation [17, 48, 53]. Indeed, AMPK coordinates the changes in the hepatic lipid metabolism and, so, regulates the partitioning of fatty acids between oxidative and biosynthetic pathways [18]. Thus, AMPK activation by metformin induces the phosphorylation and inactivation of acetyl CoA carboxylase (ACC), an important rate-controlling enzyme for the synthesis of malonyl-CoA, which is both a critical precursor for the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation [17]. In human hepatoma HepG2 cells, metformin enhances ACC phosphorylation and induces the reduction on triglycerides levels, which can be supported by increased fatty acid oxidation and/or decreased fatty acid synthesis [53]. In addition, AMPK suppresses expression of lipogenic genes such as fatty acid synthase, S14 and ACC by direct phosphorylation of transcription factors (carbohydrate response element binding protein (ChREBP) and hepatocyte nuclear factor 4 (HNF4)) and co-activators (p300) [54-59]. Metformin participates in the regulation of lipogenesis gene expression by down-regulating sterol regulatory element-binding protein-1c (SREBP-1c) gene expression [17] and by inhibiting its proteolytic processing and transcriptional activity upon AMPK-mediated phosphorylation at Ser372 [60]. A recent study by Kim *et al.* reported that metformin induces AMPK-mediated thyroid hormone receptor 4 (TR4) phosphorylation and repression of stearoyl-CoA desaturase 1 (SCD1) expression, a rate-limiting enzymes involved in the biosynthesis of monounsaturated fatty acids from saturated fatty acids [61].

Fatty liver disease is strongly associated with insulin resistance and the apparent inhibition of hepatic glucose production by metformin may be secondary to the primary improvement of hepatic steatosis and insulin resistance. This hypothesis may also offer a potential explanation for the loss of metformin effect on blood glucose levels in liver-specific LKB1 knockout mice fed a high-fat diet [37]. Thus, impaired metformin-induced AMPK

phosphorylation could fail to reduce hepatic lipid levels and to improve insulin sensitivity in liver-specific LKB1-deficient mice, impeding normalization of blood glucose levels.

B- Metformin's action on diabetic nephropathy

Despite a large panel of antidiabetic agents, it is assumed that between 20 and 40% of patients with diabetes ultimately develop diabetic nephropathy. Diabetic nephropathy is a microvascular complication of diabetes, others including diabetic retinopathy and diabetic neuropathy. Because metformin is excreted by the kidney, the reduction of metformin renal clearance is considered as an important risk factor of lactic acidosis. In consequence, it is recommended to review the dose of metformin if the serum creatinine exceeds 130 $\mu\text{mol/l}$ (or estimated glomerular filtration rate is $<45 \text{ ml/min/1.73 m}^2$) and to stop metformin treatment if the creatinine levels rises above 150 $\mu\text{mol/l}$ (or estimated glomerular filtration rate is below 30 ml/min/1.73 m^2) [1]. But recently, some data accumulate suggesting that metformin could favour a protection against the deleterious consequences of hyperglycemia in kidney. Some of these data come from rodent models of diabetes as the Zucker diabetic fatty rats. In this model, Takiyama *et al.* demonstrated that metformin treatment (250 mg/kg/d) during 9 to 39 weeks ameliorates tubular injury associated with hyperglycemia while no protective effect was observed with insulin [62]. The authors showed that metformin specifically reduces HIF-1 expression (and their specific target genes) not only by reducing ATP synthesis but also by a drop of renal oxygen consumption in renal cells. Interestingly, the beneficial effect of metformin is preserved after knockdown of AMPK α subunit and can not be reproduced by AICAR (AMPK activator) or rapamycin (mTORC1 inhibitor) [62]. This suggested that, in this case, metformin acts independently of AMPK by decreasing renal oxygen consumption. Because chronic hypoxia and consequent increased HIF-1 expression are now considered as a key event during the initiation and progression of diabetic nephropathy and kidney fibrosis, the management of renal chronic hypoxia becomes a new therapeutic strategy for the prevention of diabetic nephropathy. It has been also demonstrated that metformin prevents gentamicin-induced acute renal failure, presumably by decreasing reactive oxygen species (ROS)-mediated lipid peroxidation [63], and decreases the TGF β -induced epithelial-to-mesenchymal transition (EMT), a key event in the development of the tubulointerstitial fibrosis during the diabetic nephropathy [64].

It is now well accepted that hyperglycemia increases ROS production in diabetic podocytes, contributing to the development of diabetic nephropathy. Until now, all the

strategies used to reduce the ROS production in diabetic kidney have failed. Interestingly, Piwkowska *et al.* showed that metformin activates AMPK and decreases the NAD(P)H oxidase activity, ultimately leading to reduction of ROS production in cultured podocytes [65]. In consequence, control of ROS production by metformin could be a new optimal strategy for the management of diabetic nephropathy. Recently, the deleterious role of lipotoxicity in kidney has been recognized in Goto-Kakizaki [66] and OLETF rats [67], two rodent models of T2D. In this last one, diabetic nephropathy was correlated with the kidney triglycerides content and metformin reduces fat content by decreasing SREBP-1, FAS and ACC expression in kidney [67]. The reduction of renal lipotoxicity by metformin could thus be a new strategy for the prevention of diabetic nephropathy. Another intriguing effect of metformin is a reduction of cystic growth in the dominant polycystic kidney disease mice model [68]. This beneficial effect has been explained by AMPK activation by metformin and subsequent inhibition of both CFTR and mTOR pathways, demonstrating that the drug can modulate multiple molecular pathways in the kidney.

The reduction of kidney damage in animal models of T2D has to be confirmed in clinical studies. Taken together, a careful revision of clinical recommendations, especially the contra-indications of metformin use, may be required, in agreement with recent literature reviews suggesting that metformin treatment could probably be extended to all diabetic patients [69, 70]. Thus, because the risk of lactic acidosis is extremely low while the use of metformin results in cardiovascular, renal and survival benefits, the use of metformin should be revised accordingly to clear glomerular filtration rate cut-off or adaptation of metformin dose by determination of its plasma levels [71].

C- Metformin's action on the cardiovascular system

Ischemic heart disease (IHD) remains the leading cause of death in the patients with T2D [72]. Importantly, the UKPDS longitudinal trial demonstrated that metformin reduced by 42% the diabetes-related death (9-63, $p=0.017$), and by 36% all-cause mortality (9-55, $p=0.011$). Interestingly, in this study, metformin was used as a primary prevention and its beneficial effect was rapidly observed after a median duration of the study of 10.7 years [3]. This conclusion has been replicated in other clinical or epidemiological studies [73, 74]. Thus, the use of metformin as the first-line antidiabetic treatment in T2D patients was not only justified by the antihyperglycemic effect of the drug but also by the reduction of the mortality rate in this population. The mechanisms of such beneficial effect are not clearly

understood but data accumulated concerning some potential mechanisms of metformin action in heart including the promotion of myocardial preconditioning, the reduction of cardiomyocytes apoptosis during ischemia, the adaptation of cardiomyocytes metabolism during ischemia or the protection against the development of heart failure.

Experimental evidence suggests that metformin reduces cardiac ischemia/reperfusion injury. Indeed, Yin *et al.* showed that metformin treatment improves cardiac function (preserved left ventricular ejection fraction) and reduces the infarct size after a myocardial infarction in Sprague-Dawley rats [75]. By contrast with sham-operated rats, the metformin group was less insulin resistant and has altered myocardial AMPK phosphorylation status during cardiac remodeling. Myocardial preconditioning is now recognized as a protective mechanism that allows reducing the infarct size and the consequent risk of heart failure. Induction of such mechanism has been demonstrated in a model of rats with neonatal streptozotocin T2D treated or not by metformin 3 days prior to an ischemia-reperfusion of the heart [76]. In this study, metformin induced preconditioning was supported by a reduction of the infarct size in the treated group.

Cardioplegic-induced hypoxia/reoxygenation (H/R) injury results in cardiomyocyte apoptosis. In cardiomyocytes, metformin attenuated the production of proapoptotic proteins, increased the antiapoptotic proteins and reduced the percentage of apoptotic cardiomyocytes [77]. This effect was correlated with an activation of AMPK and reproduced by AICAR, another AMPK activator. Another property of metformin (which seemed fundamental to reduce the risk of heart failure) is the adaptation of cardiac metabolism during myocardial ischemic condition. The healthy heart gets 60-90% of its energy for oxidative phosphorylation from fatty acid oxidation [78]. The failing heart has been demonstrated to shift toward an increased glucose uptake and utilization [79]. Because utilization of fatty acid costs more oxygen per unit of ATP generated than glucose, the promotion of a metabolic shift from fatty acids oxidation to glucose utilization may improve ventricular performance and slow the progression of heart dysfunction [78, 79]. Thus, in a volume-overload model of heart failure in rats (aortocaval fistula), Benes *et al.* demonstrated that metformin normalized serum NEFAs, and modified the cardiac lipid/glucose oxidation ratio, suggesting a metabolic adaptation induced by the drug [80].

Another new area of intense research is the possibility to use metformin in patients with a history of heart failure. Metformin is classically contraindicated in patients with heart failure because this condition increases the risk of lactic acidosis. Surprisingly, recent evidence suggests that this contraindication could be revised [81]. Indeed, metformin alone or in

combination with sulfonylurea reduced the mortality and the morbidity in T2D patients with heart failure in comparison of sulfonylurea monotherapy [82-84]. This unexpected result has been found in the Reduction of Atherothrombosis for Continued Health (REACH) Registry which included 19691 T2D patients with established atherothrombosis [85]. The mortality rate was significantly lower in patients treated with metformin, including the ones in whom metformin use is not now recommended (history of congestive heart failure, patients older than 65 years and patients with an estimated creatinine clearance of 30 to 60 mL/min/1.73 m²). The cardiovascular protection in metformin-treated T2D patients seems not to be dependent of reduction in HbA1c level since it was not observed with other oral antidiabetic drugs [86], suggesting that metformin has specific properties on cardiovascular outcomes. Even if further studies are needed to better understand this specific point at the molecular level, some original mechanisms have already been proposed. Thus, dysregulated autophagy has been described as a key mechanism for the development of diabetic cardiomyopathy and heart failure. Interestingly, treatment with metformin restored impaired autophagy in OVE26 diabetic mice and prevented heart damage in this model [87]. This effect is probably dependent of cardiac AMPK activation since metformin is inefficient in cardiac-specific AMPK dominant-negative transgenic diabetic mice. In addition, Gundewar *et al.* demonstrated that metformin significantly improves left ventricular function and survival in a murine model of heart failure (myocardial ischemia induced by left coronary artery occlusion) [88]. The authors showed that metformin significantly improved myocardial cell mitochondrial respiration and ATP synthesis by an underlying mechanism requiring activation of AMPK and its downstream mediators, eNOS and PGC-1 α [88]. Similar prevention of both heart failure and mortality by metformin was observed in a dog model of heart failure [89]. In this case, other AMPK activators such as AICAR have equivalent effects than those of metformin, suggesting that myocardial AMPK activation is also required. A large proportion of T2D patients have chronic high blood pressure, which is known to induce cardiac hypertrophy and fibrosis. Metformin inhibits cardiac hypertrophy in a rat model of pressure overload (transaortic constriction) *via* a reduction of angiotensin II-induced protein synthesis and enhanced phosphorylation of AMPK and eNOS, leading to subsequent increase in NO production. All of these effects were abolished by Compound C, a non-specific AMPK inhibitor [90].

Beyond its specific effects in heart, the reduction of mortality by metformin asked also the question of how endothelial dysfunction and atherogenesis could be prevented by the drug. Endothelial dysfunction, as characterized by an impairment of endothelium-dependent

relaxation and reduced NO bioactivity, is the critical step for atherogenesis. In addition, vascular NO inhibits platelet aggregation and adhesion and can also reduce leucocytes adhesion to the vessel wall (see for review [91]). Schulz *et al.* have demonstrated that AMPK phosphorylates and activates eNOS in cultured endothelial cells, stimulates NO synthesis in response to several agonists and increases endothelium-dependent vasodilatation in animal model [92]. Taken together, such data suggested an anti-atherogenic role for the AMPK system. High glucose leads to endothelial ROS overproduction which promotes endothelial dysfunction. Metformin, decreased intracellular ROS production in aortic endothelial cells by inhibiting both NAD(P)H oxidase and the respiratory-chain complex 1 [93]. Furthermore, activated AMPK reduces hyperglycaemia-induced mitochondrial ROS production by induction of Mn-SOD and promotion of mitochondrial biogenesis through activation of the PGC-1 α pathway in HUVEC [94]. Lastly, activated AMPK largely offsets the adverse effects of palmitate on endothelial superoxide production and NF- κ B activation. Recently, two additional vascular target of metformin have been described: the advanced glycated end products (AGEs), the soluble intercellular cell-adhesion molecules (ICAMs) and the soluble vascular cell-adhesion molecules (VCAMs). AGEs are important contributors of diabetic complications by promoting cellular oxidative stress and inflammation. It has been reported that metformin can reduce AGE synthesis and their specific cell receptor expression independently of its anti-hyperglycemic effects [95]. Although done *in vitro*, this suggests that metformin can directly modulate the glycation process. In addition, excessive plasma levels of ICAM-1 and VCAM-1 are linked with an increase of cardiovascular events. Interestingly, as for AGE, metformin decreases ICAM-1 and VCAM-1 levels in T2D patients independently of its normoglycemic property [96]. These studies support the notion that activated AMPK has a beneficial effect on endothelial function by suppressing oxidative stress in endothelial cells [97]. Altogether, these data suggested that metformin has complex properties on endothelial functions, ROS production and cardiomyocytes functionality.

D- Metformin's action on the polycystic ovary syndrome

The polycystic ovary syndrome (PCOS) is a common endocrinopathy, affecting at least 5 to 15% of reproductive-aged women [98]. The revised diagnostic criteria of PCOS associated menstrual disturbance and/or hyperandrogenism and/or polycystic ovary on ultrasound [99]. It is now recognized that insulin resistance is a common but not an imperative feature in PCOS. As a consequence, insulin sensitizers have been proposed as a

pharmaceutical option in overweight women with PCOS and insulin resistance. Recently, a meta-analysis of 31 clinical trials demonstrated that metformin treatment may increase ovulation, improve menstrual cyclicity, and reduce serum androgen levels in these patients [98]. These beneficial effects of metformin are based on alleviation of insulin excess acting upon ovary and through direct ovarian effects. Insulin was shown to directly stimulate several steroidogenic enzymes in the ovary, such as CYP17, 3 β -HSD and StAR protein. By improving insulin sensitivity, metformin reduces CYP17 activity [100]. Furthermore, metformin suppresses androstenedione production by a direct effect on ovarian theca cells and decreases FSH-stimulated 3 β -HSD, StAR, CYP11A1 and aromatase activities in both rat granulosa cells and women with PCOS (with reduction of basal and of FSH-stimulated progesterone and estradiol levels as a consequence) [100]. The molecular pathways whereby metformin acts directly on the ovary remain elusive. Recently, it has been demonstrated that metformin treatment increased AMPK activity in rat granulosa cells, leading to subsequent reduction of steroid synthesis [101]. However, it is still unclear whether this effect is AMPK-dependent or not. Pharmacogenetics aspects of metformin action have to be taken into account in the effect of the drug on PCOS. Indeed, data from the Pregnancy in PCOS (PP-COS) trial indicated that a polymorphism in LKB1 gene is associated with a significant decreased chance of ovulation in PCOS patients treated with metformin [102]. Interestingly, metformin has been shown to reduce the risks of abortion in women with PCOS at high risk of pregnancy and neonatal complications by increasing some factors needed for implantation and pregnancy safekeeping, such as IGFBP-1 and glycodelin levels, or uterine artery blood flow [100]. By contrast, metformin reduces factors increasing the abortion risk, such as endometrial androgens receptor expression, plasminogen activator inhibitor-1 (PAI-1) levels and plasmatic endothelin-I (ET-1). Most of these effects are probably mediated by the metformin-induced improvement in insulin sensitivity. From a clinical point of view, metformin administration should be considered as initial intervention in (overweight or obese) PCOS patients especially when oral contraception is contraindicated or when insulin resistance was present.

E- Metformin's action on cancer

Recent prospective and case-control studies conducted on large cohorts have confirmed that T2D is associated with significantly increased risk of cancer mainly affecting breast, colon, prostate, kidney and pancreas [103]. This increased risk has been attributed to the

growth-promoting effect of chronic elevated plasma insulin levels [104]. Insulin resistance and resultant hyperinsulinemia might indeed promote carcinogenesis directly through the insulin receptor or indirectly by increasing the levels of insulin-like growth factors (IGF), steroid sex hormones, inflammatory processes and disrupting adipokines homeostasis [104]. However, additional explanations for this association may be invoked such as the role of persistent elevated plasma glucose levels [105]. Given the epidemiological evidence between T2D and increased risk of cancer, the impact of metformin therapy on cancer risks and cancer-related mortality has been evaluated in a first pilot case-control study with a cohort of 12000 T2D patients [106]. Metformin therapy was associated with a reduced risk of cancer (odds-ratio of any exposure to metformin was 0.79). Furthermore, the authors found a dose-response relationship between duration of exposure to metformin and cancer incidence [106]. Similarly, more recent retrospective and observational studies reported reduced incidence of neoplastic diseases and cancer mortality in T2D patients treated with metformin [107-109]. Importantly, metformin use has been associated with a significant decrease in the relative risk of specific cancers, such as prostate, pancreas and breast cancers [110-112]. These observations are consistent with *in vitro* and *in vivo* studies showing antiproliferative action of metformin on various cancer cell lines [113] and several cancers in animal models (Table 1).

Although the underlying mechanisms are not yet completely elucidated, the association between metformin and reduced risk of cancer in T2D patients may be simply explained through metformin action on improvement of blood glucose and insulin levels [114]. Accordingly, prevention of tumor growth in animal models with diet-induced hyperinsulinemia is attributable to reductions in circulating insulin levels [115, 116]. Given that hyperinsulinemia is associated with increased levels of IGF-1, it is possible that the metformin-lowering effects on serum insulin and IGF-1 levels might explain, at least in part, its therapeutic efficacy (Figure 3). This hypothesis is particularly relevant in light of recent studies showing that calorie restriction, which lowers insulin and IGF-1 levels, induces a dramatic decrease in the incidence of cancer in rodent models [117]. However, a decrease in insulinemia is not always correlated with metformin efficacy as shown in PTEN^{+/-}, HER-2/neu and APC^{min/+} mouse tumor models, indicating an insulin-independent antitumoral action of metformin [118-120]. Hence, metformin appears to have a direct action on tumor growth both *in vitro* and *in vivo* by a mechanism involving activation of the LKB1/AMPK pathway and subsequent modulation of downstream pathways controlling cellular proliferation (Figure 3). AMPK knock-down by siRNA or AMPK inhibitors partially revert the antiproliferative action of metformin in breast and ovarian cancer cells [121-123].

Furthermore, antitumoral action of metformin was significantly reduced in mice displaying a reduction in LKB1 expression [119]. The antineoplastic activity of metformin *via* AMPK activation is mediated through the inhibition of mTORC1 signaling, leading to inhibition of protein synthesis and cell proliferation [121, 123, 124]. AMPK inhibits mTORC1 at multiple levels through the phosphorylation of tuberous sclerosis 2 protein (TSC2) on Ser¹³⁴⁵, leading to accumulation of Rheb-GDP (the inactive form), and the phosphorylation of raptor on Ser⁷²² and Ser⁷⁹², which disrupts its association with mTOR and thereby prevents mTORC1 activation. However, recent studies revealed the existence of an alternative AMPK-independent pathway, potentially mediated by RAG GTPase, by which metformin inhibits mTORC1 signaling [125]. Of particular note is the inhibition of IGF-1-induced mTOR activity by thiazolidinediones, another class of antidiabetic drugs which activated AMPK [18], indicating that activation of the kinase could further attenuate signaling pathways downstream insulin and/or IGF-1 receptors, particularly at the level of mTOR [126]. Furthermore, it is of interest that metformin-induced activation of AMPK disrupted crosstalk between insulin/IGF-1 and G-protein-coupled receptor signaling pathways in pancreatic cancer cells [127]. Another mode of action of metformin might be through an AMPK-mediated regulation of fatty acid synthesis. Indeed, cells derived from prostate, breast and colon cancers constitutively over-express fatty acid synthase (FAS), a key enzyme for *de novo* fatty acid biosynthesis, which has been associated with the malignant phenotype. Interestingly, it has been observed that reduction of FAS and ACC expression by AMPK activation diminishes the viability and growth of prostate cancer cells [128]. Another potential mechanism is based on the positive impact of metformin on chronic inflammation [129], a major contributory factor to cancer development and progression. Emerging results showing the capacity of AMPK to inhibit the inflammatory responses [130] suggest that metformin may also target the inflammatory component present in the microenvironment of most neoplastic tissues, leading to tumor reduction. In addition, inhibition of neoplastic angiogenesis by metformin might also participate in the reduction of tumor growth [131]. Consistently, metformin has been shown to significantly decrease the levels of vascular endothelial growth factor (VEGF) and PAI-1 [132].

Although these results suggest a pivotal role of LKB1/AMPK signaling, the antineoplastic action of metformin could also be independent of AMPK activation. Indeed, metformin was reported to decrease the expression of the oncoprotein HER2 (*erbB-2*) in human breast cancer cells *via* a direct and AMPK-independent inhibition of p70S6K1 activity [133]. Metformin also exerts its anti-cancer effect through induction of cell-cycle arrest in

prostate cancer cell lines *via* a decrease in cyclin D1 protein expression [134] and an increase in REDD1 expression in a p53-dependent manner [135]. In breast cancer cells, metformin-induced cell-cycle arrest is due to enhanced binding of CDK2 by p27Kip or p21Cip in addition to cyclin D1 downregulation and AMPK activation [136]. In addition to the inhibition of cancer cells proliferation, metformin has been shown to promote cell death of some cancer cells through activation of apoptotic pathways by both caspase-dependent and caspase-independent mechanisms [137, 138]. The caspase-independent pathway involved activation of poly(ADP-ribose) polymerase (PARP) and correlates with enhanced synthesis of PARP and nuclear translocation of apoptosis-inducing factor (AIF), which plays an important role in mediating cell death [139]. Additionally, it was showed that metformin-stimulated apoptosis of colon cancer cells was associated with loss of p53-dependent enhancement of autophagy and glycolysis, an effect stimulated by nutrient deprivation. In contrast, metformin promotes apoptosis of prostate cancer cells in a p53-dependent manner in the presence of 2-deoxyglucose [140].

Metabolic adaptations are critical to maintain survival of cancer cells that are often under a variety of stress stimuli, such as hypoxia and lack of nutrients. To successfully meet their high metabolic demand, it is crucial that cancer cells engage proper adaptive responses to provide sufficient ATP supply and support survival. A recent study revealed that AMPK activation promotes the survival of cells metabolically impaired by glucose limitation in part through p53 activation [141]. It has been suggested that metformin could inhibit the growth of cancer cells by decreasing cellular energy status and force a metabolic conversion that cancer cells are unable to execute. Indeed, loss of p53 impairs the ability of cancer cells to respond to metabolic changes induced by metformin and to survive under conditions of nutrient deprivation [142]. Similarly, LKB1-deficient cells were more sensitive to metformin-induced energy stress when cultured at low glucose concentration and were unable to compensate for the decreased cellular ATP concentration causing cell death [116]. A recent report demonstrated that the combination of metformin and 2-deoxyglucose inhibited mitochondrial respiration and glycolysis in prostate cancer cells leading to a massive ATP depletion and affect cell viability by inducing apoptosis [140].

F. New therapeutic perspectives

1- Gestational diabetes

The risk of gestational diabetes (GD) is increasing in obese women and is associated with adverse pregnancy outcomes [143]. Recently, data accumulated from case control studies [144] or the Metformin in Gestational Diabetes Trial [145] suggested that women treated with metformin had less weight gain and improved neonatal outcomes compared with those treated with insulin. Although no significant adverse events were observed when metformin was administered during pregnancy, its use in overweight women with GD has to be confirmed by additional studies and new guidelines.

2- Diabetes prevention

The Diabetes Prevention Program (DPP) was a clinical trial comparing the efficacy of lifestyle modifications and metformin on glucose homeostasis in 3234 pre-diabetic patients. In this study, metformin was efficient to significantly reduce (-31%) the development of T2D [146]. Even if reduction of body weight through physical activity and hypocaloric diet is unanimously recognized as a cornerstone for a global prevention of T2D, the use of metformin in pre-diabetic population looks promising but have to be evaluated in additional studies.

3- Regulation of circadian clock

Mammalian behavior, including spontaneous locomotion, sleeping, eating, and drinking are influenced by a circadian system, composed of a central clock in the brain and subsidiary oscillators existing in peripheral tissues. Circadian rhythms are regulated by alternating actions of activators and repressors of transcription, in particular CLOCK (circadian locomotor output cycles kaput), BMAL1 (brain and muscle ARNT-like protein 1), PER (Period) and CRY (Cryptochrome) [147]. Um *et al.* [148] have recently proposed a molecular mechanism by which metformin causes a dramatic shift in the circadian phase of peripheral tissues. It was indeed shown that metformin-induced AMPK activation promotes phosphorylation of Ser 386 on casein kinase 1 (CK1), one of the key circadian regulators, thereby enhances the CK1-mediated phosphorylation of PER2, leading to its the degradation and ultimately to the shortening of the period length. Accordingly, PER2 accumulates to higher levels in organs of mice lacking the catalytic subunit $\alpha 2$ of AMPK [148]. Recent evidence indicates that dysregulation of circadian functions could underlie, at least partly, the development of obesity and insulin resistance. Caton *et al.* recently examined the effect of metformin on the dysregulation of clock genes in adipose tissue of obese *db/db* mice and in mice fed a high-fat diet [40]. Interestingly, metformin markedly enhanced expression of the

core clock components CLOCK, BMAL1 and PER2 through induction of AMPK-NAMPT-SIRT1 signaling and was associated with reduction of hyperglycemia and hyperinsulinemia in *db/db* mice. Taken together, the apparent beneficial association between targeted modulation of the circadian system and whole-body metabolic state suggests that chronotherapy could be a promising approach for the treatment of obesity and T2D.

4- Metformin and pharmacogenetics

Metformin is a hydrophilic base which exists at physiological pH as an organic cation (pKa 12.4). Consequently, its passive diffusion through cell membranes is very limited. Indeed, it has been shown that metformin only negligibly permeate the plasma membrane by passive diffusion [29] and cationic transporters such OCT1/2 are, to date, the main transporters identified to be involved in the intracellular internalization of the drug [15, 149]. It is worth noting that most of the experiments using metformin were performed in immortalized cell lines treated with drug concentrations far above those reported to accumulate in tissues after oral administration of metformin [149]. It may be related to the low levels of cationic transporters at the surface of these cell lines. Interestingly, uptake of metformin into immortalized cell lines is very low and can be greatly enhanced by ectopic expression of organic ion transporter cDNAs [150, 151]. Thus, the key determinant for metformin's action appears to be a balance between concentration and time of exposure which can in fact reflect the tissue/cell-specific abundance of organic transporters

Understanding the link between genetic variation and response to drugs will be essential to move towards personalized medicine. Metformin requires the organic cation transporters (OCTs) to be transported into the liver and the gut (OCT1) and the kidney (OCT1 and OCT2) [152]. In contrast, the multidrug and toxin extrusion 1 protein (MATE1) facilitates metformin excretion into bile and urine [152]. OCT1 and OCT2 are encoded by the *SLC22A1* and *SLC22A2* genes, respectively, and MATE1 by the *SLC47A1* gene. In OCT1^{-/-} mice, hepatic metformin concentration in the liver and in the gut is lower than in control mice suggesting that OCT1 is essential for the uptake of the drug in these tissues [15]. Recently, Shu *et al.* showed that *SLC22A1* variants reducing OCT1 function increased the area under the curve of glucose during OGTT after metformin treatment in healthy volunteers compared to subjects with wild type alleles [15]. In contrast, the loss-of-function variants R61C and 420del have no consequence on HbA1c level achieved during metformin treatment in T2D patients [153]. Urinary excretion of metformin is preserved in OCT1^{-/-} mice indicating that renal excretion

of metformin is dependent of OCT2. This last point has been challenged by Tzvetkov *et al.* which demonstrate a significant OCT1 expression in human kidney and a reduction of metformin renal clearance depending on OCT1 polymorphisms (Arg61Cys, Gly401Ser, 420del or Gly465Arg) [154]. OCT2 polymorphisms are also known to modify metformin renal clearance. Indeed, 14 genetic variants of *SLC22A2* gene were identified of which the 808G>T polymorphism was associated with a reduced metformin tubular clearance and prevented the tubular secretion of metformin by cimetidine [155]. Other important OCT2 variants for the renal elimination of metformin have been described in healthy volunteers (see for review [156]). Nevertheless, the clinical relevance of such variants in T2D patients remains to be determined in a large scale studies. In addition, other candidate genes may be involved in the therapeutic response to metformin in diabetic population. Thus, in a genome-wide association study investigating glycemic response to metformin, Zhou *et al.* have found a locus associated with treatment success and containing the ataxia telangiectasia mutated gene (ATM), a gene involved in DNA repair and cell cycle control [157]. Interestingly, it was found that inhibition of ATM markedly reduced AMPK activation by metformin indicating that ATM acts upstream of AMPK and is probably required for mediating metformin effect [157].

Conclusion

Metformin is currently used as an antihyperglycemic agent. It is accepted that the main effect of this drug is to decrease hepatic glucose production through a mild inhibition of the mitochondrial respiratory-chain complex 1. As a consequence, the resulting transient decrease in cellular energy status promotes activation of AMPK, a well known cellular energetic sensor. Consequently, metformin-induced AMPK activation is believed to promote transcriptional inhibition of hepatic gluconeogenic program. Nevertheless, our recent findings showing that inhibition of hepatic glucose production by metformin is preserved in liver-specific AMPK knockout mice strongly suggests that other mechanism(s) are involved. Thus, the decrease in hepatic energy status following inhibition of the respiratory-chain complex 1 by metformin is probably the central explanation for the acute reduction of hepatic gluconeogenesis by the drug. Additionally, AMPK-dependent mechanisms linked to the action of metformin on hepatic lipid metabolism are also proposed, notably for explaining its beneficial effect on hepatic steatosis and insulin resistance, leading to the normalization of blood glucose levels. Interestingly, metformin demonstrated protective properties against

diabetic complications, especially by reducing the diabetes-related death rate. Although still unclear, multiple molecular mechanisms were proposed, including modulation of myocardial preconditioning, reduction of cardiomyocytes apoptosis during ischemia, metabolic switch during ischemia, protection against the development of heart failure or protection of endothelial functions. Most of them require AMPK activation while others (as the anti-oxidative properties of metformin on endothelial cells) seems to be kinase-independent. Metformin appears to impact ovarian function in PCOS in a dual mode, by decreasing insulin resistance and by direct ovarian effects. Finally, the risk of lactic acidosis is relatively low in comparison of the multiple benefits of metformin treatment, explaining the exponential development of its therapeutic use. New clinical indications of metformin are awaiting large scale clinical studies before to be recommended. Among them, the use of metformin in cancer therapy and for large scale prevention in pre-diabetic populations looks the most promising ones. Finally, new data from pharmacogenetics studies will also provide new findings to predict or adapt the dose of metformin in personalized medicine.

Table 1 : Protective effect of metformin in animal models for cancer.

Cancer Model	Tumor type	Treatment	Route	Ref.
Xenograft	Pancreas	250 mg/kg	IP	[127]
	Lung	50 mg/kg	Oral	[158]
	Colon	250 mg/kg	IP	[142]
	Prostate	200 µg/ml 1 mg/day	Oral IP	[134]
	Breast	2 mg/ml	Oral	[138]
	Leukemia	250 mg/kg	IP	[124]
	Cancer stem cells	100 µg/ml	IP	[159]
chemically-induced	Breast	50-500 mg/kg	Oral	[160]
	Lung	250 mg/kg	IP	[161]
	Colon	250 mg/kg	IP	[162]
	Pancreas	320 mg/kg	Oral	[115]
HER2/neu mice	Breast	100 mg/kg	Oral	[118]
PTEN ^{+/-} mice	All	300 mg/kg	Oral	[119]
APC ^{Min/+} mice	Gastrointestinal polyps	250 mg/kg	Oral	[120]

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Figure legends

Figure 1. The mitochondrial respiratory-chain complex 1 is the primary target of metformin. Due to its high acid dissociation constant ($pK_a=12.4$) metformin exists in a positively charged protonated form under physiological conditions and, as a result, can only marginally cross the plasma membrane by passive diffusion. Thus, its intracellular transport is mediated by different isoforms of the organic cation transporters (OCT) depending of the tissue considered (*e.g.* OCT1 in liver or OCT2 in kidney). Once inside the cytosolic compartment, mitochondria then constitute the primary target of metformin. The positive charge of metformin was proposed to account for its accumulation within the matrix of energized mitochondria, driven by the membrane potential ($\Delta\phi$), whereas the apolar hydrocarbon side-chain of the drug could also promote binding to hydrophobic structures, especially the phospholipids of mitochondrial membranes [31]. Although the exact mechanism(s) by which metformin acts at the molecular level remains unknown, it has been shown that the drug inhibits mitochondrial respiratory-chain specifically at the complex 1 level without affecting any other steps of the mitochondrial machinery. This unique property of the drug induces a decrease in NADH oxidation, proton pumping across the inner mitochondrial membrane and oxygen consumption rate, leading to lowering of the proton gradient ($\Delta\phi$) and ultimately to a reduction in proton-driven synthesis of ATP from ADP and inorganic phosphate (Pi).

Figure 2: Potential molecular mechanisms of metformin action on hepatic gluconeogenesis. After hepatic uptake through OCT1, the mitochondria is the primary target of metformin which exerts specific and AMPK-independent inhibition of respiratory-chain complex 1. The resultant mild decrease in energy status leads to acute and transient inhibition of energy-consuming gluconeogenic pathway. In addition, through AMPK-dependent and -independent regulatory points, metformin can lead to the inhibition of glucose production by disrupting gluconeogenesis gene expression. In parallel, the LKB1-dependent activation of AMPK triggered by ATP depletion could reduce hepatic lipogenesis and exert an indirect effect on hepatic insulin sensitivity to control hepatic glucose output.

Figure 3: Control of cell proliferation and tumor growth by metformin. The anti-neoplastic action of metformin appears to be exerted by several pathways. Metformin inhibits the growth of cancer cells by the reversal of hyperglycemia, insulin resistance and

hyperinsulinemia, resulting in reduced levels of glucose, insulin and IGFs and activation of growth signaling pathways through their respective receptors. The anti-tumor effects of metformin seems regulated by both AMPK-dependent or -independent mechanisms leading to the inhibition of mTOR signaling, cell cycle by decrease of cyclin D1 level, stimulation of p53/p21 axis, fatty acid synthesis, angiogenesis and inflammation. Adapted from [104] and reproduced from [163].

Figure 1

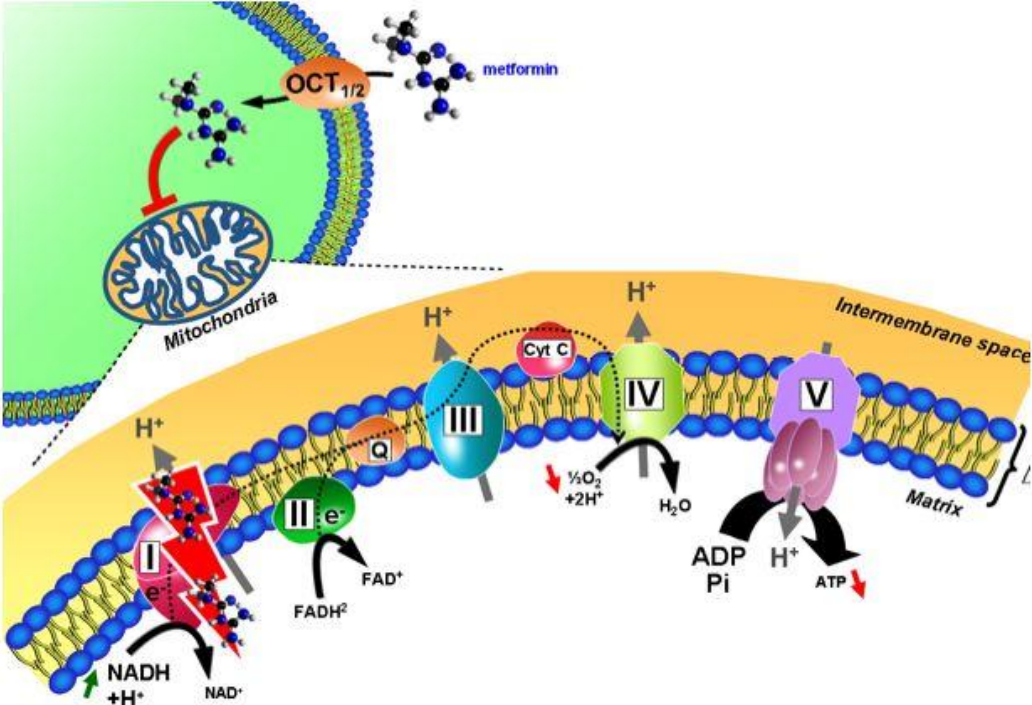


Figure 2

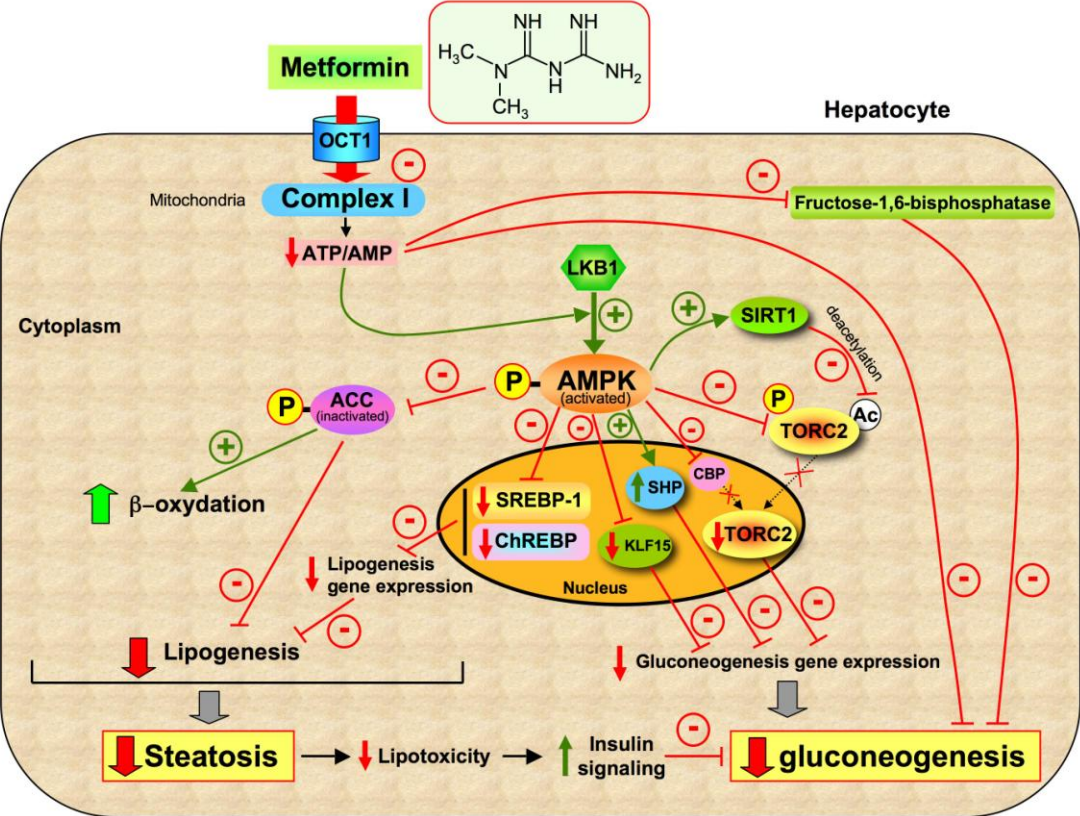


Figure 3

