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REVIEW ARTICLE

Entering the Sugar Rush Era: Revisiting the Antihyperglycemic Activities of Biguanides after a Century of Metformin Discovery

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Abstract: The development of clinically viable metformin analogs is a challenge largely to be overcome. Despite being an extremely efficient drug for the treatment of type 2 diabetes mellitus, multiple studies were conducted seeking to improve its hypoglycemic activity or to ameliorate aspects such as low oral absorption and the incidence of gastrointestinal side effects. Furthermore, efforts have been made to attribute new activities, or even to expand the pre-existing ones, that could enhance its effects on diabetes, such as pancreas-protective, antioxidant, and anti-inflammatory activities. In this paper, we describe the analogs of metformin developed in the last three decades, highlighting the lack of computationally based rational approaches to guide their development. We also discuss this is probably a consequence of how unclear the mechanism of action of the parent drug is and highlight the recent advances towards the establishment of the main molecular target(s) for metformin. We also explored the binding of metformin, buformin and phenformin to the mitochondrial respiratory chain complex I through molecular docking analyses and reviewed the prospects of applying computational tools to improve the success in the development of such analogs. Therefore, it becomes evident that the wide range of molecular targets and the multiple activities displayed by metformin make this drug a promising prototype for developing novel entities, particularly for treating type 2 diabetes mellitus.

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1. INTRODUCTION

When Werner and Bell first synthesized metformin (dimethylbiguanide) (Fig. **1**, comp. **1**) a century ago [1], they probably would have never imagined the impact this simple molecule could have on our lives. Metformin was not the first biguanide synthesized since Rathke had already reported the synthesis of a biguanide in 1879, and was not also the first guanidine drug marketed, considering Synthalin A (**2**) was launched in Europe in 1926 by Schering AG to be withdrawn only two years later because of renal and hepatotoxicities [2, 3]. In fact, the drug has received little attention in the first three decades since it was less potent in lowering blood glucose levels than other further marketed biguanides such as buformin (**3**) or phenformin (**4**), which were later discontinued due to the high risk of causing lactic acidosis [4]. The drug only became available in the UK in 1958, in Canada in 1972, and received USA FDA approval in 1998 [5].

Nevertheless, metformin became the first-line drug for treating patients with type 2 diabetes mellitus (T2DM), holding high efficacy, low cost, and no cardiovascular risk, rarely associated with hypoglycemia or weight gain [6]. Unlike phenformin or buformin, lactic acidosis is a very rare side effect of metformin (*i.e.*, about 1 per 20,000), mostly occurring in diabetic patients with severe renal failure [7].

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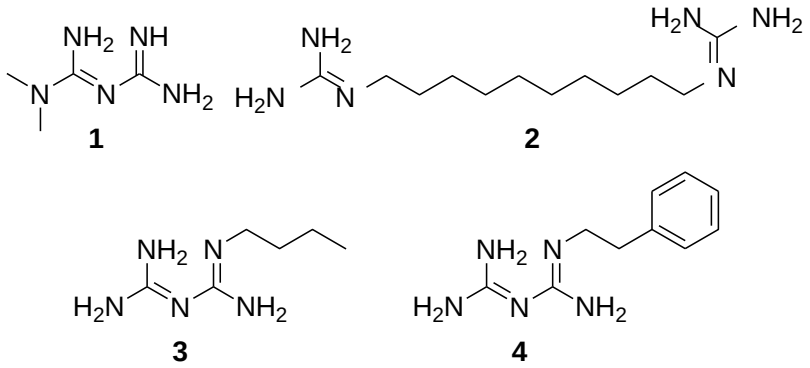


Fig. (1). Structures of metformin (**1**), Synthalin A (**2**), buformin (**3**), and phenformin (**4**).

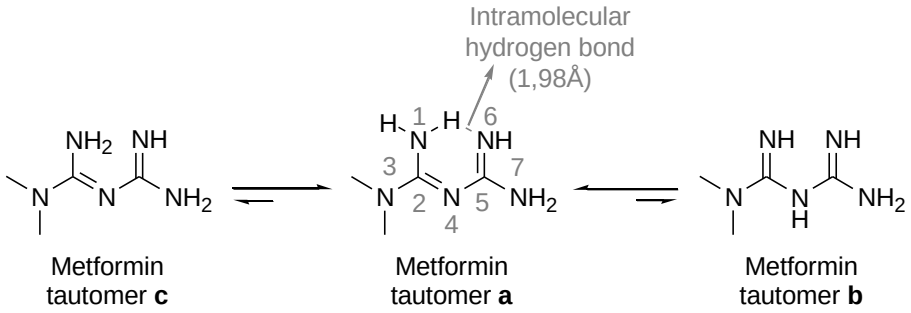


Fig. (2). Tautomeric forms of metformin: (**a**) major form, (**b**) minor form.

Although metformin is a centenary antihyperglycemic agent, which has also served as a prototype for the development of several anticancer biguanides [8], the general 2D structures of metformin and most of its derivatives continue to be misrepresented. Although biguanides are ubiquitously represented as analogous to diketones (Fig. **2,** tautomer **b**), crystallographic studies suggest the representation with conjugation across the $-C_2=N_4-C_5=N_6$ bonds as the more appropriate (Fig. **2,** tautomer **a**). Additionally, an intramolecular hydrogen bond between N_1 and N_6 can be inferred from the nearly planar structure observed, which results in a higher-than-expected melting point (135ºC). Analogously, the prevalence of a tautomer **a** as in metformin is also supported by crystallographic/theoretical studies for buformin and phenformin [9], but such information generally lacks for most other biologically active biguanides.

Concerning the pharmacokinetic properties of metformin, since the small substituents at the side chain add little to the total lipophilicity of the molecule, passive diffusion through cell membranes tend to be small. In this concern, the design of prodrugs with better oral absorption than metformin is still a matter of interest. The absorption of metformin first occurs in the small intestine [10], where the plasma membrane monoamine transporter (PMAT) and the organic cation transporter 3 (OCT3) are expressed and play a role in its absorption. The drug leaves the enterocytes using the organic cation transporter 1 (OCT1), and its uptake in the hepatic cells also occurs mainly through the OCT1 [11, 12]. Accordingly, Wang *et al.* reported that OCT1 knockout mice displayed metformin levels in the liver 30 times lower than in control mice [13].

Approximately one quarter of the patients taking metformin suffer from gastrointestinal side effects (including nausea, vomiting, and diarrhea), while the administration of the drug must be suspended in 5% of the cases. In patients with reduced OCT1 function, which consequently display increased levels of metformin on the intestines, intolerance was about four times more likely to develop. Accordingly, OCT1 inhibitors (tricyclic antidepressants, proton-pump inhibitors, α-adrenoreceptor antagonists, and calcium-channel blockers) also increase the risk of gastrointestinal intolerance [14].

As the concentration of metformin in the intestinal mucosa is higher than in the other tissues, the gastrointestinal tract is suggested as an important site of action for metformin [15]. Studies have shown the drug increases the glucose uptake and use by the intestine,

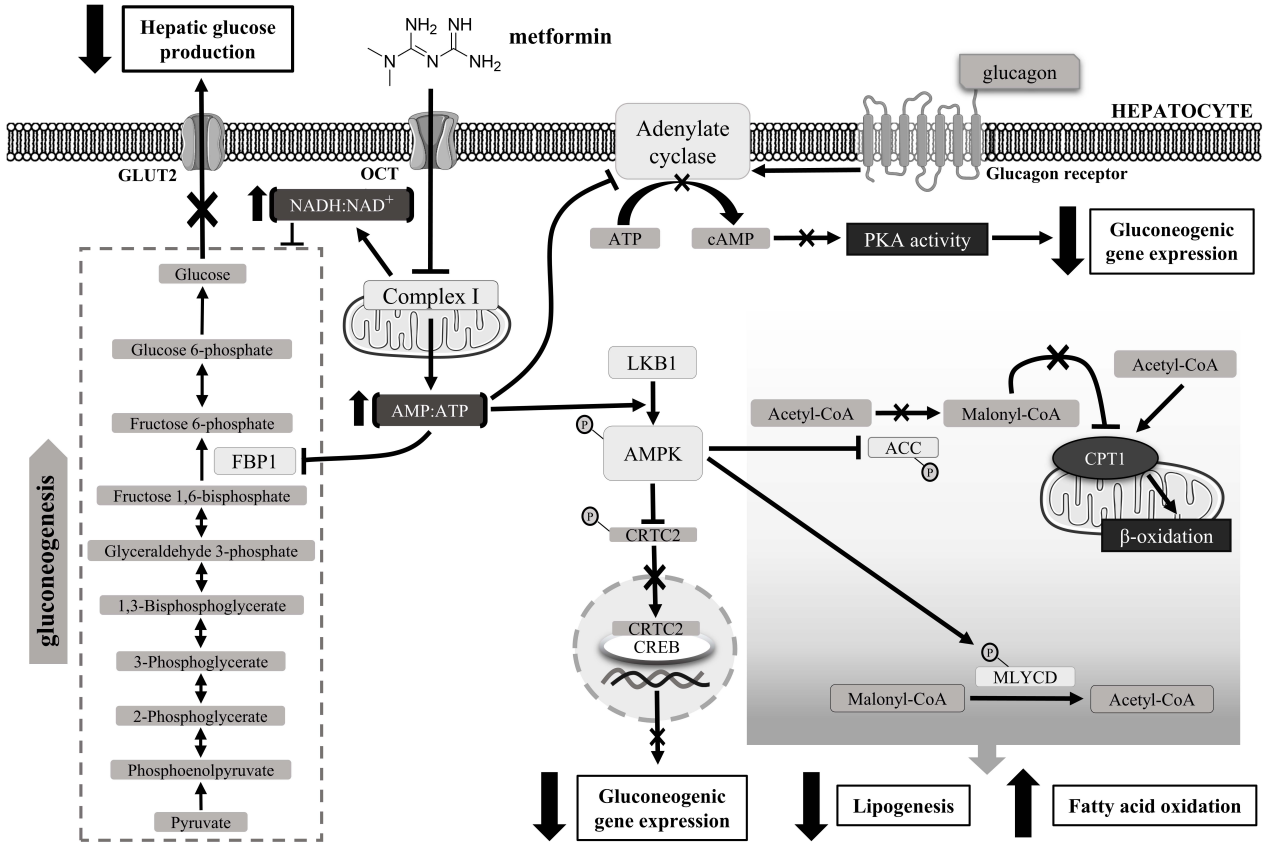


Fig. (3). AMP-dependent mechanisms of metformin-induced gluconeogenesis inhibition. Metformin is transported into the hepatocyte mainly through OCT1 and accumulates within the mitochondria. Metformin inhibits the complex I activity, causing a decrease in the ATP generation by mitochondrial oxidative phosphorylation and a consequent accumulation of AMP. Gluconeogenesis is then reduced as a result of ATP and NAD^+ deficit. AMP increased levels also inhibit the FBP1, a critical gluconeogenic enzyme. AMP binding to AMPK leads to AMPK phosphorylation by LKB1. AMPK's activation reduces ACC activity, an enzyme that catalyzes acetyl-CoA conversion to malonyl-CoA. AMPK also increases MLYCD activity, an enzyme that catalyzes the conversion of malonyl-CoA to acetyl-CoA. As malonyl-CoA is an allosteric inhibitor of CPT1, when there are lower levels of malonyl-CoA, CPT1 activity is increased, resulting in the entry of long-chain fatty acyl-CoA into the mitochondria and increased β oxidation. Malonyl-CoA is also a precursor of fatty acid synthesis; therefore, its lower content impairs lipogenesis. AMPK activation prevents the CRTC2 from entering the nucleus to activate CREB, a critical component in the induction of gluconeogenesis in the liver. Increased AMP levels inhibit adenylate cyclase, responsible for cAMP production, reducing the activity of PKA and downstream signaling involved in the glucagon signaling pathway. Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CPT1, carnitine palmitoyltransferase; CREB, cAMP response element-binding protein; CRTC2, CREB-regulated transcription coactivator 2; FBP1, fructose-1-6-bisphosphatase; GLUT2, glucose transporter type 2; LKB1, liver kinase B1; MLYCD, malonyl-CoA decarboxylase; OCT1, organic cation transporters; PKA, protein kinase A. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

mainly through the stimulation of anaerobic metabolism of glucose, which would increase the lactate production in the intestinal enterocytes and could also contribute to the intolerance for the drug [16]. However, as we will discuss below, there are several alternative hypotheses concerning the mechanism of action of the drug, a fact that considerably hinders the use of target-centered structural approaches for the development of metformin analogs.

1.2. Mechanism of Action

1.2.1. Inhibition of the Mitochondrial Respiratory Chain Complex I

Metformin lowers the rate of glucose production by reducing gluconeogenesis [17]. Since the 1950s, it has been suggested that biguanides would exert their effect through the interaction with mitochondria as some studies showed metformin could inhibit mitochondrial oxidative phosphorylation (Fig. **3**) [18, 19]. However,

it was only in this century that two independent research groups reported the pharmacological effects of metformin are mediated through the inhibition of the respiratory chain. They proposed its primary mechanism of action was through direct [20] or indirect [21] inhibition of the complex I of the respiratory chain, which leads to a decrease in ATP generation and accumulation of AMP in hepatocytes.

Bridges *et al.* suggest metformin as a reversible non-competitive inhibitor of complex I. The authors theorize that such inhibition results from an interaction of the drug with the matrix loop of the subunit ND3, an amphipathic region at the interface of the hydrophilic and membrane domains. This would lead the enzyme to be trapped in an open-loop catalytic inactive conformation, resulting in the inhibition of NADH-linked respiration [22].

There is still a lack of consensus on whether metformin concentration is higher within the mitochondria of hepatocytes or not. According to Wang *et al.*, metformin accumulates in the cytosolic fraction of the hepatocytes rather than in the mitochondria, so only supra-pharmacological concentrations of metformin could disrupt mitochondrial respiration [23]. However, another study states the positive charge of metformin allows its accumulation within the matrix of the energized mitochondria driven by the membrane electrochemical potential [20], which would account for the significant effect of the drug on this organelle.

The wide range of doses used in these studies and the diversity of experimental models (animals, cell culture or isolated organelles), though understandable and relevant, end up leading to conflicting results regarding the exact mechanism of action of the drug. Adapting *in vitro* experiments to simulate as much as possible the *in vivo* conditions is one of the most challenging aspects of physiology experimentation. Therefore, considering the lack of uniformity in the methods and the resulting apparent discrepant results, it is hard to achieve a consensus about the exact mechanism through which metformin exerts its therapeutic effects.

In this context, although some studies suggest only metformin concentrations higher than the therapeutic dose could inhibit the mitochondrial respiratory chain complex I [24-26], most of the publications in this field support the idea that metformin indeed acts by directly inhibiting complex I, which would account for most of its anti-hyperglycemic effect [20, 27, 28]. Owen *et al.* demonstrated through *in vitro* and *in vivo* studies the direct inhibition of complex I of the respiratory chain by using 50 μM of metformin [20], the same drug concentration found in treated patients, especially in the liver and intestine, which contain the higher amounts of the drug than in the general circulation (*i.e.*, 40 -70 μM after 60 min) [15, 29, 30].

Moreover, complex I remains the best elucidated metformin target from the structural point of view. Boukalova *et al.* describe complex I as a molecular target for the triphenylphosphonium analogs of metformin, the so-called Mito-Mets, but the focus of the study was the optimization of the structure of metformin for the treatment of pancreatic cancer. According to their docking simulations, performed with the crystallographic structure of the complex I from *Yarrowia lipolytica* (PDB ID 4WZ7), the predicted binding site for these compounds is placed at the peripheral arm (Q-module) of complex I [31]. The biological significance of such interaction can be inferred from the fact the oxidation of NADH by the flavin mononucleotide (FMN) generates a flow of electrons through a chain of iron-sulfur clusters, which ends at the terminal Fe-S cluster (N^2) in the Q-module, then promoting a conformational change that will affect the intermembrane pump modules [32].

Interestingly, Rahman *et al.* used a different docking method to map the most probable interaction sites for metformin at the human complex I (PDB ID 5XTD) and they also observed the Q-module as the main interaction site for the drug. According to the authors, the main residues involved in such interaction are Trp^{114} (chain H), Ser^{63}, Val^{64}, Pro^{65} and Pro^{66} (chain I), Phe^{81} and Tyr^{245} (chain P) and Pro^{393}/Gly^{394} (chain Q). However, the information is limited since the authors focused their discussion mainly on the transport of metformin across the membranes *via* OCT1 and the probable connection with the development of lactic acidosis [33].

Considering the convergence of the studies of Boukalova *et al.* [31] and Rahman *et al.* [33] in suggesting the Q-module of complex I as the probable binding site for the expression of the hypoglycemic, antitumoral and effects related to the lactic acidosis triggered by metformin, we used a third independent docking approach, aiming to reproduce the findings observed by Rahman *et al.* In this concern, we first processed the structure of the human complex I (PDB ID 5XTD) with the Dock Prep module of the UCFS Chimera [34] which, with a total resolution of 3.7 Å [35], is very prone to display the sidechains of amino acids with wrong rotamers [36]. By analyzing the geometry of the metformin binding pocket [37], one may notice this site, formed by residues from the chains H, I, P and Q, as described by Rahman *et al.*, but also from the L chain based in our analyses, could accommodate larger ligands than metformin (Fig. **4**).

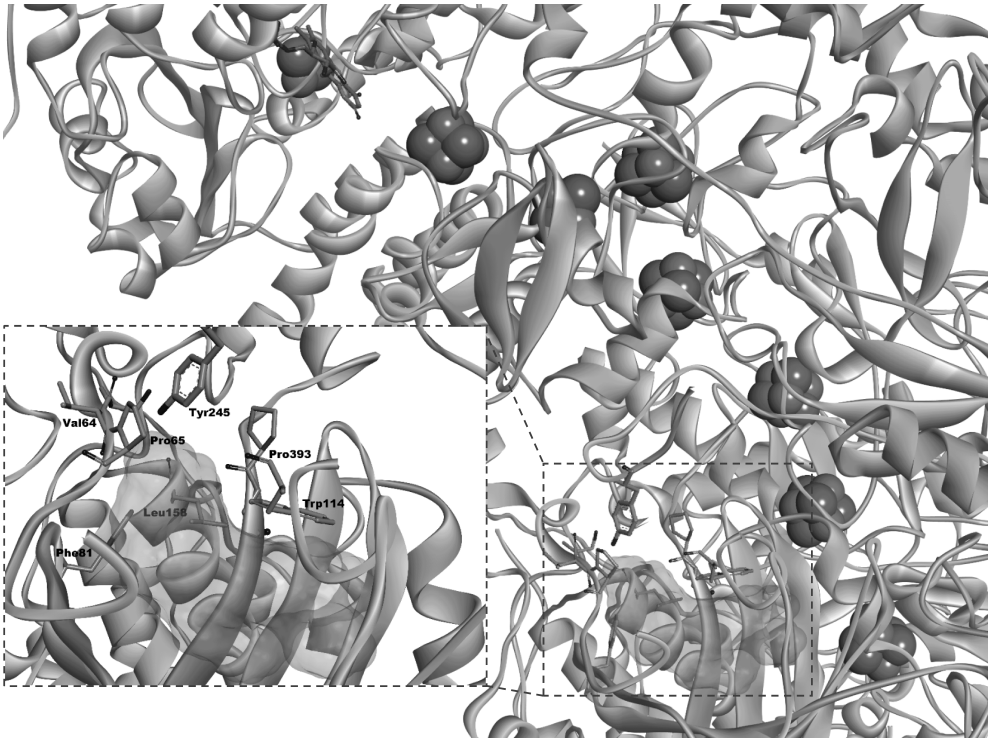


Fig. (4). Tridimensional structure of the hydrophilic (membrane-protruding) segment of the human complex I (PDB ID 5XTD). Highlighting, in the expansion, the segments that compose the putative binding pocket for metformin. The putative residues involved in metformin binding in the expansion were defined as reported by Rahman *et al.* [33]. The surface contours of this putative binding pocket, as identified *via* cavity prospection [37], are also highlighted in the expansion. The structures of the iron-sulfur clusters (on the right side) are described as space-filled models. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Performing the ligands and docking simulations as we previously reported [38, 39], assessed in a 10 Å radius docking sphere from the O atom of Val^{64}, we could reproduce the binding mode of metformin as reported by Rahman *et al.* [33]. We have chosen the oxygen of Val^{64} to define the binding site because the authors reported that this residue is involved in a hydrogen bond performed with metformin, while all the remaining interactions are of hydrophobic nature. In fact, we confirmed this interaction, but we also observed two additional H-bonds (Fig. **5A** and comp. **6**) performed with Tyr^{245} and Pro^{393}, a triad responsible for the establishment of H-bonds also predicted for buformin (Fig. **5B** and comp. **6**) and phenformin (Fig. **5C** and comp. **6**).

The docking poses, ranked using the Piecewise Linear Potential (PLP) fitness function [40], displayed docking scores for metformin (31.68), buformin (36.75) and phenformin (47.86) that correlate both with their cytotoxicities against HepG2 cells and the relative potencies in inhibiting the mitochondrial respiration [41]. While the strongest interactions observed for metformin are only the above-mentioned H-bonds (Fig. **5A**), these interactions are reinforced in phenformin by two additional π-π stacking interactions, performed with Trp^{114} (Figs. **5C** and **6**). While the corresponding interaction in buformin is a weaker π-akyl interaction (Fig. **5B**), the overall number of hydrophobic interactions is higher than in metformin (Figs. **5A** and **6**), which probably contributes to a better affinity for the target.

To design biguanides with improved pharmacokinetic properties, one could benefit from an early assessment of how structural modifications would impact the overall antihyperglycemic activity and the risk of triggering adverse effects. Considering the molecular docking results here presented and the available information from the literature, it seems clear that molecular docking studies with complex I can be valuable tools both to predict the antihyperglycemic effect of a particular biguanide as to estimate its potential to induce lactic acidosis since the occurrence of lactic acidosis increases with the compound's ability to inhibit complex I. Phenformin, for example, is 20-fold more potent than metformin in inhibiting complex I and was withdrawn from the market in several countries for causing lactic acidosis [42].

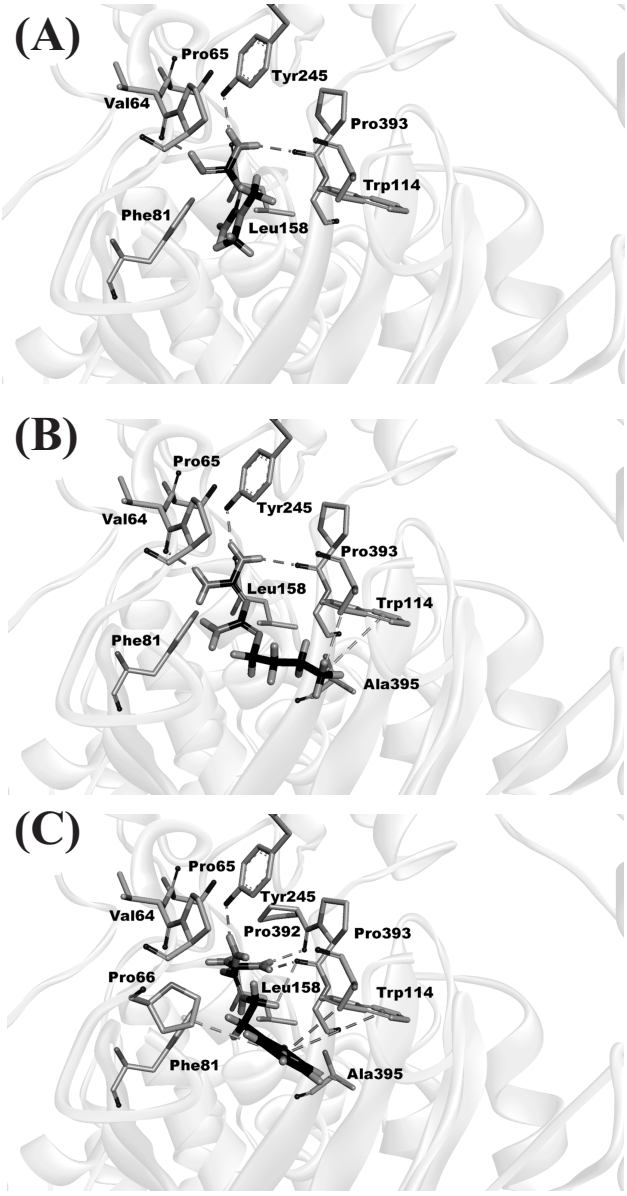


Fig. (5). Proposed binding modes for metformin (**A**), buformin (**B**) and phenformin (**C**) at the hydrophilic segment of the human complex I (PDB ID 5XTD). Hydrogen bonds are represented as dashed lines between N/O atom pairs for the three compounds, while the π-akyl (buformin) and π-π (phenformin) interactions are depicted as a dashed line between the aromatic indole ring of tryptophan and the terminal CH or phenyl ring of the drug. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

At first glance, biguanides that are strong inhibitors of the complex I seems unlikely to reach clinical use unless one could dissociate their risk of inducing lactic acidosis from the antihyperglycemic effects. However, a possible measure to tackle the lactic acidosis induced by potent complex I inhibitors would be the co-utilization of drugs that could reverse the shift to the anaerobic metabolism. Methylene blue has been reported to effectively treat a patient with metformin-associated lactic acidosis, likely due to its ability to act as an artificial electron carrier, providing electrons to cytochrome c and restoring the aerobic metabolism despite of the complex I inhibition [43].

Methyl succinate warranted a similar effect, serving as a substrate for complex II when converted to succinate [44]. Later *in vitro* assays were unable to replicate the clinical achievement of methylene blue but attested the effectiveness of succinate prodrugs [45], the latter being thoroughly discussed as therapeutic alternatives following their promising results [46]. Therefore, the development of therapeutic alternatives to avoid lactic acidosis may have a deep impact on the syntheses of novel antidiabetic biguanides, making the design of clinically viable potent complex I inhibitors possible through the use of computational docking studies with the target.

Alternatively, novel drug candidates could be planned to better inhibit less established targets of metformin, such as mGPDH [47], AMP deaminase [48] and SHIP2 [49], all of which have been reported to be directly inhibited by metformin and could be connected to its hyperglycemic activity due to involvement in glucose metabolism. Although no attempts of drug development have been made in this sense, the inhibition of complex I appears to account for most of the activity and would be preferably targeted.

1.2.2. AMP-dependent Mechanisms of Metformin-induced Gluconeogenesis Inhibition

AMP-activated protein kinase (AMPK) is a sensor of cellular energy and nutrient status and is activated in response to increased ADP:ATP and/or AMP:ATP ratios. Zhou *et al.* suggested for the first time the pharmacological effects of metformin could be related to the activation of AMPK in hepatocytes, an important element in the regulation of the metabolism of glucose and lipids [50]. This activation was later related to the inhibition of mitochondrial respiratory chain complex I by metformin, which causes a decrease in the ATP level while increasing ADP and AMP intracellular levels, leading to the activation of AMPK [51]. In the skeletal muscle cells, the activation of AMPK by metformin stimulates glucose uptake by inducing the GTPase Rab4

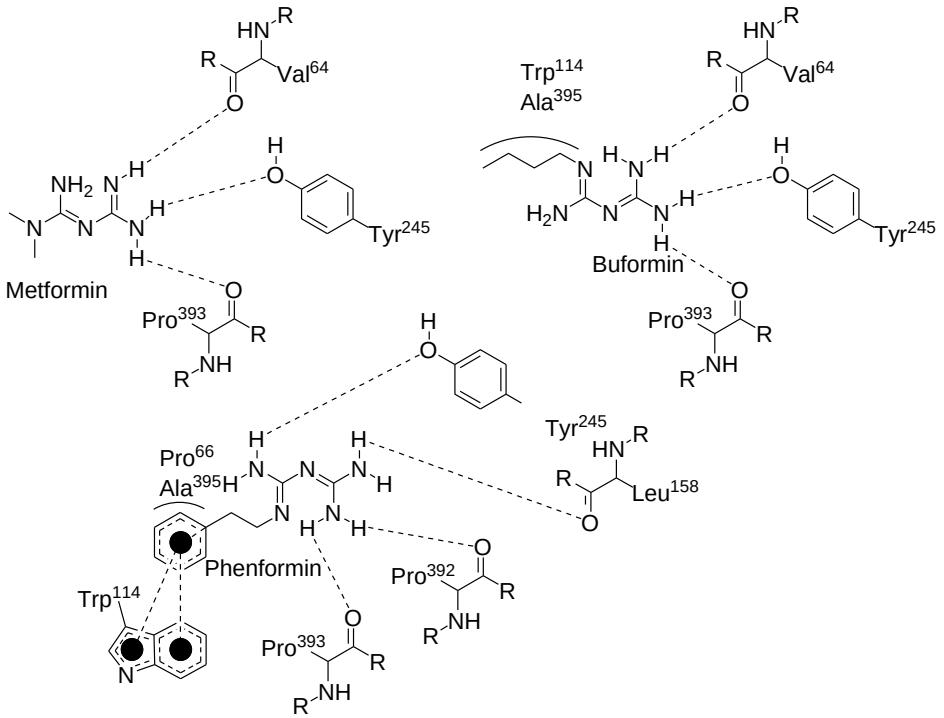


Fig. (6). Two-dimensional diagrams of the ligand-protein interactions based on the outputs generated using PoseView [39]. Dashed lines correspond to hydrogen bonds or π-π interactions (in this case, aromatic features are indicated as black circles), while solid arches highlight the hydrophobic interactions. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

expression *via* the AMPK-AS160-PKCζ pathway leading to translocation of vesicles containing glucose transporter type 4 (GLUT4) to the plasma membrane [52].

The activation of AMPK by metformin in the liver results in the induction of fatty acid oxidation and in the suppression of the expression of lipogenic enzymes. It also reduces the acetyl-CoA carboxylase (ACC) activity, an enzyme that catalyzes the conversion of acetyl-CoA to malonyl-CoA, and increases the activity of malonyl-CoA decarboxylase (MLYCD), an enzyme with the opposite effect of ACC. Together, these effects reduce the intracellular content of malonyl-CoA, an important precursor of fatty acid biosynthesis and exerts allosteric regulation in inhibiting the carnitine palmitoyltransferase (CPT1) activity, an enzyme responsible for the entry of long-chain fatty acyl-CoA into the mitochondria. Therefore, lower levels of malonyl-CoA promote β-oxidation at the same time as it suppresses the syntheses of fatty acids [50].

AMPK also phosphorylates the Ser^{171} residue of cAMP response element binding protein (CREB)-regulated transcription coactivator 2 (CRTC2, previously called TORC2). Consequently, CRTC2 is sequestrated from the nucleus to the cytoplasm by the 14-3-3 protein and becomes unable to activate CREB. As CREB is a critical component in the induction of gluconeogenesis in the liver, this mechanism could work as a metabolic checkpoint. Furthermore, liver kinase B1 (LKB1), an enzyme that phosphorylates and activates AMPK, was believed to be required for the hypoglycemic effect of metformin [53, 54].

Interestingly, the same research group also reported the inhibition of hepatic gluconeogenesis by metformin as independent of LKB1 and AMPK. The drug inhibited glucose production even after the forced expression of gluconeogenic genes through the superexpression of peroxisome proliferators-activated receptor gamma (PPARγ) co-activator 1α (PGC-1α), indicating metformin inhibits the activity of gluconeogenic enzymes rather than their gene expression [51].

Based on a series of *in vivo* and *in vitro* experiments, Miller *et al.* proposed that metformin antagonizes the action of glucagon by inhibiting the mitochondrial respiratory chain complex I, which decreases the ATP levels and accumulates AMP. As gluconeogenesis is a highly energy-consuming metabolic pathway, the reduction of ATP levels by itself is enough to reduce glucose production. Additionally, the accumulation of AMP leads to inhibition of adenylate cyclase

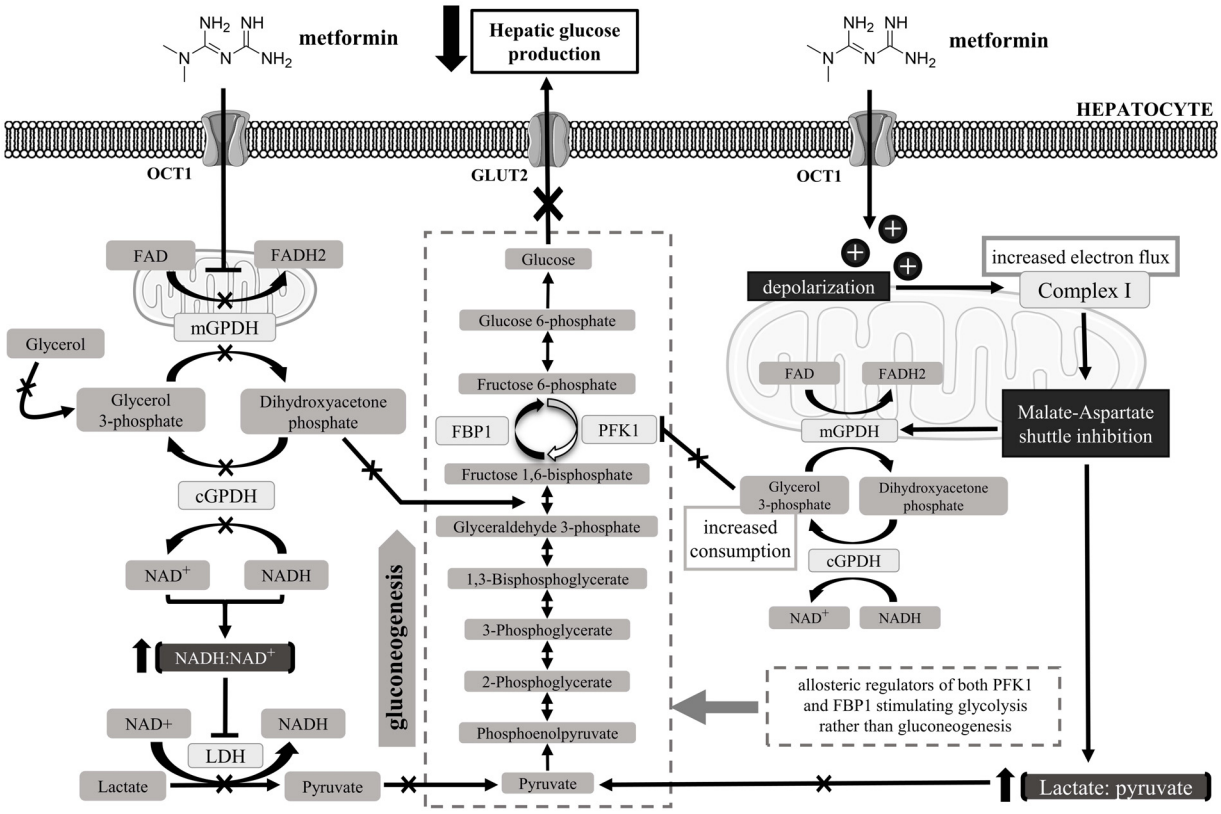


Fig. (7). AMP-independent mechanisms of metformin-induced gluconeogenesis inhibition. Metformin inhibits the enzyme mGPDH, involved in the GP-shuttle. Glycerol is phosphorylated by the glycerol kinase to glycerol-3-phosphate to promote glucose production in the liver and then is converted to dihydroxyacetone phosphate by the mGPDH. The inhibition of mGPDH impairs glucose production from glycerol and leads to the accumulation of cytosolic NADH, which impairs the conversion of the gluconeogenic precursor lactate to pyruvate by the enzyme LDH. Another proposed mechanism is that the accumulation of metformin in mitochondria causes the depolarization of the mitochondrial membrane, possibly increasing the flux through respiratory chain complex I and leading to the inhibition in electrogenic transport of the MA-shuttle. This shuttle inhibition would then stimulate the glycerol-phosphate shuttle (as a compensatory mechanism), causing a decrease in glycerol 3-phosphate level. As glycerol 3-phosphate is a potent allosteric inhibitor of PFK1, its reduction stimulates the flux towards glycolysis versus gluconeogenesis. The inhibition of MA-shuttle would also be the reason for the increased lactate/pyruvate ratio reported after metformin treatment, contributing to the gluconeogenesis inhibition. **Abbreviations:** cGPDH, cytosolic mitochondrial glycerol 3-phosphate dehydrogenase; FBP1, fructose-1-6-bisphosphatase; GLUT2, glucose transporter type 2; GP-shuttle, glycerol 3-phosphate shuttle; LDH, lactate dehydrogenase; mGPDH, mitochondrial glycerol 3-phosphate dehydrogenase; OCT1, organic cation transporters; PFK1, Phosphofructokinase-1. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

and, therefore, to decreased levels of cAMP, then reducing the activity of protein kinase A (PKA) and downstream signaling. Since glucagon signaling depends on adenylate cyclase activation, followed by the production of the second-messenger cAMP and the activation of PKA, metformin would inhibit gluconeogenesis through the suppression of the glucagon pathway [55]. However, another study contradicts this hypothesis as it suggests metformin is unable to inhibit glucagon-stimulated endogenous glucose production in humans [56]. These clinical results indicate that the decrease in gluconeogenesis by metformin seems to be not related to glucagon production, at least not for the dose used in the study. Therefore, metformin would act by modulating the gluconeogenesis pathway at the enzyme level or by limiting its substrates. As mentioned before, these conflicting results are caused by differences in the doses and in the experimental models, as the first study was carried out in isolated primary hepatocytes from mice and the second one measured hormones and metabolites from human patients.

The enzyme fructose-1-6-bisphosphatase (FBP1) is a key component in controlling the rate of hepatic gluconeogenesis, and, accordingly, FBP1 deficiency has

been shown to impair glucose production from all the gluconeogenic precursors, thus causing hypoglycemia and acute crises of lactic acidosis [57]. Hunter *et al.* showed an increased AMP concentration induced by metformin causes allosteric inhibition of FBP1, which significantly contributes to the glucose-lowering effect of metformin [58].

1.2.3. AMP-independent Mechanisms of Metformin-induced Gluconeogenesis Inhibition

Madiraju *et al.* reported that acute and chronic treatments with metformin promote the non-competitive inhibition of the mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH) in rats. All biguanides reduced mGPDH activities by 30-50%. To promote glucose production in the liver, glycerol is phosphorylated by the glycerol kinase to glycerol-3-phosphate and then converted into dihydroxyacetone phosphate by the mGPDH. Therefore, inhibition of mGPDH would prevent glycerol from contributing to gluconeogenic flux. Besides, the inhibition of mGPDH affects the glycerol 3-phosphate shuttle (GP-shuttle), which is one of the mechanisms that transfer reducing equivalents from the cytoplasm to the mitochondria regenerating NAD^+ consumed during glycolysis. This inhibition leads to the accumulation of cytosolic NADH, which impairs the conversion of lactate into pyruvate by lactate dehydrogenase (LDH). Based on these findings, the authors concluded that the inhibition of the mGPDH alters the hepatocellular redox state, reducing the conversion of both lactate and glycerol to glucose, which, consequently, decreases hepatic gluconeogenesis (Fig. **7**) [48].

They later provided evidence that rats treated with clinically relevant doses of metformin displayed gluconeogenesis inhibition that could not be explained by any of the previously proposed mechanisms for the drug. However, the study also concluded that the inhibition of hepatic glucose production by metformin was a consequence of the cell redox state, considering that only the gluconeogenesis from lactate and pyruvate (substrates dependent on cytosolic NADH) were suppressed [59].

A study by Alshawi and Agius also suggested that metformin could inhibit gluconeogenesis independently from the inhibition of mGPDH or mitochondrial respiratory chain complex I. The researchers proposed the accumulation of the drug in the mitochondria causes the depolarization of the mitochondrial membrane and leads to the inhibition of the malate-aspartate shuttle (MA-shuttle). MA-shuttle and GP-shuttle, together with the production of lactate by the enzyme LDH, are the three significant mechanisms in the liver that are involved in the regeneration of NAD^+. Therefore, they propose the membrane depolarization caused by metformin inhibits the MA-shuttle resulting in GP-shuttle stimulation as a compensatory mechanism that would cause a decrease in glycerol-3-phosphate content, a potent allosteric inhibitor of the enzyme phosphofructokinase 1 (PFK1). This mechanism, together with the action of other allosteric regulators of PFK1 and FBP1, would direct the flux towards glycolysis and inhibition of gluconeogenesis [60].

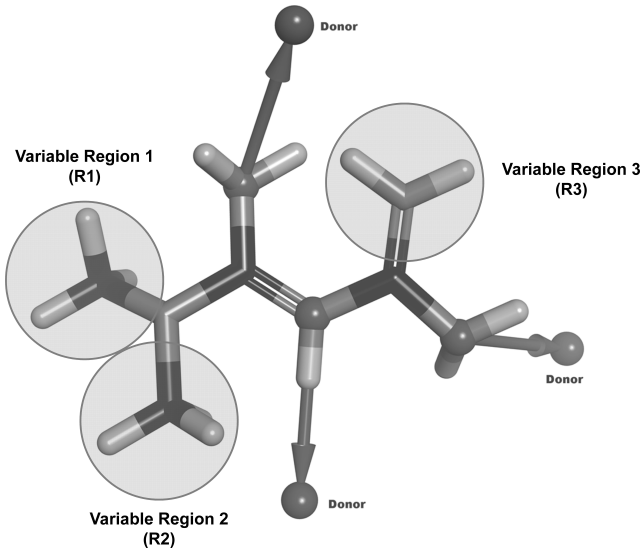


Fig. (8). The variable substituents explored in the acyclic biguanides are revised in this study. Despite these substituents, N_1, N_4 and N_7 tend to act as hydrogen donors or acceptors depending on the interaction with the target. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

2. ANTI-DIABETIC ANALOGS OF METFORMIN

In the book "Oral anti-diabetics", published in 1996 by Kuhlmann and Puls, the authors compiled the information for biguanides with anti-diabetic activity, reported from 1971 to 1996. In this context, the authors describe the structure-activity relationships for metformin analogs where the R_1 and R_2 (Fig. **8**) are alkyl/ aralkyl substituents. The highest effect on the blood sugar content was observed for R_1 = *n*-pentyl (please notice that in buformin, R_1 = *n*-butyl), with a continuous decrease in the activities from *n*-octyl to *n*-decyl (no activity). However, the best activities were observed when R_2 = H, biguanides with a methyl group (*e.g.*, metformin) were also active. For biguanides with a single aralkyl substituent, the highest activity was observed for the phenethyl analog (*i.e.*, phenformin) [2].

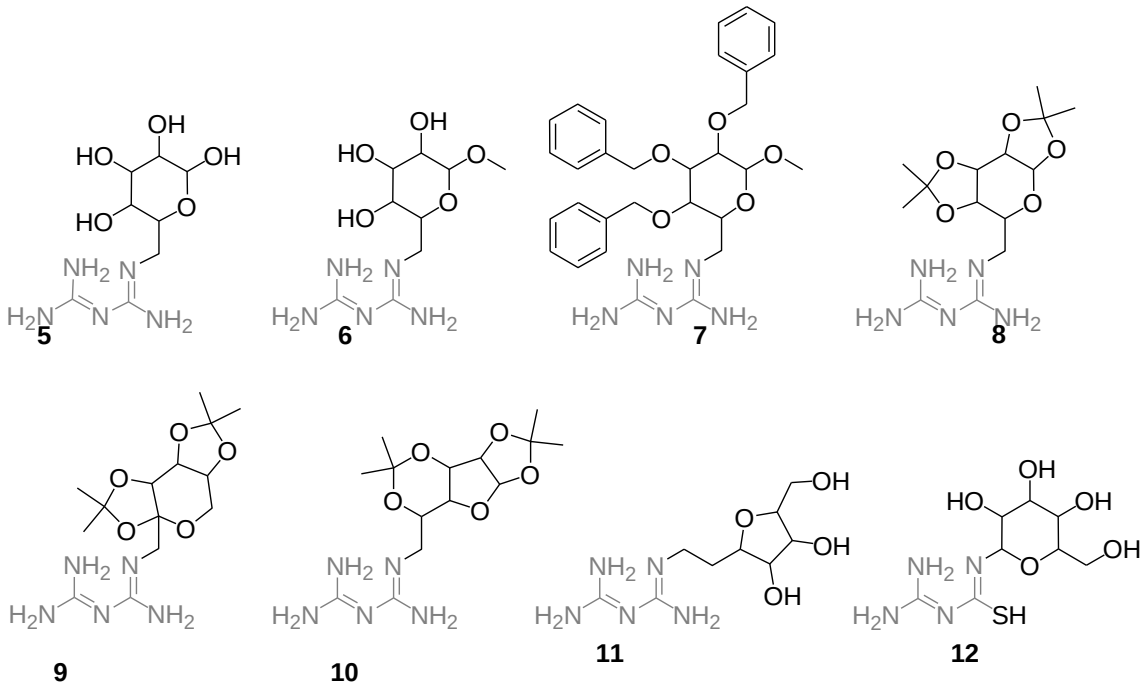


Fig. (9). Structures of metformin analogs reported by Reitz *et al.* (1989) [61].

Although we will depict the 2D structures of the subsequent biguanides based on the most prevalent tautomer of metformin (Fig. **2, b**) [9], the reader must have in mind they are indeed misrepresented in the original referenced papers. Additionally, we have pointed out in Fig. (**8**) that the R_3 substituents are linked to the imine nitrogen (N_6) because this is supported by the current data reported for buformin and phenformin (Fig. **1**) [9] but, again, in the original papers they are (mis)represented as N_7 substituents.

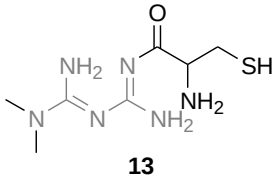


Fig. (10). Structures of a metformin analog reported by Liu *et al.* (2008) [62].

In 1989, Reitz *et al.* idealized and synthesized carbohydrate biguanides (Fig. **9**, comps. **5-12**), which were tested as hypoglycemic agents *in vivo* (*i.e.*, they were subjected to glucose tolerance tests in non-diabetic rats). The idea behind the design of these compounds was based on the evidence that biguanides inhibit the uptake of monosaccharide by the cells. Therefore, by coupling a biguanide to different monosaccharides, the authors expected an improvement in specificity, then carrying the biguanides directly to the ligand site of the hexose transporter to increase their hypoglycemic activity. Two *O*-protected glucose biguanide derivatives (**7** and **10**) provided satisfactory biological results, comparable to those obtained for metformin. However, when the authors doubled the concentration of these two analogs in the oral glucose tolerance test, they observed a dose-independent activity [61].

To design an analog with enhanced activity and additional pancreas-protecting effects, Liu *et al.* synthesized a hybrid from metformin and cysteine (Fig. **10**, comp. **13**), a compound with a potentially improved liposolubility and, consequently, a better bioavailability for an anti-diabetic drug. Additionally, cysteine has been reported to exert protective effects on beta cells, similarly to its precursor, *N*-Acetylcysteine. Compound **13** promoted a hypoglycemic effect superior to the observed for metformin while maintaining a similar hypolipidemic effect. The levels of insulin in the blood of alloxan-induced (insulin-deficient) animals were increased upon treatment, whereas in streptozotocin-induced animals fed with high lipid diet (hyperinsulinemic), the same parameter was decreased, indicative of improvement in beta cell function. The drug reduced oxidative stress (similarly to cysteine) and restored the activity of several key antioxidants (superoxide dismutase, reduced glutathione, catalase and the pancreatic exocrine enzyme amylase). In contrast, metformin and

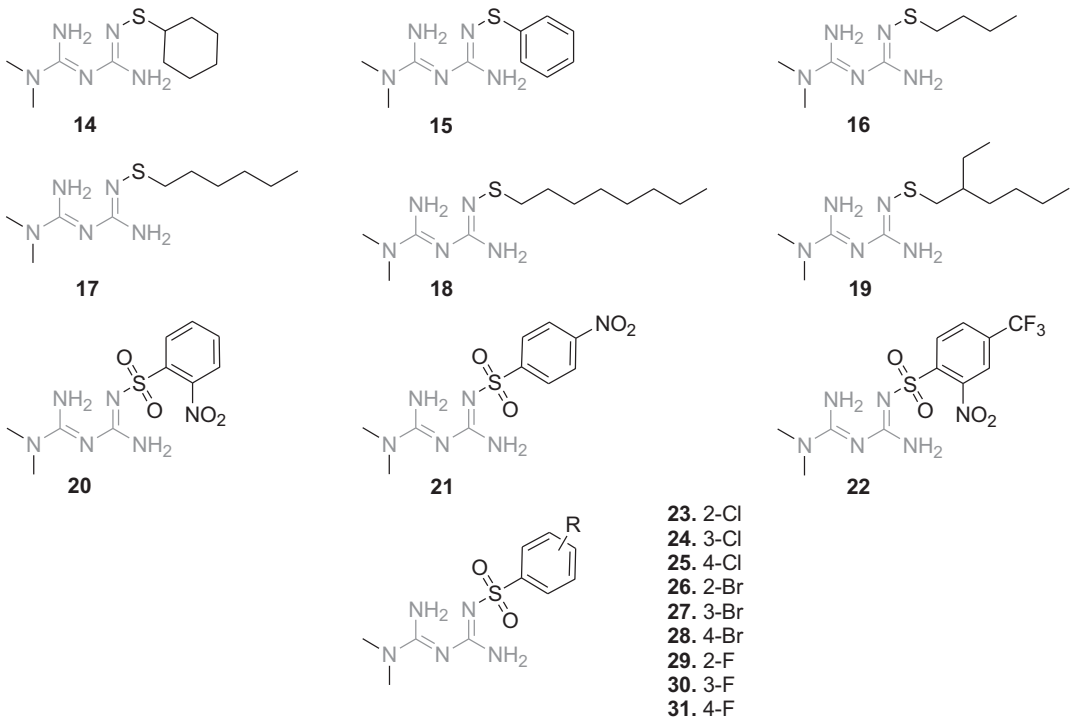


Fig. (11). Structures of sulfenamide and sulfonamide pro-drugs of metformin.

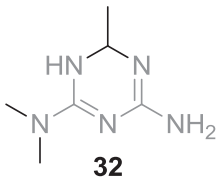
cysteine had their influence limited to superoxide dismutase. The suppression of edema in diabetic rats was the final evidence reported by the author to suggest **13** as a robust protective agent against oxidative stress and a potential tool for the treatment of both type 1 and 2 diabetes [62].

Huttunen and colleagues published the first of many studies concerning the development and characterization of sulfenamide and sulfonamide pro-drugs. This alteration intended to mask hydrogen bonding groups from the parent molecule with a bioreversible modification, therefore improving the lipophilicity and the bioavailability of the active compound. This would allow a decrease in the therapeutic dose, reducing the probability of gastrointestinal side effects. In the first studies, two sulfenamides (Fig. **11**, comps. **14** and **15**) were tested *in vitro* and *in vivo* and displayed improved lipophilicities and oral bioavailabilities when compared to metformin [63, 64].

A later work conducted at the same research group explored the design and syntheses of sulfonamides (**20-22**), aiming to achieve compounds that could be bioactivated after oral absorption, a characteristic not observed for most sulfenamides. Bioconversion tests revealed only **22** was able to release metformin after incubation with liver homogenates. The authors argue this is likely to occur because of the *para*-trifluoro group, which decreases the electron density of the ring as is also performed by the nitro group, then easing the bioconversion by the glutathione-*S*-transferase. In order to further evaluate the bioconversion of this prodrug, kinetic studies demonstrated a good affinity for the glutathione-*S*-transferase [65].

The group continued to explore sulfonamide derivatives of metformin by designing and synthesizing nine new benzenesulfonamide derivatives with halogen substituents (**23-31**). Considering diabetic patients have an increased risk for cardiovascular diseases, the authors studied the anti-coagulation properties of these compounds. Interestingly, the *ortho*-derivatives (**23**, **26,** and **29**) prolonged the prothrombin time and the partially activated thromboplastin time. Additionally, the bromo- (**26**) and fluoro- (**29**) substituted compounds induced fibrin polymerization and produced an increased thrombin time. Satisfactory results were obtained for the *ortho*-derivatives (**23**, **26** and **29**) and the *para*-fluorine substituted (**31**) in the clot formation and fibrinolysis test. The authors selected benzenesulfonamides with *ortho*-substituents to be further studied as anti-dia-

betic agents with anti-coagulant properties, particularly considering they displayed low cytotoxicity at concentrations up to 1.5 μmol/ml [66].



32

Fig. (12). Structure of imeglimin, (6*R*)-3,6-Dihydro-N_2, N_2,6-trimethyl-1,3,5-triazine-2,4-diamine.

Among the metformin analogs, imeglimin (TWYMEGG®, Fig. **12**, comp. **32**) is one of the most broadly explored [67-70] and the only one that achieved clinical practice, with recent approval for use in Japan [71]. Although closely resembling and being inspired by metformin [72], **32** does not belong to the biguanide family but is classified as a glimin, which arises from the cyclization of the biguanide into a dihydro-1,3,5-trizine [73]. The drug displayed a variety of different mechanisms through which it acts in glucose homeostasis, including increased insulin sensitivity in muscle and hepatic tissues [74], decreased hepatic glucose production [73], decreased liver steatosis [74], improved glucose-stimulated insulin secretion in beta cells [73-75], and protective effects on endothelial and beta cells from cell death induced by glucotoxicity [73-76]. In clinical trials, **32** positively improved glycemic control in humans as monotherapy or combined with traditionally used drugs, including metformin [77-80]. Like metformin, its precise mechanism of action is not completely elucidated, but some studies have associated its effects with the modulation of mitochondrial bioenergetics [70, 74, 76].

The anti-diabetic activities of three novel 1-substituted-biguanide derivatives (Fig. **13**, comps. **33-35**) were described by Abbas *et al.*, while this study evaluated the effects of the compounds on rats previously induced to develop T2DM. Multiple analyses were performed to characterize these compounds concerning their hypoglycemic activities, cholesterol- and triglyceride-reducing properties, liver enzyme activities (aspartate aminotransferase and alanine aminotransferase), kidney function (urea and creatinine) and their protective actions on liver, pancreas, and kidney. Although all the three compounds displayed significant improvements concerning the studied activities when compared to metformin, the 2,4-Dimethoxyphenyl-biguanide (**33**) was the most promising derivative of the series [81].

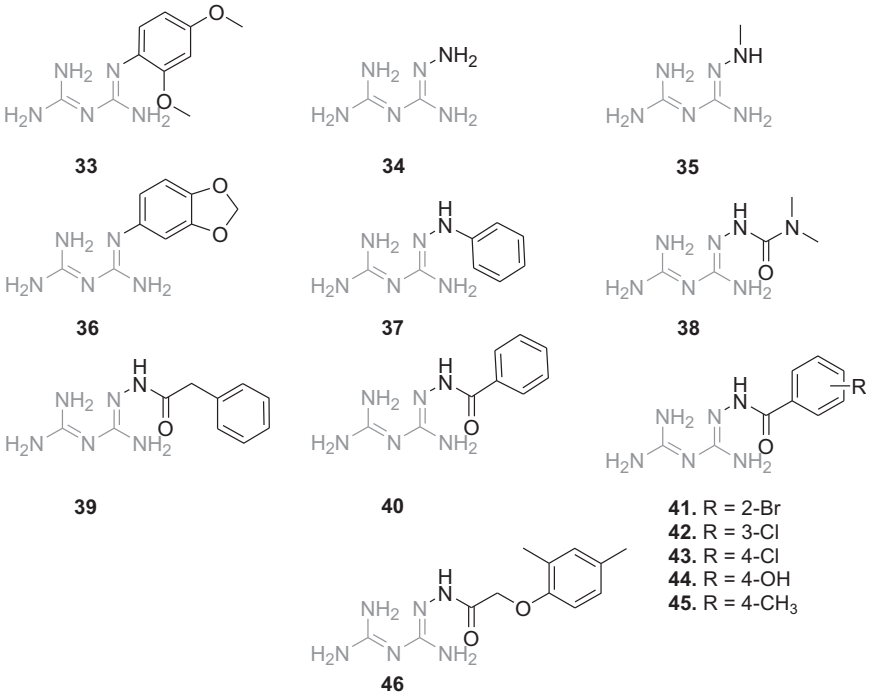
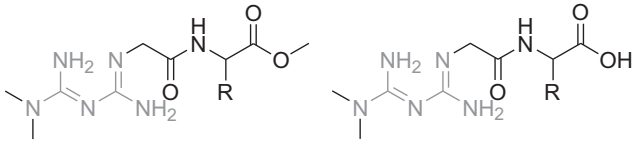


Fig. (13). Structures of metformin analogs reported by Abbas *et al.* (2016) and Basyouni *et al.* (2017) [81-83].

In a second study conducted by Abbas *et al.*, five new biguanides (**36-40**) were tested concerning their hypoglycemic activities. Although none was superior to metformin, all promoted some reduction in mean glucose levels. Similar results were obtained concerning liver enzyme activities and the analyses of lipid profiles. All the analogs improved the activities of the enzymes aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase and reduced the levels of triglycerides and cholesterol but not as efficiently as metformin. Although none of these compounds displayed improved anti-hyperglycemic effects, they were superior to metformin concerning their antioxidant properties and protective effects on the morphology of the pancreas, liver, and kidney [82].

The same group envisioned the synthesis of six new 1-arylamidebiguanide derivatives (**41-46**). In addition to the same properties explored in the previous work, the authors evaluate the expression of adhesion molecules [intercellular (ICAM) and vascular (V-CAM)], inflammatory markers [tumor necrosis factor alfa (TNF-α) and interleukin 10 (IL-10)], and a growth factor [vascular endothelial growth factor (VEGF)]. These compounds also displayed superior antioxidant properties and additional pancreas protective effects when compared to metformin [83].



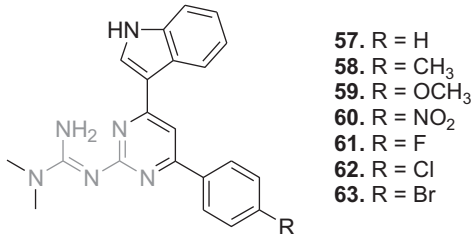
47. R = H
48. R = CH_3
49. R = CH_2-Phe
50. R = prolin residue
51. R = gaba residue

52. R = H
53. R = CH_3
54. R = CH_2-Phe
55. R = prolin residue
56. R = gaba residue

Fig. (14). Structures of metformin analogs reported by Mahdi *et al.* (2016) [84].

Aiming to improve oral bioavailability, Mahdi and colleagues synthesized ten analogs (Fig. **14**, comps. **47-56**) by linking amino acids or methyl ester derivatives of amino acids to the structure of metformin, using glycolic acid as a spacer. Considering the presence of di/tripeptide transporters in the membrane of enterocytes, the insertion of the amino acid moieties would theoretically increase the absorption and bioavailability of the anti-diabetic drugs. In the *in vivo* anti-hyperglycemic activity tests, compounds **48**, **49**, **51**, and **54** were superior to metformin in reducing blood glucose levels after 5 hours (reduction of 58-69%, when compared to a 50% decrease promoted by metformin), while compounds **50**, **51** and **53-56** produced profound effects after 24 hours (50-59%, compared to 41% for metformin). These results validate the approach proposed by the authors, while **51** was the most promising compound, promoting a reduction in the blood glucose levels of 58%-59% from 5 to 24 hours [84].

More recently, Ramya and colleagues conducted a study on seven new (*E*)-2-(4-(1*H*-indol-3-yl)-6-*p*-substituted phenylpyrimidin-2-yl) dimethylguanidine derivatives (Fig. **15**, comps. **57-63**) as anti-diabetic drugs. The analog with the unsubstituted phenyl ring (**57**) displayed particularly good results. The authors assessed these compounds concerning their *in vitro* antimicrobial activities, which are uncommon for metformin analogs. After measurement of the minimum inhibitory concentrations and the diameters of the zones of inhibition, all analogs exhibited good antimicrobial activities against one or more classes of microorganisms (Gram-positive, Gram-negative bacteria and fungus). Subsequently, the authors demonstrated all the seven new biguanides inhibit two carbohydrate-hydrolyzing enzymes *in vitro* (alpha-amylase and alpha-glucosidase, both related to postprandial hyperglycemia). Finally, the *in vivo* anti-diabetic activity assays revealed **57** and **63** as the most promising analogs of the series [85].



57. R = H
58. R = CH_3
59. R = OCH_3
60. R = NO_2
61. R = F
62. R = Cl
63. R = Br

Fig. (15). Structures of metformin analogs reported by Ramya *et al.* (2017) [85].

The strategy of hybridization was used for the development of three structurally distinctive compounds, which contain a 1,3,5-triazine ring derived from the cyclization of metformin (Fig. **16**, comps. **64-66**). The new ring was linked to berberine (**64** and **65**) or magnolol (**66**), natural products used in traditional Chinese medicine for the treatment of diabetes, cancers, inflammations, and hyperlipidemia. These compounds were tested for their anti-inflammatory activities by measuring the expression of cyclooxygenase-2 and the production of prostaglandin E2 by monocyte/macrophage cells *in vitro*, which were both effectively reduced after treatment with the synthesized compounds. A second analysis was performed to evaluate their effects on insulin secretion, which was successfully stimulated in insulinoma cells *in vitro* [86].

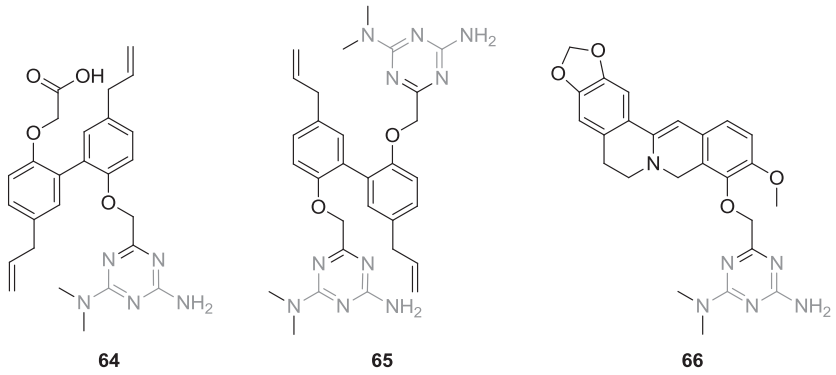


Fig. (16). Structures of metformin analogs reported by Cao *et al.* (2017) [86].

Although all three compounds have great potential as anti-inflammatory and anti-diabetic agents, the berberine analog MD568 (**65**) was selected for the assessment of anti-obesity activity. Obese rats treated with the hybrid compound reduced body weight, blood lipid contents, white adipose tissue, and liver mass compared to the control group. The *in vitro* results revealed that MD568 inhibits lipogenesis and lipid accumulation in 3T3-L1 pre-adipocytes. The authors applied microarray to investigate the mechanism of action responsible for the activity of MD568 and concluded this compound can regulate genes related to lipid metabolism both *in vitro* and *in vivo* [87]. Using obese rats with T2DM as a model, the authors demonstrated that MD568 reduces body weight, plasma glucose levels, total cholesterol, triglycerides, and low-density lipoprotein cholesterol levels, while also reduces the insulin resistance in obese T2DM rats [88].

The magnolol analog BMH473 (**66**) was also selected for studies regarding its therapeutic effects on obese T2DM animals. Besides displaying notable anti-hyperglycaemic and anti-hyperlipidemic activities, no significant toxicity was observed. Moreover, BMH473 promoted a considerable reduction in the inflammatory response in cells of the white adipose tissue by down-regulating the expression of p-NF-κB and pro-inflammatory cytokines to control the inflammatory process in obese T2DM rats. Although the authors displayed evidence of increased AMPK activity, the specific molecular mechanisms by which the compound works are still unknown. Overall, the results suggest its potential in maintaining glucose and lipid homeostasis in obese T2DM rats, displaying better effects than the prototypes [89].

Gutierrez-Lara *et al.* designed a series of ten cycloalkyl/alkarylbiguanides based on the structure of metformin and phenformin (Fig. **17**, comp. **67-76**). The biguanide group, which represents the pharmacophore, was maintained, while the dimethyl and

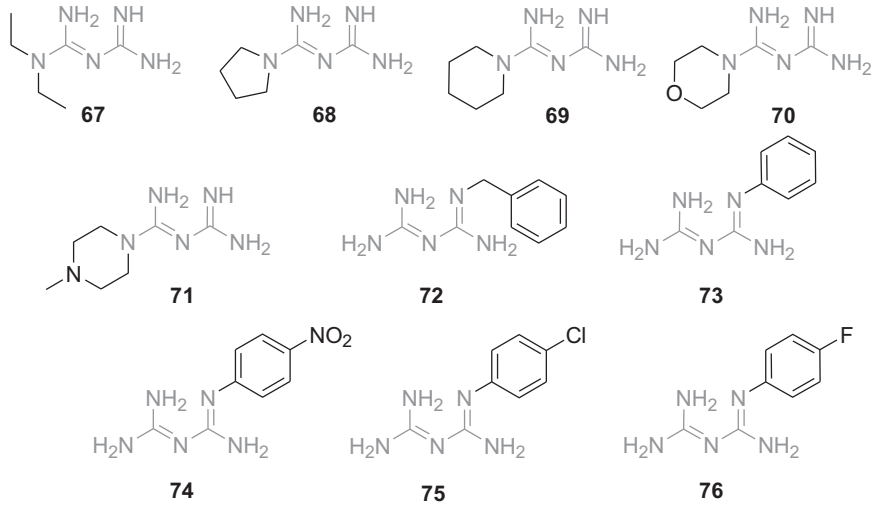


Fig. (17). Structures of metformin and phenformin analogs reported by Gutierrez-Lara *et al.* (2017) [90].

phenylethyl side chains were replaced by the cycloalkyl/alkaryl substituents. The principle of chain-ring transformation was also used to produce conformationally-constrained analogs (**67** *vs* **68-71**). The pyrrolidine (**68**) and piperidine (**69**) analogs were based on the concept of homologous series of alkyl substituents, while the morpholine and 4-methylpiperazine moieties were selected as isosteric replacements for piperidine (**70** and **71**). Analogs containing benzyl or 4-substituted phenyl rings were designed as homologues (**71-75**) of phenformin [90].

All compounds were tested *in vitro* for their potential to activate AMPK in hepatocytes, and most were inactive, including the aliphatic, alicyclic, and aromatic compounds. However, three compounds (**70-72**) displayed similar or better effects when compared to metformin. Docking analyses were then performed to evaluate the putative binding modes of these three compounds at the regulatory γ-subunit of AMPK. The simulations indicated these analogs access the nucleotide-binding pocket of the enzyme and interact with amino acid residues essential for its activation. Among the compounds designed, EGL-6M (**72**) displayed the best docking results, which agreed with both the *in vitro* and the posterior anti-diabetic *in vivo* assays. Although all three compounds reduced the plasmatic glucose levels, only the most promising was evaluated in a glucose tolerance test, providing similar results to metformin. Besides characterizing the therapeutic aspects of these three molecules, the authors also assessed the *in silico* potential for toxicity and monitored the *in vivo* models for lactic acidosis. Both analyses confirmed the analogs as promising anti-diabetic drugs [90].

CONCLUSION

After nearly a century, metformin remains the only biguanide used for the treatment of diabetes. The absence of analogs with clinical usefulness probably reflects the lack of consensus information concerning the molecular target of the drug. As a considerable amount of new information has been published, including the crystallographic structures of some targets, *in silico* techniques can now be explored more thoroughly. Although multiple targets are suggested for metformin in the literature, most of its biological activities are ascribed to the inhibition of the mitochondrial respiratory chain complex I. Herein, we performed a detailed exploration of this target using molecular docking simulations, and we could effectively correlate the *in vitro* potencies of metformin, buformin and phenformin with their predicted affinities for the complex I. In this context, we provided further evidence for the inhibition of the Q-module of complex I by metformin and its main analogs, possibly serving as a guide for future research seeking to develop antidiabetic metformin analogs.

LIST OF ABBREVIATIONS

ACC	=	Acetyl-CoA carboxylase
AMPK	=	AMP-activated protein kinase
CPT1	=	Carnitine palmitoyltransferase
CREB	=	cAMP response element binding protein
CRTC2	=	cAMP regulated transcription coactivator 2
FMN	=	Flavin mononucleotide
FBP1	=	Fructose-1-6-bisphosphatase
GLUT4	=	Glucose transporter type 4
GP-shuttle	=	Glycerol 3-phosphate shuttle
IL-10	=	Interleukin 10
LDH	=	Lactate dehydrogenase
LKB1	=	Liver kinase B1
MA-shuttle	=	Malate-aspartate shuttle
mGPDH	=	Mitochondrial glycerol 3-phosphate dehydrogenase
MLYCD	=	Malonyl-CoA decarboxylase
OCT1	=	Organic cation transporter 1
OCT3	=	Organic cation transporter 3
PFK1	=	Phosphofructokinase 1
PKA	=	Protein kinase A
PLP	=	Piecewise linear potential
PMAT	=	Plasma membrane monoamine transporter
PPARγ	=	Peroxisome proliferators-activated receptor gamma
T2DM	=	Type 2 diabetes mellitus
TNF-α	=	Tumor necrosis factor alfa
VEGF	=	Vascular endothelial growth factor

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIALS

Supplementary material is available on the publisher's website along with the published article.

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