A tale about perfect partners:

New horizons in glimepiride and metformin Mechanisms of action

Una historia sobre socios perfectos: Nuevos horizontes en el mecanismo de acción de la Metformina y la Glimepirida

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Abstract

The sustainability of the effects, the improvement of directly damaged and associated organ dysfunction, acceptable oral tolerability and lower side effects are probably the desired outcomes of any pharmacological therapy, especially for one that is used for long periods of time, such as T2DM pharmacotherapy. Biguanides and Sulfonylureas have a common development history, both associated with the economic difficulties associated with both World Wars and the impact caused by the discovery and application of Insulin for diabetes management. Glimepiride, the third generation sulfonylurea, is a KTP channel modulator, which also happens to influence plasma membrane dynamics, pro-inflammatory cytokine secretion and PPAR-π activation. The biological effects of metformin are widening every day, and they are not only related with the activation of AMP-dependent Kinase, but also with mitochondrial bioenergetics, glycogen and monocarbon metabolisms, epigenetic silencing and cell death pathways. The individual effects of glimepiride added to metformin´s could very much improve the patient´s metabolic and cardiovascular profiles, especially when such benefits are obtained with lower dosages than the standard. Such properties not only could guarantee adhesion to the oral medication but could enhance the prognosis of T2DM patients. The purpose of the following review is to describe the effects of glimepiride and metformin from a biological standpoint, including their recently pleiotropic effects.

Key words: metformin, glimepiride, lipid rafts, AMP-dependent Kinase, K_{ATP} channel, mitochondrial bioenergetics, glucose transporters.

Resumen

La sostenibilidad de los efectos, la mejora de la disfunción de orgánica, una tolerabilidad oral aceptable y pocos efectos secundarios son probablemente los resultados deseados de cualquier terapia farmacológica, especialmente, para una que se utiliza durante largos períodos de tiempo como los es la farmacoterapia con DM2. Las biguanidas y las sulfonilureas tienen una historia de desarrollo común, ambas asociadas con las dificultades económicas asociadas con ambas guerras mundiales y el impacto causado por el descubrimiento y la aplicación de insulina para el manejo de la diabetes. La glimepirida, la una sulfonilurea de tercera generación, es un bloqueador del canal KTP que también influye en la dinámica de las membranas plasmáticas, la secreción de citocinas pro-inflamatorias y la activación de receptores PPAR-gamma. Los efectos biológicos de la metformina se están ampliando cada día, y no sólo están relacionados con la activación de la quinasa dependiente de AMP, sino también con bioenergética mitocondrial, el metabolismo de glucógeno, silenciamiento epigenético y vías de muerte celular. Los efectos individuales de la glimepirida añadida a la metformina podrían mejorar mucho el perfil metabólico y cardiovascular del paciente, especialmente cuando estos beneficios se obtienen con dosis menores que las estándar. Tales propiedades no sólo podrían garantizar la adhesión al tratamiento, sino que podrían mejorar el pronóstico de los pacientes con DM2. El propósito de la siguiente revisión es describir los efectos de la glimepirida y la metformina desde un punto de vista biológico, incluyendo sus efectos pleiotrópicos recientemente descubiertos.

Palabras clave: metformina, glimepirida, balsas lipídicas, quinasa dependiente de AMP, canal KATP, bioenergética mitocondrial, transportadores de glucosa.

Mechanism of action of sulfonylurea ~ not as simple as it seems

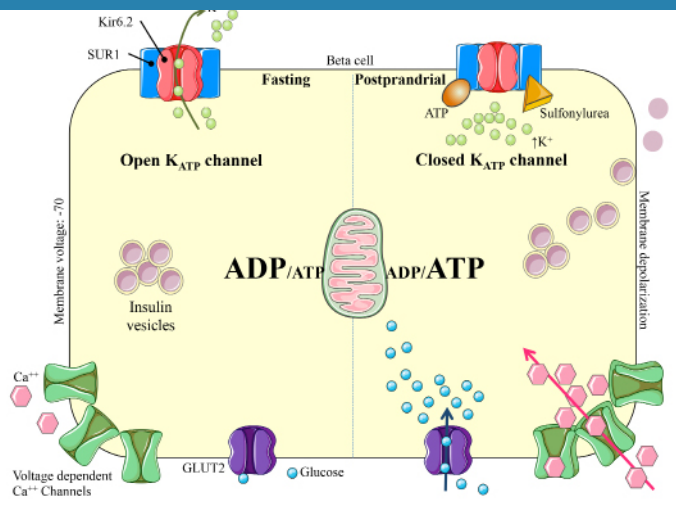
The primary effect of sulfonylureas is to stimulate pancreatic insulin secretion[1] and this property varies according to the generation of this drug class. The first developed sulphonylureas – called First Generation – were tolbutamide, acetohexamide, chlorpromide and tolazamide[2]. Second Generation class were gliclazide, glibenclamide (gliburide), glipizide, and Third Generation is currently glimepiride[3]. The drugs from the second and third generation are 20-50 more potent in their effects, with a prolonged half-life time when compared with chlorpromide[4]. These new sulfonylureas are associated with less side effects, included hyponatremia and disulfiram effect due to inhibition of hepatic alcohol dehydrogenase[5].

As previously mentioned, insulin stimulation effect varies according to sulfonylurea, and it's due to structural changes which would modify binding affinity to its receptor localized in the plasma membrane. The Sulfonylurea Receptor (SUR) belongs to the ATP-Binding Cassette family, described in Aguilar-Bryan et al.[6] as a 140-170 kDa membrane protein. The SUR is structurally related to another protein called Inwardly Rectifying Potassium Channel and together they constitute the K_{ATP} channel, one of the major metabolic sensors in pancreatic beta cells[7]. This ion channel is an octamer of 4 SUR subunits and 4 Kir6.2 subunits, where the latter units are the porous aspect of the channel and the former are the regulatory elements[8]. The basic layout for sulfonylurea-mediated insulin secretion is briefly as follows (**Figure 1**). The SUR subunit acts via switch mechanism, so when a sulfonylurea is bound to SUR the ion channel closes, and when the sulfonylurea dislodges from the receptor, the channel opens again. The net result is increased concentrations of intracellular potassium, which progressively depolarizes the cell and induces insulin release[9,10]. Depolarization induces Voltage-dependent calcium channels, increasing calcium influx towards the beta cells, activating cytoskeletal changes that end in the exocytosis of insulin vesicles[10].

Sulfonylureas depend on genetically-determined receptors, where binding time and intensity of the effect varies with each drug generation. The SUR receptor family can be divided in 3 types[11]: a) SUR1, located mainly in the beta cell plasma membrane; b) UR2A and SUR2B derived from alternative splicing, are expressed in cardiac and skeletal muscle. SUR1-related polymorphisms have been associated with hyperinsulinemic hypoglycemia[12] and neonatal diabetes mellitus[13]. In regards to receptor binding affinity, each drug class has peculiar properties. glibenclamide has 2.5-3 times more binding affinity than glimepiride[14], which results in higher insulin secretion in humans and in dogs[15]. This higher affinity enhances the risk of oxidative stress, endoplasmic reticulum stress, and beta cell apoptosis, proven in dogs and humans[16-18]. Prolonged use of sulfonylureas (especially those with higher affinity to SUR) has been associated with less beta cell survival rate, suggesting that insulin replacement therapy is actually a better choice regarding preservation of pancreatic islet[19].

Nevertheless, evidence has suggested that glimepiride may participate in the preservation of insulin secretion capacity during hyperglycemic states[20]. In fact, when glimepiride is combined with sitagliptine, islet diameter and proliferation markers seem to improve when compared with monotherapy[21]. Moreover, a clinical study conducted by our laboratory concluded that low dosages of glimepiride (0.5 mg/day) combined with metformin enhanced beta cell function, without an enhanced insulin secretion or downregulation of insulin receptors[22]. These findings show that glimepiride at low doses could exert non-pancreatic effects which influence beta cell function and improved 100% its performance. Therefore, we proposed that the extrapancreatic effects of sulfonylureas

Figura 1



The Sulfonylurea receptor in the pancreatic beta cell. The drawing is divided into two periods: fasting and postpandrial; added to the latter the use of sulfonylurea. During fasting, there is low glucose influx via GLUT2 due to low plasma glucose; ergo there are higher levels of ADP inside the cells, allowing the K_{ATP} channel to be open. Once feeding has occurred, there is an increased intracellular glucose level via GLUT2 and steady production of ATP, which closes the K_{ATP} ion channel, increasing intracellular potassium concentration, leading to cellular depolarization, opening of voltage-dependent Calcium Channels and exocytosis of insulin. Sulfonylureas can induce insulin secretion due to closing of the K_{ATP} channel, imitating the glucose-induced insulin secretion pathway.

like glimepiride deserve further investigation, especially when combined with a pleiotropic drug such as metformin.

Non-Pancreatic effects of Glimepiride

The hypoglycemic effect of sulfonylureas has been mainly attributed to acute insulin secretion[10], albeit, some extra-pancreatic effects have surfaced over the years, especially with glimepiride, which suggest novel pathways in the control of hyperglycemia in diabetic patients. First off, in vitro studies have confirmed that glimepiride intervenes in glycogen metabolism. Experiments in Hep-G2 cells have confirmed that the drug enhances glycogen production by 30-40% when used with insulin, apparently induced by increased insulin receptor recycling and Protein Kinase-C (PKC) pathway activation[23]. Moreover, a similar effect has been observed in myotubes mediated by Phosphatidylinositol-3-Kinase (PI3K), an effect that is not shared with glibenclamide[24]. Evidence has suggested that PKC might be related to this phenomenon[23], especially with PKCε[25] which is associated with insulin resistance in liver. In fact, PCKε is considered the culprit of lipid-induced insulin resistance via downregulation of insulin receptor expression[26].

Other related miscellaneous effects have been described over cytokine production, such as the one reported by Mori et al.[27], where glimepiride improved glucose tolerance and blunted expression of TNF-α in retroperitoneal adipose tissue, suggesting that glimepiride participates in the control of low grade inflammation and adiposopathy. Müller et al.[28] published that glimepiride induced the expression of mRNA for Peroxisome Proliferator-Activated Receptor-gamma implying that this sulfonylurea is truly capable of modifying the expression on insulin-sensitizing factors such as adiponectin, subsequently enhancing insulin signaling in its dependent tissues[28]; these aspect will be further discussed in the next section. These two cytokines are important in the adiposopathy-related microenvironment, since they actively partake in the shaping of such inflamed tissue. TNF-α is known to phosphorylate and inhibit IRS-1/PI3K/Akt pathways which is immediately activated after insulin binds its receptor[29]. Meanwhile, adiponectin exerts the opposite effect, by activation of AMP-dependent Kinase (AMPK) which phosphorylates serine and theonine residues in IRS-1, improving insulin´s intracellular signaling[30].

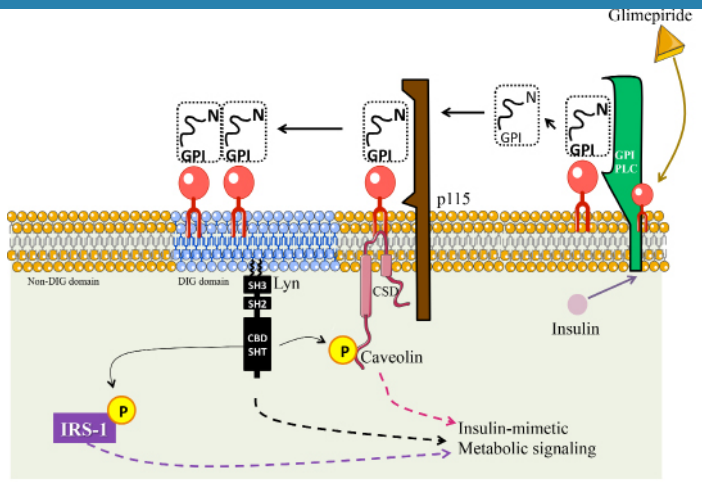
Glimepiride´s Insulin-like activity

The insulin-like effects of glimepiride have been investigated for the past 2 decades in adipose, skeletal muscle and liver cells. This sulfonylurea stimulates glucose influx in rat adipocyte, being associated with enhanced translocation of GLUT1 and GLUT towards the plasma membrane[16]. Interestingly, this effect is also observed in insulin resistant adipocytes and cardiomyocites[31], partially explaining its benefits in T2DM patients[31] and in ischemic cardiopathy[32]. Moreover, Müller et al.[33] published that glimepiride also modulates the activity of several enzymes associated with glucose metabolism like cAMP-specific phosphodiesterase 3B and Protein Kinase A. Likewise, the drug is known to modify lipid metabolism by inhibiting lipolysis, as shown by the in vitro studies[28]. Finally, Takada[34] reported that glimepiride induces PI3K and Akt activity, and these results were higher when compared with glibenclamide and tolbutamide. Enhancement of insulin downstream pathway seems vital in the pleiotropic effects of this sulfonylurea.

Glimepiride and plasma membrane dynamics

During the search for glimepiride´s long lost receptor, several in vitro studies were conducted using [^{3}H]Glimepiride[33,35], and during the analysis of several possible targets, plasma membrane proteins were marked with the tracer. Glycosylphosphatidylinositol 1/2 (GPI-1/2) were found to be covalently bound to glimepiride and preferably located in lipid raft domains[36]. These lipid raft domains contain GPI-anchored proteins in the outer side of the membrane, as well as Src kinases and prenilated small G Proteins[36]; **Figure 2**. GPI-anchored proteins are main components of the lipid rafts, and such structure can be disassembled via GPI-specific Phospholypase C (GPI-PLC)[37]. Interestingly, GPI-PLC is activated by glimepiride and to a lesser extent by insulin, interrupting such signaling cascades[38-40] and enhancing glucose transporter translocation[86]. In fact, these effects are also observed during exercise, resulting in lower insulin secretion and decreased levels of C peptide[41].

Figura 2



Insulin mimetic properties of Glimepiride. As proposed by Müller [70], glimepiride seems to modify plasma membrane dynamic, by stimulating GPI-associated PLC, reorganizing lipid raft composition, activating non-receptor tyrosin kinases like Lyn and activating downstream cascades that mimic insulin´s metabolic effects. CSD: caveolin scaffolding domain; DIG: Detergent-Insoluble glycolipid enriched raft domains.

The exact mechanisms for lipid rafting membrane control are still not completely understood, especially the regulation of outer membrane GPI-proteins and dually acylated signaling proteins (also known as NRTK) in the inner membrane locations[28]. It seems that it all depends on the electrochemical interactions between the fatty acid long chains of GPI-proteins and the dually acylated proteins which involves caveolin domains as scaffolds[42,43]. The caveolin scaffolding domain (CSD) conveys the presence of caveolin-1 fragments associated with cholesterol rich membranes, which are essential in receptor platforms where homo-oligomerization is key[44].

If this theory is correct, then insulin/insulin receptor signaling must has a complex lipid raft like this, which would aid in the homo-phosphorylation of more insulin receptors (amplification) and coordinates the hetero-phosphorylation of IRS-1/2 as second messengers. Müller et al.[45] published that insulin signaling required a dynamic lipid raft composed of NRTKs, Lyn and Fak, in raft microdominions called hydrophobic detergent-insoluble Glycolipid-enriched raft microdomain (DIG)[46]. It seems that when insulin binds its receptor, caveolin is phosphorylated while Lyn gets dissociated from the raft, and this phenomenon is also imitated by glimepiride. The NRTK are Non-receptor Tyrosine Kinases that act as switch mechanisms controls for several signaling platforms, commonly called cytoplasmatic enzymes[47]; the family comprises 32 NRTK in human genome, including Lyn and Fak. The former, is part of the Src family of cytoplasmatic enzymes, intimately associated with PI3K and Phospholypase Cγ2 [48]. The latter, Fak (Focal Adhesion-binding domain Kinase), is associated with plasma membrane shaping, focal adhesions and cytoskeleton remodeling[49].

Further research has demonstrated that glimepiride and caveolin are also associated in insulin secretion stimulation, where depletion of caveolin-1 in beta cells blunts sulfonylurea-induced insulin secretion, suggesting that caveolin-rich microdomains might also be associated with SUR/Kir6.2 assembly[50]. Sun and Hu[51] reported that the cardiac K_{ATP} channel required caveolin-3 scaffolding domains to properly assemble with SUR2A. Likewise, Davies et al.[52] K_{ATP} Kir6.1/SUR2B activity in vascular smooth muscle cells depends on caveolin-1 rich microdomains. This information suggests that KATP control is a two-way street, relying not only on the peptidic structure of its ensemble, but also on the lipid-based structure which is embedded in the plasma membrane, further enhancing the role of lipid raft in metabolic control[53].

Novel anti-diabetic effects of glimepiride – beyond the plasma membrane and onwards to the nucleus

The PPAR´s belong to a subfamily of nuclear receptors, with 3 different isoforms coded by 3 separate genes: PPARα (PPARA), PPARβ/δ (PPARD) and PPARγ (PPARG)[54,55]. PPAR receptors control gene expression for several gene clusters involved in processes such as adipogenesis[56,57], lipid metabolism[58], inflammation[58,59] and bone turnover[60]. These receptors are activated by lipophylic ligands like fatty acids and derived metabolites, heaving as an intracellular lipid content sensor, and ergo capable of redirecting intermediary metabolism[61].

Receptor activation is similar for all 3 PPAR proteins. After the ligand has bound to the receptor it heterodimerizes with another nuclear receptor, the X-Retinoid receptor, forming the PPAR-RXR complex. This dimeric complex colocalizes to the promoter site of target genes, activating assembly and progression by recruitment of several transcription coactivators[62,63]; nevertheless, all PPAR isoforms have specific tissue distribution and different roles in energy metabolism. For example, PPARα are expressed in skeletal muscle, liver, heart muscle and kidneys, whose principal function is lipid and lipoprotein metabolism control[64]. Likewise, the PPARβ/δ are expressed ubiquitously albeit with lower levels in liver, and has been associated with energy balance in adipose tissue and skeletal muscle[65]. Finally, the PPARγ has two alternate splicing products, PPARγ1 and PPARγ2. The former is expressed in adipose tissue, small intestine and hematopoietic cells, while the latter is expressed in white and brown adipose tissue[66-70]. Endogen ligands for PPARs include fatty acids and prostanoids such as 15-deoxy-12,14-prostaglandin-J2, 9- and 13-cis-hydroxy-octadecaenoic acids, and lysophosphatidic acid, acting as weak agonists when compared with thiazolidinediones (TZD), certain NSAIDs and the Angiotensin II receptor blocker Telmisartan[71-78]. A possibility is open concerning the existence of an endogenous ligand with higher affinity than those previously mentioned, and perhaps this type of ligand performs in a “promiscuous” manner in order to detect small changes in intracellular lipid concentrations[79,80].

In humans, these receptors are incredibly important in order to regulate glucose and lipid homeostasis in liver, muscle and adipose tissue, the differentiation process of adipocytes, especially from pre-adipocytes to mature adipocytes, and achieving adipocyte functional differentiation maturity by controlling the expression of major cytokines such as ADPN, leptin, resistin, TNF-α, and insulin signaling platform-related proteins such as GLUT4 and CAP[81-89]. Interestingly, PPARγ participates in the immune system, particularly in antigen presenting cells such as macrophages and dendritic cells, controlling lipid partition, inflammation and cell proliferation. In fact, such anti-atherosclerotic properties have also been observed in animal models using TZDs, demonstrating expression pattern changes in TNF-a, IL-1b and IL-6, suppression of inducible Nutric Oxide Synthase (iNOS) and reduction of free radical production[90-92].

As mentioned in previous sections, several sulfonylureas have been proven to generate extra-pancreatic effects, especially with glimepiride[93]. Most cited articles show sensitizing effects with increased glucose uptake in skeletal muscle and adipose tissue via higher density of GLUT4 in plasma membrane[94,95]. Even though these studies proved the association, they did not show how these effects came to be until the XXI Century, when reports of glimepiride, gliben-

clamide and telmisartan agonist activity of PPARγ surfaced. Fukuen et al.[96] published a classic manuscript describing how glimepiride was capable of inducing PPAγ activity (25% potency of Pioglitazone) in HEK293 cells, with parallel increase in DRIP205 co-activator participation and dissociation of co-repressors NCoR/SMRT. Likewise, this study also revealed that glimepiride assembles complexes with these receptors, competing with Rosiglitazone for its binding site. Moreover, this glimepiride-PPARγ complex modified mRNA levels of PPARγ-gene targets in 3T3-L1 adipocytes, including ADPN[96]. Such findings were confirmed by Tsunekawa et al.[97] which reported that ADPN expression is enhanced in T2DM patients when treated with glimepiride.

An important aspect concerning sulfonylureas, it's the pro-apoptotic effects reported with glibenclamide, glimepiride and repaglinide treatment in in vitro studies with pancreatic beta cells[98-100]. In fact, the UKPDS (United Kingdom Prevalence Diabetes Study) published that glibenclamide and chlorpropamide were associated with better glycemic control and lower incidence of microangiopathic complications. However, even though improved HOMA-βcell indexes are observed, further down the line there is a progressive decline of beta cell function with literal fatigue of this cell, observed in almost all sulfonylureas[10]. In vitro and animal studies have suggested that beta cell decline is associated with activation of Akt, NADPH oxidase and increased oxidative stress, and these outcomes seem to de dose-dependent[102,103]. Del Guerra et al.[104] analyzed that glucose stimulated insulin secretion, insulin content, islet apoptosis and GLUT1 expression in human Langerhans islets exposed to glimepiride (10 μM), glibenclamide (10 μM) and chlorpropamide (600 μM). Insulin content diminished after the exposure with the three drugs, albeit glucose stimulated insulin secretion was steady on the cells exposed to glimepiride, not with the other 2 sulfonylureas[104].

In fact, Remedi and Nichols[105] evaluated prolonged pancreatic hyperexcitability with implants that were able to measure depolarization impulses in beta cells when exposed to glibenclamide. They reported a progressive decline in production of and insulin secretion, effects that were reverse after glibenclamide treatment was removed. Moreover, immunostaining of pancreatic islet cells demonstrated normal-sized α and β cells when further evaluating them using TUNEL technique (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling)[106]. These findings are similar to those found on our laboratory using low doses of glimepiride in combination with metformin[22,106]. Taking all this information into account, it's conceivable that glimepiride´s biological profile is more favorable than other sulfonylureas, especially due to its insulin-mimnetic effects, anti-inflammation properties and recently published anti-apoptotic effects via Bcl/Bcl_{XL} and protein 14-3-3ε [103,107-109].

Metformin – the oldest new kid on this block

As previously mentioned, metformin´s IUPAC name is N,N-Dimethylimidodicarbonimidic diamide (**Figure 1**), is positively charged at physiological pH, with a pKa of 2.8 and 11.51 [110]. Its molecule has five nitrogen groups, making it electronically possible to form square planar complexes with transition metals like copper and nickel by acting as a bidentate ligand coordinator in a 1:2 ratio manner[111]. Such structural conformations require the two imino groups, serving as primary, secondary and tertiary amino groups as electron donors during the conformation of the π bond[111]. Since copper bears functional importance due to mitochondrial bioenergetics[112], coordinated complexes with copper have extensively analyzed. Zhu et al.[113] published that deprotonated metformin forms a stable complex with deprotonated copper, described as $[Cu(C_4H_{10}N_5)_2]\cdot 8H_2O$, with a resulting copper atoms between a twofold rotation axis held together by van der Wall forces[114].

Absorption and distribution and metformin is quite complex and requires the intervention of small intestine, the liver and kidneys. The drug has an oral bioavailability of 40-60% and is completely absorbed during the first 6 hours after ingestion[115], with plasma levels between 54 – 4133 ng/mL[116]. The varying plasma levels are associated with a variety of plasma membrane transporters belonging to 3 transporter families, whose affinity guarantee metformin´s level of absorption (**Table 1**). Once inside the target cell, metformin will influence several aspects of its functionality, including mitochondrial energetics and DNA metabolism. Sun et al.[117] published a signaling pathway network analyzing which genes/proteins were relevant in metformin´s network. They found that the drug targets 65 upstream genes and some 355 downstream genes, and includes 7 fundamental genetic targets that are partially responsible for its antidiabetic and anticancer effects: CDKN1A, ESR1, MAX, MYC, PPARGC1A, SP1 and STK11[117]. The signaling cascades that are needed to activate these target proteins can be academically divided in two major groups: AMP-Dependent Kinase (AMPK)-dependent and AMPK-independent; which will be explained shortly.

Table 1. Plasma membrane transporters associated with metformin´s absorption.				
Name	**Class/Type**	**Locus**	**Structure**	**Associated SNPs**
Plasma Membrane Monoamine Transporter (PMAT)	**PMAT**: SLC29A4 [OMIM 609149].	7p22.1	PMAT: 11 transmembrane domains, 530 amino acids. Molecular mass of 58 kDa.	
Organic Cation Transporters (OCT)	**OCT1**: SLC22A1 [OMIM 602607].	6q25.3	OCT1: 12 transmembrane domains, 554 amino acids. Molecular mass ~47 kDa.	OCT1 420del: reduced metformin transport [191]. OCT1 R61C: reduced plasma membrane distribution [191].
	OCT2: SLC22A2 [OMIM 602608].		OCT2: 12 transmembrane domains, 555 amino acids. Molecular mass ~47 kDa.	OCT2 T199I, -T201M, -A270S: decreased metformin transport [192].
	OCT3: SLC22A3 [OMIM 604842]		OCT3: 12 transmembrane domains, 556 amino acids. Molecular mass ~47 kDa.	OCT3 T44M: metformin transport increases >50% [193]. OCT3 T400I, -V423F: significant reduction of metformin transport [P]. The SNPs is related to structural differences in the pore itself or membrane spanning helixes [193].
Multidrug And Toxicity Extrusion Protein (MATE)	**MATE1**: SLC47A1 [OMIM 609832].	17p11.2	MATE1: 12 transmembrane domains, 570 amino acids. Molecular mass 62 kDa.	MATE1 rs2252281: better glucose tolerance responses [194]. MATE1 loss-of-function variants are associated with higher liver concentrations and lactic acidosis [195]. MATE1 rs2289669: associated with reduction of HbA1c levels [196].
	MATE2: SLC47A2 [OMIM 609833].		MATE2: 12 transmembrane domains, 602 amino acids. Molecular mass 62 kDa.	MATE2 rs12943590: higher renal clearance and lower glucose tolerance responses [194]. MATE2 P103R: higher cargo transportation due to higher concentration on plasma membrane [197]. MATE2 Y273C: lower plasma membrane localization with lower metformin transport [197].

AMP-Dependent Kinase (AMPK)-dependent mechanism of action of Metformin

The Adenosin Mono-Phosphate-dependent Kinase (AMPK) is perhaps the most important piece in metformin´s intracellular signaling pathways. This αβγ heterotrimeric complex is constituted by 3 subunits: alpha-subunit which is the catalytic fraction beta-subunit[118] which functions as a scaffold protein[119]; and the gamma-subunit which is the AMP/ATP sensor[120]. When metformin enters the target cell, it activates AMPK and its subsequent downstream signaling which includes the following net effects: lower Acetyl-CoA Carboxylase (ACC) activity, lower expression of lipogenic enzymes and induction of fatty acid oxidation[121]. The activation of AMPK relies basically in two pathways: Mitochondrial-dependent and -independent mechanisms. Even though the mechanisms will be explain separately, they are indeed intertwined and will be discussed further.

The Mitochondrial dependent pathway requires the uncoupling of respiratory chain from oxidative phosphorylation, modifying overall cellular energetics[122]. Metformin inhibits Complex I due to interaction with deprotonated cupper using molecule chelating mechanisms[113,114], and such property has been proposed as a viable anticancer therapy due to cancer cell´s upregulation of OCT1 and weaker intracellular redox status[122,123]. Moreover, inhibition of this Complex was associated with enhanced superoxide production by 260%, increasing oxidative cellular damage and apoptosis induction, key processes in cancer control[124]. Uncoupling between the respiratory chain and Complex V shifts cellular bioenergetics, increasing glycolysis and lactate production[122], with a resulting overall decrease of ATP levels and progressive elevation of intracellular AMP. Once the ATP/AMP ratio shifts, the gamma-subunit from AMPK changes the cargo in its adenosine-binding sites, switching from ATP to AMP (considered a primed inactive state)[122], which loses the beta-subunit myristoylated tail from the alpha-subunit, making it susceptible to phosphorylation from upstream AMPK kinases like LKB1 and CaMKK (activated state)[122,125,126]; **Figure 4**. In accordance to this line of thought, AMPK non-catalytic subunits are more important than previously considered, suggesting that actually beta-subunit is the gatekeeper during metabolic stress sensing and AMPK activation[127].

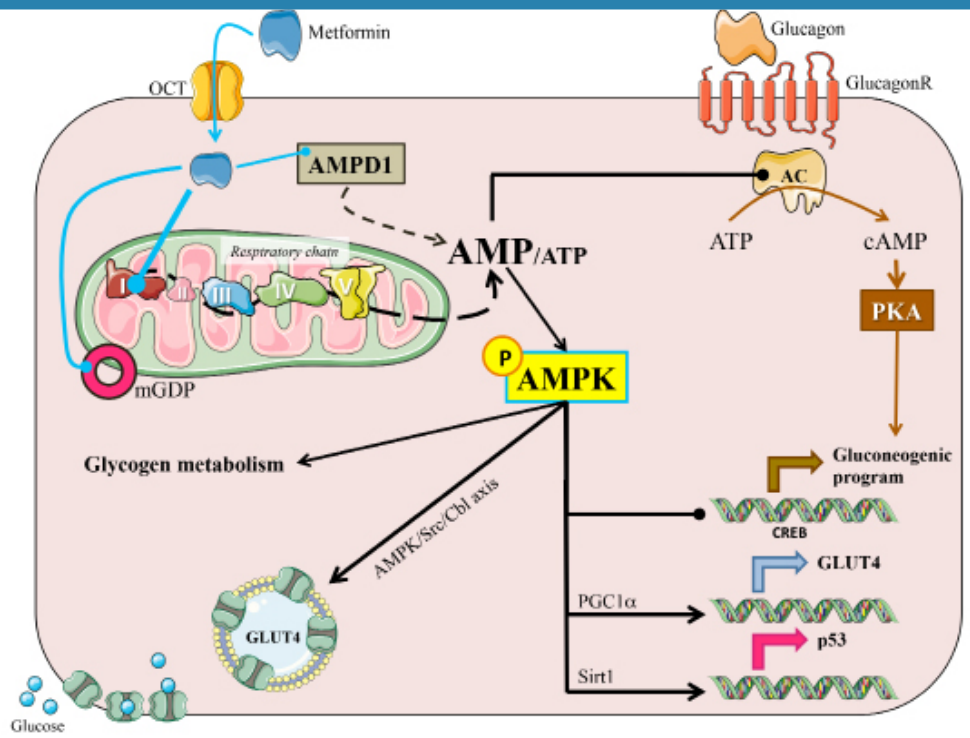
Among the Mitochondrial-independent proposed mechanisms for AMPK activation, Ouyang et al.[128] published that metfotmin inhibited the enzyme ADP Deaminase, responsible for the conversion of AMP to inosine monophosphate (IMP), increasing the availability of AMP. These findings were corroborated by Vytla and Ochs[129] that this biguanide increases free AMP and ADP availability, enhanced fatty acid oxidation and gluconeogenesis inhibition, placing the AMPD1 enzyme in a novel role as insulin sensitizer and probable pharmacological target[130]. In fact, modulation of the one-carbon metabolism and interference with folate availability, suggests that metformin actually mimics antifolate drugs by accumulating

5-formimino-THF[131,132]. The sudden increase in AMP levels has been also associated with inhibition of the Adenilate cyclase (AC) family of enzymes (EC 4.6.1.1). Miller et al.[133] published that the inhibition of AC by AMP[134] results in a blunted Protein Kinase A signal and therefore abrogates glucagon hyperglycemic signals and favors metabolic homeostasis in insulin resistant and diabetic patients[134], especially those with hyperglucagonemia[135].

AMPK downstream signaling

Once this "secondary messenger" has been activated, the end results can be classified into 2 groups: a) nuclear effects, which include the modulation of genetic expression of specific set of genes, induction of sirtuins and autophagia, and final modification of metabolic memory programming, and b) cytosolic effects, encompassing glucose transporter-4 (GLUT4) mobilization towards the plasma membrane; **Figure 3**.

Figura 3



Metformin: AMPK-dependent and –independent effects. Metformin has mainly two mechanisms of action. The first, is via its "second messenger" AMPK, which exerts nuclear effects over gluconeogenic genetic program, glucose transporters and housekeeping genes such as p53. Likewise, AMPK is capable of other cytosolic effects such as mobilization of GLUT4 vesicles towards the plasma membrane and interaction with glycogen-related enzymes in order to modify glycogen deposits. The second mechanism is using an AMPK-independent pathway, which included the uncoupling of the respiratory chain and oxidative phosphorylation, inhibition of mitochondrial glycerol phosphate shuttle and the enzyme AMP Deaminase. Interestingly, these mechanisms induce AMP elevation, which is known to allosterically inhibit Adenilate Cyclase (AC), blunting cAMP production and later activation of Protein Kinase A (PKA), literally shutting down Glucagon´s signaling pathway.

Overall Nuclear Effects

The Cyclic-AMP (cAMP) responsive element (CRE)-binding protein depends on the phosphorylation of Ser[133] (active state) in the KID domain of the protein via PKA[136]. The CRTCs (cAMP-regulated transitional Co-activators) are cytosolic proteins which mobilize towards the nucleus after cAMP induces dephosphorylation via inhibition of SIK[137]. These co-activators have multiple phosphorylation sites, including one for AMPK, the Ser[171] which serves as a negative modulator[136]. The CREB and its DNA binding site CRE are responsible for the expression control of gluconeogenic genes such as Pyruvate Carboxylase, Phosphoenolpyruvate Carboxykinase 1 and Glucose-6-phosphatase[136-140]. AMPK is a major genetic modulator, having major participation in several protein expression machineries, including transporting families, cytoskeleton-related proteins, enzymes, and housekeeping genes.

Indeed, several have made the assumption that AMPK and suirtuins may work hand in hand in managing metabolic stress, starvation and proper response[141]. In fact, AMPK and Sirtuin-1 share some common functions, such as inducing GLUT4 translocation[142] and fatty acid oxidation induction[143], both scenarios being associated with the activation of Peroxisome Proliferator-Activated Receptor-gamma Coactivator 1-alpha. It´s been demonstrated that metformin itself induces SIRT1 activity by incrementing intracellular NAD+ levels, resulting in increased SIRT1 activity, including three especial targets: PGC1α, Forkhead Box O1 and Forkhead Box O3 [144]. The biguanide´s actions rely on AMPK´s ability to increase the expression of Nicotinamide Phosphoribosyltransferase, the key enzyme during NAD+ salvage pathways[145], favoring sirtuin activity. Finally, a novel pathway between AMPK and SIRT1 has been described, and it relates to the decreased availability of p53 via metformin-mediated inhibition of MDM2 (murin double minute 2), the ubiquitin-ligase responsible for p53 ubiquitinization and destruction[146]. Metformin induces AMPK activity in high and low glucose concentrations, yet SIRT1 is only induced by the drug during high-glucose condi-

tions[146]. Such effect on p53 could impact in the activation of senescence, DNA repair and aging mechanisms associated with hyperglycemic states. As a side note, another sirtuins seems to participate.

In regards to glucose uptake via insulin-dependent tissues, the Facilitated Glucose Transporter member 4, otherwise known as GLUT4, is perhaps the most important glucose transporter during the postprandial phase, since it correlates with insulin sensitivity and glucose deposition[147]. Grisouard et al.[148] have proven that metformin increases the expression of GLUT4 mRNA but not GLUT1 mRNA via AMPK, favoring glucose uptake in insulin-dependent tissues such as adipose and skeletal muscle tissues. Almost 20 years ago, Lenzen et al.[149] demonstrated that metformin could modify the expression of glucose transporters in the small intestine, with increased expression of GLUT5 and SGLT1. By 2005 Walker et al.[150] reported that AMPK induced GLUT2 translocation towards the brush-border membrane (BBM), suggesting that metformin might be involved in other glucose transporter systems. Five years later, Sakar et al.[151] confirmed that metformin via-AMPK does redistribute glucose transporters GLUT2 towards the BBM, whereas reducing SGLT1´s concentration in this cellular region.

Overall Cytosolic Effects

The translocation of GLUT4 to the plasma membrane has been reviewed elsewhere[152], but certain aspects will be detailed in order to describe the effects of metformin in this process. Insulin downstream pathways depict the phosphorylation and activation of Phosphoinositide-3-kinase, key enzyme in insulin metabolic effects. Once PosphoInositol-3,4,5 is generated, this serves as an anchor and allosteric activator for the serine/threonine kinase 3-Phosphoimositide-dependent Protein Kinase and PDK2. Both PDK enzymes have Akt/Protein Kinase B as target, but they phosphorylate different targets[153,154]: PDK2 associates with mTOR/Rictor in order to phosphorylate Ser^{473}, and afterwards, PDK1 phosphorylates Thr^{308}. Once Akt is activated, phosphorylates AS160, a GTPase protein which is known to block VAMP2-vesicles towards the plasma membrane by favoring the generation of Rab-GDP[155]. Lee et al.[156] reported that metformin increases phosphorylation of AS160 and enhances the activity of Rab4-GTPase activating protein and even increases phosphorylation of Protein Kinase C-zeta (PKCζ), modulating insulin-dependent GLUT mobilization towards the plasma membrane and overall improvement of glucose uptake, notions that were previously reported by Thong et al. in 2007 [157]. The PKCζ is known to be involved in the dynamics of vesicle traffic via actin remodeling[158], suggesting that metformin also influences cytoskeleton networking. Moreover, Lee et al.[159] also published that metformin stimulates an alternate vesicle-traffic inducing pathways, the AMPK/Src/Cbl axis. The Cbl-CAP-CrkII-C3G-TC10 pathway is an alternate pathway for GLUT4-vesicle mobilization towards the plasma membrane[160].

As a final note, there are other miscellaneous effects attributed to metformin, including modulation of mitochondrial shuttles, like the Glycerol phosphate shuttle. This mechanism of reducing equivalent mobilization is important during keep the respiratory chain active using Complex II as the catapult[161]. Madiraju et al.[162] confirmed that metformin inhibits the Glycerophosphate Dehydrogenase, which modifies hepatic redox states, decreasing the rate of gluconeogenesis: albeit increasing the chance for lactoacidosis since the conversion of lactate to glucose is seriously blunted in a dose-dependent manner. Similarly, this shuttle seems to be prone to electron leak and could be considered an important site for ROS production using intermediaries such as flavin or semiquinone[163]. Moreover, in 2003 Otto et al.[164] reported that hepatocytes incubated with metformin had impaired gluconeogenesis and interrupted glycogen synthesis as well. AMPK´s activity seems to be modulated by carbohydrate-binding via its beta-subunit, and this chemical property seems to be important in glycogen metabolism. During metabolic stress, acute exposure to AMPK is bound to block glycogen synthesis in favor of glucose oxidation and ATP production by phosphorylating Glycogen Synthase at Ser^{8} [165]. However, chronic activation of AMPK has been known to increase glycogen synthesis due to increased availability of glucose-6-phosphate[166]. In fact, AMPK is also known to esoterically associate with Glycogen-phosphorylase[164] and Glycogen debranching enzyme[167] and such interactions seem to depend on the beta-subunit and autophosphorylation of βThr148 residue[168].

Together is better

The use of glimepiride and metformin in this day and age of new anti-diabetic drugs such as SGLT2 inhibitors[169], can still be justified by the numerous synergistic effects of these “old school” drugs, especially considering their respective pleiotropic effects, albeit considerable lesser side effect. Using an animal model of streptozotocin + high fat diet to induce diabetes in rats, Saad et al.[170] reported that metformin increased ADPN levels, while glimepiride was more powerful when concerning lowering nonesterified fatty acids, suggesting that both drugs exerted cardiovascular protection functions. In fact, metformin/glimepiride combination showed lower crude incidence rated of cardiovascular mortality (20.7 [19.7-21.7]) and lower rates of cardiovascular death (9.6 [8.9-10.3]) when compared with metformin/glibenclamide and metformin/glipizide[171]. When comparing effect over HbA1c between metformin/glimepiride fixed doses vs. metformin tittering doses, the combination was significantly superior in lowering basal levels[172], even when metformin was up to 2550 mg/day[173]. In fact, there´s a plateau effect of metformin at 1500 mg, after which no additional response has been observed[174,175].

The combination of low dose of glimepiride and up to 1500mg of metformin has been proven to be effective in managing not only insulin resistance in T2DM, but to properly wield pleiotropic effects that enhance glycemic control. Our laboratory published in 2007 [22] that 0.5 mg/day of glimepiride with 1500

mg/day of metformin daily is associated with powerful reduction of fasting, postpandrial levels, associated with improved HOMA-IR and HOMA-βcell indexes. These findings have been observed in further clinical studies such as the one from Keiko et al.[176], reporting significant and sustained glycemic control when using these drug quantities, even when compared with triple-medication therapy (sitagliptine, metformin and sulfonylurea). Likewise, González-Ortiz et al.[177] reported that 1 mg of glimepiride in combination with metformin (500 mg/day) was more efficacious than glibenclamide (5 mg/day) with metformin in achieving glycemic control and sustained management of uncontrolled T2DM.

Concluding remarks

The sustainability of the effects, the improvement of directly damaged and associated organ dysfunction, acceptable oral tolerability and lower side effects are probably the desired outcomes of any pharmacological therapy, especially for one that is used for long periods of time, such as T2DM pharmacotherapy. Several anti-diabetic drugs have been approved[178], new ones are recently[169] and certainly more will be devised. The pharmacological goal is to standardize which is a better monotherapy and how to choose add-on medication onwards, reflected in the latest management guide sponsored by the American and European diabetes agencies[179]. The individual effects of glimepiride over metformin could very much improve the patient´s metabolic and cardiovascular profiles, especially when such benefits are obtained with lower dosages than the standard[170-177]. Such properties not only could guarantee adhesion to the oral medication but could enhance the prognosis of T2DM patients, especially in the younger patients.

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