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Biguanides drugs: past success stories and promising future for drug discovery

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ABSTRACT

Biguanides have attracted much attention a century ago and showed resurgent interest in recent years after a long period of dormancy. They constitute an important class of therapeutic agents suitable for the treatment of a wide spectrum of diseases. Therapeutic indications of biguanides include antidiabetic, antimalarial, antiviral, antiplaque, and bactericidal applications.

This review presents an extensive overview of the biological activity of biguanides and different mechanisms of action of currently marketed biguanide-containing drugs, as well as their pharmacological properties when applicable. We highlight the recent developments in research on biguanide compounds, with a primary focus on studies on metformin in the field of oncology. We aim to provide a critical overview of all main bioactive biguanide compounds and discuss future perspectives for the design of new drugs based on the biguanide fragment.

Keywords:

Biguanide
Antidiabetic
Antimalarial
Antiseptics
Anticancer

Contents

1. Introduction
2. Properties of Biguanides
3. Antidiabetic agents
 - 3.1. Metformin
 - 3.1.1. Mode of action
 - 3.1.2. Molecular mechanism of action
 - 3.2. Phenformin
 - 3.3. Bufornin
4. Antimalarial agents
 - 4.1. Mode of action
 - 4.2. Proguanil
 - 4.3. Clorproguanil
 - 4.4. Clociguanil
 - 4.5. WR99210
 - 4.6. PS-15
5. Biguanides as antiseptics and disinfectants
 - 5.1. Chlorhexidine
 - 5.1.1. Mode of action
 - 5.2. Alexidine
 - 5.2.1. Mode of action
 - 5.3. Picloxydine
 - 5.4. Polymeric biguanides
 - 5.4.1. Mode of action
6. Antiviral activity of biguanides
7. Anticancer activity of biguanides
 - 7.1. Metformin transport and organic cation transporters

- 7.2. Modes of action
 - 7.2.1. Indirect mechanism
 - 7.2.2. Direct mechanism
 - 7.2.2.1. AMPK dependent
 - 7.2.2.1.1. Inhibition of lipogenesis
 - 7.2.2.1.2. AMPK induced autophagy
 - 7.2.2.1.3. Downstream effects of the mTORC1 inhibition
 - 7.2.2.1.4. Inhibition of NF- κ B pathway
 - 7.2.2.1.5. Inhibition of cancer cell invasion and migration
 - 7.2.2.2. AMPK dependent
 - 7.2.2.2.1. Cell cycle arrest
 - 7.2.2.2.2. Inhibition of unfolded protein response (UPR)
 - 7.2.2.2.3. Immune response
 - 7.2.2.3. Mechanisms via other pathways
- 7.3. Past and recent clinical studies
- 8. Conclusions and Outlook

1. Introduction

Biguanide – or amidinoguanidine – is a purely synthetic chemical function derived from guanidine, in which two guanidines are linked to each other with a common nitrogen atom. Although being related to guanidine, biguanide is a very distinct chemical function with its own physicochemical and pharmacological properties that render it a particularly valuable pharmacophore in medicinal chemistry. Since the first demonstration of the antihyperglycemic properties of the biguanide derivative metformin a century ago,[1] biguanide drugs experienced several waves of intense research interspersed with long periods of dormancy. The two decades following World War II saw the discovery of key pharmacological applications of biguanides as antidiabetics[2-4], anti-malaria[5], disinfectants[6-9], and anti-virals[10], and the emergence of the most famous biguanide drugs (**Figure 1**). Further progressing hurdles for the discovery of new anticancer drugs led scientists and clinicians to look closer at already approved biguanides. Such repurposing studies proved to be fruitful for the main class representative metformin, injecting new momentum into biguanide research.

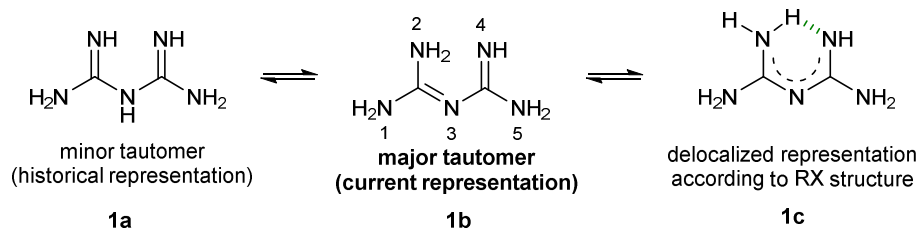
Currently, seven biguanide drugs are commercially available in the USA, and three additional ones are available on other national markets. Among those, drugs: metformin, chlorhexidine, and proguanil are included in the WHO List of Essential Medicines[11]. Despite the importance and uniqueness of biguanides as promising drugs with a broad range of activities, the literature on this class of compounds is relatively sparse in terms of the number of drugs with this motive. This review aims at spotlighting the very particular biguanide function by presenting the undeniable past successes of biguanide drugs in various therapeutic fields, in particular in oncology research for metformin. With this review, we would like to draw attention to the value of the biguanide motive as a potential pharmacophore and suggest its incorporation in the design of future drugs in oncology, as well as other fields.

Given the existence of an extensive overview of biguanides from the perspective of medicinal chemistry[12], as well as comprehensive reviews on the synthesis and reactivity of biguanides[13, 14], this work focus on the different mechanisms of action of biguanides. It presents concisely the most important biological and clinical data reported for biguanides sorted by their therapeutic indications. The discovery of different therapeutic agents is put into historical context and follows chronological order. It spans from century-dated anti-diabetes reports of properties to an emerging interest in anticancer potential. The mode of action and pharmacological parameters of marketed biguanide drugs and the most promising drug candidates are discussed and compared. Special emphasis is given to metformin, a molecule sometimes praised to be a “magic drug” against metabolic disorders, age-related diseases, and cancer.

Finally, we do not attempt to detail every single aspect of the multifaceted field of metformin anticancer research, that has been extensively covered in other works.[15-18] Rather this review is aimed at highlighting the most important findings in the clinical application of biguanides and their mode of action, as well as providing critical analyses and an outline of the future perspectives for the design of new biguanide-based drugs.

2. Properties of Biguanides

Biguanide structure was presented similarly to diketones (**Scheme 1, 1a**). However, this structural representation failed to explain some aspects of biguanide reactivity. In 1977 X-ray analysis revealed the absence of hydrogen in position 3[20], suggesting that structure **1b** (**Scheme 1**) should be used instead. Furthermore, it was found that the length of C-N bonds lays between 1.297-1.387Å. This value falls in between the length of a single C-N (1.42Å) and a double C=N (1.28Å) bond. Judging from these values, the most realistic representation of biguanide would be a delocalization of the π -electron density along the molecule, together with an intramolecular hydrogen bond (**Figure 1, 1c**).[21] Later it was established that protonated biguanides should be treated differently from their neutral form. Computational studies reported by Bharatam *et al.*[22] show that the protonated biguanides potentially behave like nitrones (divalent N1 compound) with coordination bonds rather than conjugated systems. In this article biguanides are presented in their major neutral tautomeric form **1b** with the conjugated system -C=N-C=N-, and the numbering of the different atoms will be established as depicted in **Scheme 1**.



Scheme 1. Tautomeric forms of biguanide.

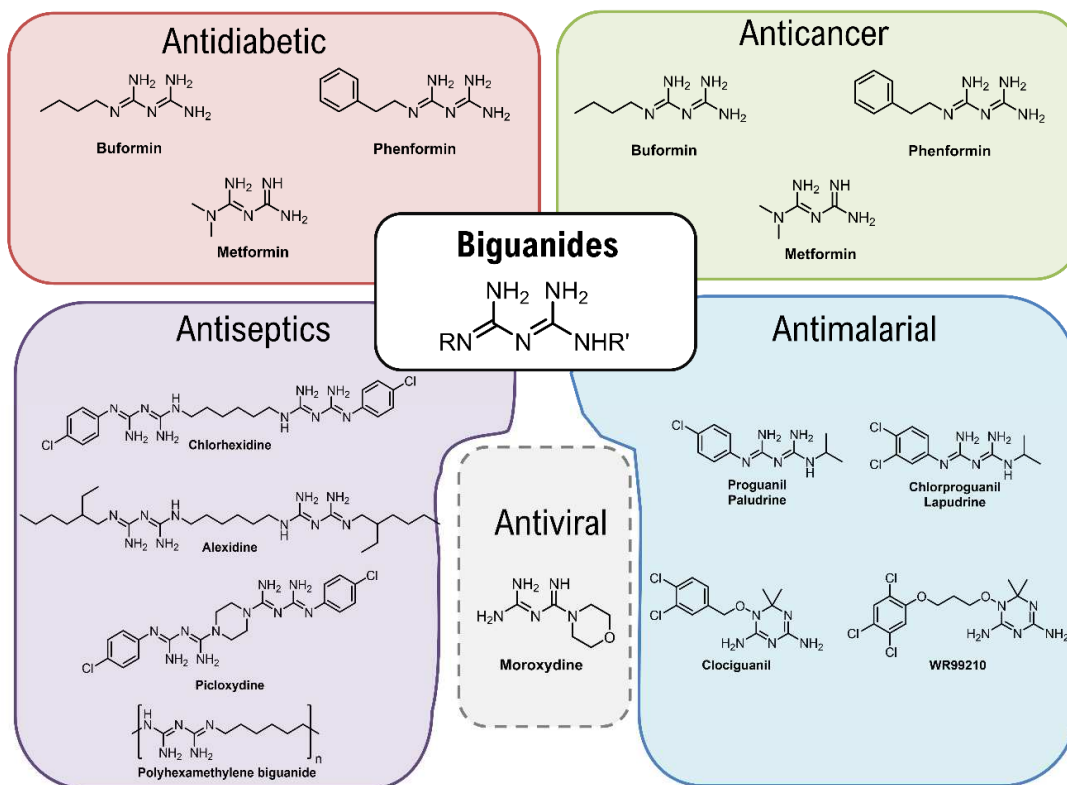


Figure 1. The main clinically used biguanide drugs.

3. Antidiabetic agents

The use of guanidine derivatives as sugar lowering medicine dates back to medieval times when the toxic plant *Galega officinalis* (French lilac) was used in traditional medicine.[35] The active components of its extract were identified in the 1920s as galegine (or isoamylene-guanidine) and synthalin (decamethylene-bisguanidine).[36, 37] While these guanidine compounds demonstrate substantial hypoglycemic properties,[38-40] significant hepato-, and renal toxicities curtailed their medical application.[37] Further efforts into the preparation of guanidine derivatives resulted in the synthesis of metformin (**Figure 1**) by Werner and Bell[41] in 1922. Despite early reports on chemical synthesis and anti-hyperglycemic activity[1, 42], antidiabetic properties of biguanides were shadowed by the side-effects of synthalins and increased availability of insulin in the 1920s-1930s. The revival of interest in metformin took place a decade later, fueled by the success of another biguanide – proguanil – as an antimalaria agent. Extensive investigations conducted in the 1950s by Sterne and Duval[43] eventually brought metformin to the clinic in 1957. Subsequently, a vast number of biguanides analogous to metformin were developed, two of which were commercialized: buformin and phenformin (**Figure 1**).

The use of metformin and its analogs in diabetes therapy remains one of the biggest success stories within the biguanide family.[44] The following sub-sections contain an overview of the pharmacological properties of those biguanides, which brought them to the market as antidiabetic drugs. The current understanding of the antihyperglycemic mode of action is demonstrated on metformin. Some molecular pathways involved in glucose regulation play an equally important role in anticancer activity displayed by metformin.

3.1 Metformin

Described in the 1920s, metformin has been fully studied only 30 years later, after the withdrawal of the synthetic guanidine derivatives synthalins[45]. Metformin became available on the market for the treatment of diabetes first in France in 1957,[45] later in Europe and Canada (until the 1970s), and the Food and Drug Administration of the United States approved it only in 1994.[46] Since 2011, metformin is on the List of Essential Medicines approved by the World Health Organization and is part of the composition of 113 different drug brand names.[37] It has unique properties to fight type II diabetes, which cannot be treated with insulin. Metformin lowers blood glucose levels by improving insulin sensitivity, particularly in the liver. Some studies have also shown decreased food intake and body weight upon metformin treatment. Based on a meta-analysis of 31 trials, encompassing a total of 4570 participants, Salpeter *et al.* demonstrated that metformin treatment results in a decrease of the body mass index (BMI) by 5.3% (95% CI, 4.0-6.7) compared with placebo or no treatment after a minimum of 8 weeks of treatment.[47] Besides, Glueck *et al.* reported 15% weight loss in a group of 31 non-diabetic morbidly obese patients after 28 weeks of metformin treatment.[48] Further research and analyses revealed that patients taking metformin present reduced risks of cardiovascular diseases.[49] Actually, metformin positively influences multiple cardiovascular risk markers, including lipid profile[47, 50] and fatty liver,[51] as well as inflammatory markers.[52, 53] An initial report about a possible link between reduced risk of cancer and metformin treatment was published by Evans *et al.*[54] in 2005. This particular property of metformin is discussed in detail in the following dedicated section of the review.

Metformin is a non-toxic safe drug, with relatively few side effects. As a result, it can be prescribed at dosages up to 3g daily.[55] The major limitations of metformin are gastrointestinal side effects (mostly diarrhea), which usually diminish over treatment time and can be minimized by dose adjustment, absorption during meals, or by using extended-release formulations of metformin.[56] Another concern in biguanide treatment is the appearance of lactic acidosis, which is a substantial issue with buformin and phenformin (*vide supra*). However, the incidence of lactic acidosis for metformin is low with 0.05 cases per 1000 patients/year.[57, 58] Moreover, another study revealed that this incidence was similar in diabetic patients on metformin compared to diabetic patients not treated by metformin.[59] Overall, metformin presents an exceptionally good safety profile with an LD₅₀ of 1000 mg/kg in rats[60]. Its high therapeutic index prompted numerous research groups to conduct clinical trials for the management of other pathologies other than diabetes, such as polycystic ovarian syndrome,[61, 62] metabolic syndrome,[63] and many forms of cancer.[64] In polycystic ovarian syndrome, metformin can be used as second-line therapy for restoring ovulation, improving fertility, sustaining weight loss, and decreasing the frequency of both early pregnancy loss and gestational diabetes mellitus (GDM).[65] Metformin appears to be safe to use during pregnancy with no evidence of obvious adverse consequences reported so far.[66] Moreover, due to the fact that metformin affects 9 main hallmarks of aging in different organisms such as loss of proteostasis, deregulated nutrient sensing, stem cell exhaustion, epigenetic alterations, genomic instability, telomere attrition, mitochondrial dysfunction, cellular senescence, and altered cell-to-cell communication, metformin was also proposed as “anti-aging drug”.[67, 68]

3.1.1. Mode of action

The antihyperglycemic action of biguanides is mainly related to the inhibition of liver gluconeogenesis, resulting in lowering of glucose levels in blood with no risk of induction of hypoglycemia in type 2 diabetic patients (**Figure 2**).[69-72] In the liver, where it accumulates, metformin has a dual effect. First, it increases the activity of the insulin receptor and enhances glucose uptake by increasing the translocation of glucose transporters (GLUT-1) to the plasma membrane.[73] Thus, metformin shows insulin-mediated inhibition of gluconeogenesis. Second, it reverses the gluconeogenic action of glucagon.[74, 75] These cumulated effects result in the inhibition of gluconeogenesis and the stimulation of glycolysis. Overall, metformin was reported to reduce the hepatic glucose output by up to 75%.[76] In skeletal muscles, metformin improves insulin sensitivity (disturbed in type II diabetes) and insulin-mediated glucose uptake. This effect is achieved through an increase in the tyrosine kinase activity of the insulin receptor[73] and through enhanced activity and translocation of glucose transporters (GLUT-4) to the plasma membrane.[77] Given that metformin action is associated with modified insulin sensitivity rather than increased insulin concentration, it, therefore, has very little potential for inducing hypoglycemia.[78] In the pancreas, metformin has been shown to increase circulating levels of glucagon-like peptide-1 (GLP-1). GLP-1 acts as a glucose regulator by increasing insulin levels and decreasing glucagon secretion.[79-82] At the same time the study on glucose transport and metabolism in human adipocytes reported that metformin did not alter GLUT-1 but increased GLUT-4 mRNA expression and cellular protein content.[83] It was also noted that metformin treatment resulted in decreased activity of dipeptidyl peptidase-4 (DPP-4), the enzyme principally responsible for inactivating GLP-1 in tissues and blood.[44, 84] Further studies showed that metformin did not directly inhibit DPP-4 activity *in vitro*. [79, 85-87] It was proposed that metformin may also induce upregulation of the expression of GLP-1 receptors on the surface of pancreatic β -cells, and therefore may provide modest support to the function of β -cells.[88, 89] Those studies are somewhat controversial to the reports of progressive increase in apoptotic β -cells upon prolonged metformin exposure (>24 h).[90] At present it is believed that under normal conditions metformin does not either promote[91] or inhibit[92] insulin secretion. In summary, the ability of metformin to reduce circulating glucose levels can be explained by at least four different mechanisms: (1) increased glucose uptake in the liver and muscles, (2) reduced gluconeogenesis, (3) improved GLP-1 and (4) reduced glucagon functions. Nevertheless, the exact molecular mechanisms by which metformin achieves these effects are still largely debated.

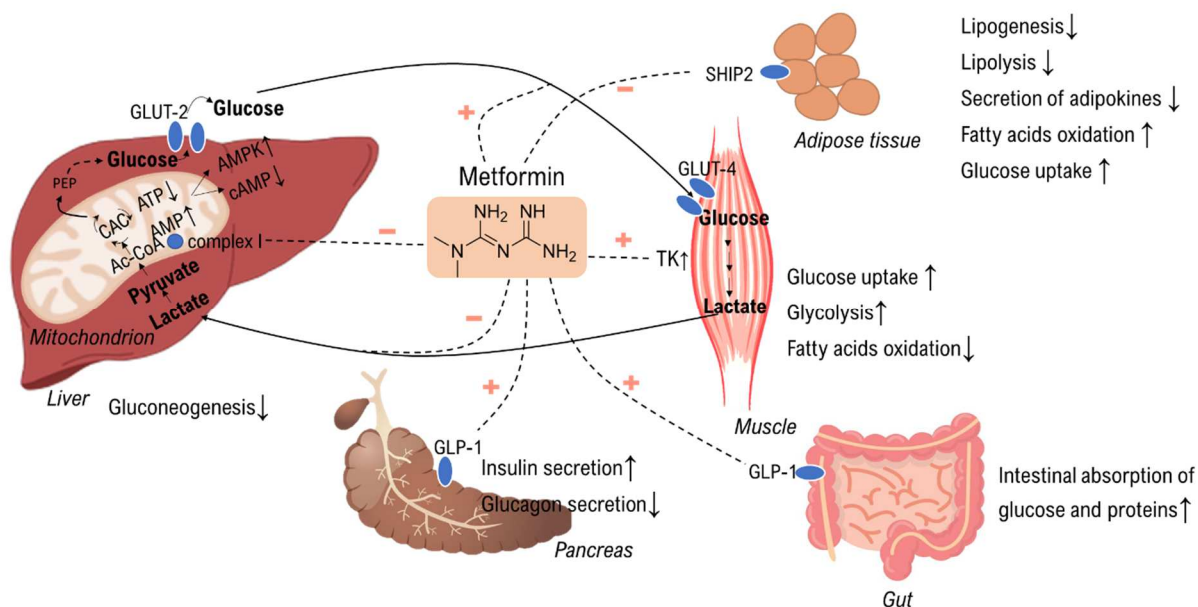


Figure 2. Mode of action of metformin. Metformin decreases hepatic gluconeogenesis by inhibiting complex I of the mitochondrial respiratory chain. Mitochondrial impairment leads to an increase of AMP concentrations and a decrease of ATP, which in turn activates (AMPK) and inhibits the synthesis of cAMP. Metformin suppresses gluconeogenesis from several substrates, including lactate and pyruvate. In skeletal muscles, metformin facilitates glucose uptake by enhancing glucose transporter GLUT-4 and by increasing tyrosine kinase activity of insulin receptors. Another mechanism of action of metformin is the stimulation of GLP-1 receptors in the pancreas, which increases the secretion of insulin and decreases the secretion of glucagon in response to high glucose levels.

3.1.2. Molecular mechanism of action

The molecular mechanisms of action of metformin on hepatic gluconeogenesis have not been fully elucidated yet [93, 94], but some studies suggested that at least partially mitochondrial complex I inhibition is involved. Owen *et al.* showed that metformin caused time-dependent inhibition of complex I in isolated mitochondria [95]. This observation was supported by further reports on the reduction in the activity of complex I measured in tissue homogenates exposed to metformin [96]. Being positively charged at neutral pH, metformin is attracted by the mitochondrial membrane potential, which appears to be its primary organelle target [97]. Thus, it accumulates in the mitochondrial matrix at concentrations up to 1000 times higher than in the extracellular medium. In the mitochondria, it transiently inhibits complex I (NADH: ubiquinone oxidoreductase) [98] which results in decreasing ATP production by oxidative phosphorylation [99] and increasing AMP levels. This initiates, in turn, two major pathways: inhibition of glucagon-induced cAMP synthesis, as demonstrated in the liver [100], and activation of 5'-AMP-activated protein kinase (AMPK) [101] which switches on the cellular metabolism toward energy production and switches off anabolic, energy-consuming pathways. In addition, AMPK phosphorylates and increases the activity of insulin receptors and IRS-2 and enhances the translocation of glucose transporters [73, 102]. Inhibition of fatty acid synthesis and increased rates of β -oxidation [103] have also been linked to AMPK activation [104]. Nonetheless, the hypothesis that AMPK activation would be the main driving force behind the decrease of hepatic gluconeogenesis lacks solid evidence. The main contradiction comes from the results of Fortez *et al.* [105] in which metformin lowered glucose production in the liver of transgenic mice that lacked AMPK or its upstream activator LKB1. Also, metformin-treated animals showed improved vascular responsiveness to insulin, improved insulin-stimulated muscle Akt phosphorylation, while no detectable levels of metformin in plasma or

change in muscle AMPK phosphorylation was reported.[102] Furthermore, metformin, at clinically relevant plasma concentrations, does not appreciably alter energy charge or AMP concentration suggesting that metformin mechanism for the downregulation of gluconeogenesis could be AMPK-independent. In 2012 it was suggested that metformin could stimulate glucose uptake in muscle cells via an AMPK-independent mechanism that involves some novel/conventional protein kinase C.[106] Later Shulman *et al.* reported that metformin non-competitively inhibits the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase.[72] More recently the same group provided evidence that metformin inhibits hepatic gluconeogenesis in a redox-dependent manner independently of reductions in citrate synthase flux, hepatic nucleotide concentrations, acetyl-CoA carboxylase activity, or gluconeogenic enzyme protein expression.[107] For more detailed information on metformin's cellular and molecular mechanisms of action readers are referred to the most recent literature reviews[108-111]

3.2. Phenformin

Phenformin or phenethylbiguanide is a biguanide derivative that contains a phenyl-ethyl part attached to the biguanide group (**Figure 1**). Phenformin was first synthesized in 1958 by Shapiro,[112] together with buformin, and was released on the market in 1959. It exhibits the most potent glucose-lowering properties in the biguanides drug class.[113] For example, the minimal active subcutaneous dose in fasted guinea pigs is 7.5-10 mg for phenformin compared to 20 mg for buformin and 75 mg for metformin. It exhibits higher lipophilicity, a higher affinity to human organic cation transporter 1 (hOCT), and therefore faster uptake kinetics compared to metformin.[114, 115] Both drugs are believed to have similar modes of action but phenformin shows a higher affinity to the mitochondrial membrane.[116] In recent studies, phenformin similarly to metformin was found to elicit a significant therapeutic effect through a redox-dependent but complex I-independent mechanism. Phenformin inhibits mitochondrial glycerophosphate dehydrogenase (mGPD), a component of the glycerophosphate shuttle, and causes elevations of intracellular NADH content.[117] To compensate for the mitochondrial impairment, glycolysis rates are accelerated. Indeed, the preservation of ATP levels despite phenformin exposure has been reported.[97] Increased glycolysis rates result in higher glucose uptake and yield lactate. These high lactate effluxes into the system cannot be fully oxidized, and result in increased lactate concentration in the plasma,[76] a phenomenon known as lactic acidosis. This latter leads to respiration inhibition and could be *in fine* lethal. Adverse effects due to lactic acidosis led to phenformin withdrawal from most drug markets in 1978, although it is still available in Brazil, Uruguay, China, Poland, Greece, and Portugal.[118, 119]

3.3. Buformin

Buformin is another biguanide with glucose-lowering properties, presenting an *n*-butyl group on the biguanide structure (**Figure 1**). Buformin has also been first synthesized in 1958 and was commercialized the year after, simultaneously with phenformin.[112] It presumably has similar mechanisms of action to metformin and phenformin.[120] It delays the absorption of glucose from the gastrointestinal tract, increases insulin sensitivity and glucose uptake into cells, and inhibits gluconeogenesis in the liver. Similarly to phenformin, buformin treatment correlates with an elevated risk of lactic acidosis, the risk ranking being as follows:

phenformin>buformin>>metformin.[121] Buformin was therefore withdrawn from the market in many countries, although it is still available in Japan, Taiwan, Romania, and Hungary.[122]

4. Antimalarial agents

Ironically, currently renowned for hypoglycemic and antiseptic properties, biguanides first came to the drug market not as biocides or diabetes medication, but as a cure for one of the deadliest infectious diseases in human history – malaria. By the end of the 20th century, malaria claimed between 150 million and 300 million lives, accounting for 2 to 5 percent of all deaths.[123] Access to antimalaria drugs proved to be a vital asset during the two World Wars. As a result, several classes of antimalarial drugs were developed by the 1940s. They include 4- and 8-aminoquinolines, amino-alcohols, sulfonamides and sulfones, diaminopyrimidines, sesquiterpene lactones, tetracycline, and macrolide antibiotics, naphthoquinones, and biguanides.[124] The latest are acting as nucleic acid synthesis inhibitors more specifically known as antifolates. They can be further subdivided into two types: inhibitors of dihydropteroate synthase (DHPS), known as type I antifolates, and inhibitors of dihydrofolate reductase (DHFR), known as type II antifolates. Proguanil, the first biguanide reported as an antimalarial antifolate agent, and all anti-malarial biguanide drugs developed since then (chlorproguanil, clociguanil, WR99210, and PS-15) are type II antifolates (**Figure 3**). Given that the combination of DHFR and DHPS inhibitors is synergistic,[125-127] the dual administration of type I antifolates (like sulfachrysoidine, sulfalene, and dapsone) and biguanides is nowadays the preferred strategy for the treatment and prevention of malaria.

4.1. Mode of action

All mentioned antimalarial biguanide drugs act as antifolates by disrupting the folate metabolism. In most species, tetrahydrofolic acid (THA) is essential for the biosynthesis of thymine, purine nucleotides, and some amino acids (Met, Gly, Ser, Glu, and His). While humans obtain THA from pre-formed dihydrofolic acid through nutritional intake, pathogenic microorganisms including *Plasmodia* are synthesizing dihydrofolic acid *de novo* from simple precursors. Two key enzymes of the folate biosynthetic pathway of the malaria parasite, dihydropteroate synthase (DHPS), and dihydrofolate reductase (DHFR) are either completely absent in humans or differ significantly from the human variants allowing for selective inhibition. Biguanides act as class II antifolates by inhibiting the parasitic dihydrofolate reductase (DHFR).[128] This hinders the parasite recycling of dihydrofolate back to

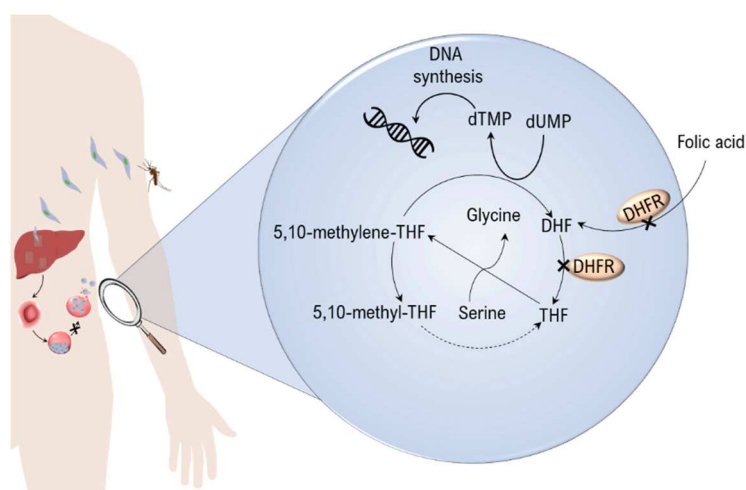
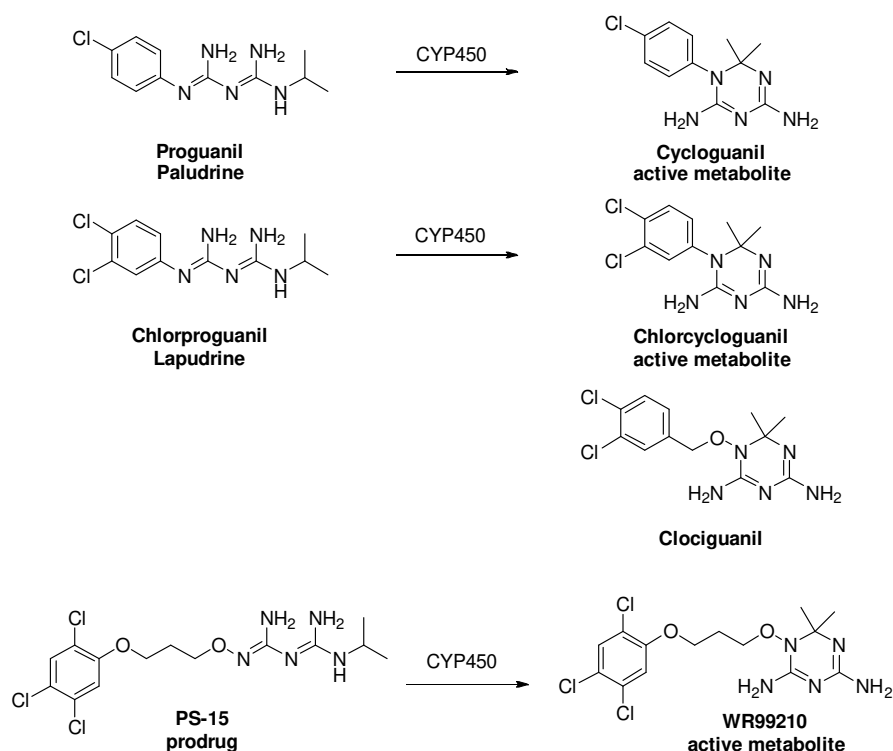


Figure 3. Mode of action of biguanides used in malaria treatment. Biguanides with antimalaria properties act on folate metabolism in erythrocytic stages of *Plasmodium* parasites. Disruption of folate synthesis by inhibition of DHFR, a critical enzyme of the folic acid cycle, leads to decreased levels of fully reduced tetrahydrofolate (THF), a necessary cofactor in important one-carbon transfer reactions in the purine, pyrimidine, and amino acid biosynthetic pathways. The lower levels of THF result in decreased conversion of serine to glycine, reduced methionine synthesis and lower thymidylate (dTMP) levels, and subsequent arrest of DNA replication.

tetrahydrofolate (THF) as well as converting folic acid to dihydrofolate (**Figure 3**). Therefore, DHFR inhibition blocks the biosynthesis of purines and pyrimidine in *Plasmodia* species, which are essential for DNA synthesis and cell multiplication. *In fine*, this leads to nuclear division failure at the time of schizont formation in erythrocytes.[129]

4.2. Proguanil

Proguanil was the first reported antimalarial biguanide with an antifolate mode of action, also known as Paludrine®. The molecule presents a biguanide core flanked by a *para*-chlorophenyl on the N₁ nitrogen and an isopropyl group on the N₅ nitrogen. The first mention of proguanil dates back to 1945 when Curd *et al.*[5] reported a better therapeutic index and a higher activity against malaria than quinine. In this study, very encouraging activities measured in bird models both as prophylactic treatment and after infection prompted its use in humans. Future investigations into the mode of action of proguanil revealed that it acts as a prodrug. In the liver, the cytochrome P450 enzymes convert proguanil into the active metabolite cycloguanil (**Scheme 2**), which inhibits parasite DHFR.[128] Proguanil could be used as a monotherapy or in combination with chloroquine as preventive measures,[130] but it is mainly used in combination with atovaquone,[131, 132] an inhibitor of electron-transport to the cytochrome bc₁ complex (coenzyme Q). In this combination, proguanil exhibits a different mode of action compared to monotherapy. It was found that in combined atovaquone-proguanil therapy, proguanil acts *via* its biguanide form, rather than as a prodrug of cycloguanil. Instead of inhibiting DHFR, proguanil shows a synergetic effect with atovaquone by enhancing its ability to destroy parasite mitochondrial membranes.[133] Thus, the addition of proguanil has been used to reduce resistance to atovaquone and re-sensitize the atovaquone ability to provoke a mitochondrial apoptotic cascade.[134] However, this atovaquone-proguanil combination failed in several reports to afford malaria chemoprophylaxis.[135, 136] The discovery of proguanil has triggered investigations into other biguanide analogs with antimalaria activity. Consequently, several more effective analogs such as chlorproguanil, clociguanil and WR99210 were developed.



Scheme 2. Chemical structure of biguanide compounds with antimalarial activity. The structures of the biguanide prodrugs and the active metabolites after oxidation by CYP450 cytochromes are presented.

4.3. Clorproguanil

Chlorproguanil, also known as Lapudrine®, is a dichlorinated analog of proguanil developed in 1950.[137] Chlorproguanil has been shown to present similar pharmacological properties to those of proguanil, but with more persistent action.[138] The half-life of chlorproguanil in the human body after oral administration is $t_{1/2} = 35$ h compared to 16 h for proguanil.[139] Likewise proguanil, chlorproguanil is metabolized by cytochrome P450 into an active cyclic metabolite: chlorcycloguanil (**Scheme 2**). Recommended doses for the prophylaxis of malaria as a monotherapy are 20 mg, compared to 100 mg for proguanil.[140] Nonetheless, despite great hopes of diminishing the side effects of proguanil, the complications observed with chlorproguanil were similar. As a result, the compound was less used than its predecessor.[141-143] Recently, chlorproguanil has been proposed in combination therapy to limit resistance apparition against other antifolates. Specifically, it has been proposed in bitherapy with the antifolate sulfone drug dapsone (sold commercially as Lapdap®), or as therapy with dapsone and the DNA-damaging agent artesunate (abbreviated as CDA). CDA entered Phase III clinical trials in 2006, but those were terminated due to intrinsic toxicity of dapsone leading to an increased risk of hemolytic anemia in patients with glucose-6-phosphate dehydrogenase deficiency, a genetic polymorphism particularly prevalent in malaria-endemic areas.[144] Lapdap has been withdrawn from the drug market since 2008 for similar reasons.[145] However, these higher risks of hemolytic anemia were suspected to be a result of dapsone toxicity rather than those of chlorproguanil.[146]

4.4. Cloriguanil

After it became evident that the active metabolite of proguanil and chlorproguanil is a cyclic product of P450 oxidation, researchers attempted to directly produce the active cyclic forms of this class of drugs. This led to the synthesis of clociguanil – an analog of chlorcycloguanil with a methylene-oxy linker between the phenyl ring and the heterocycle (**Scheme 2**) developed in 1965.²⁰² The mode of action of this drug proved to be similar to cycloguanil and it has shown activity against *Plasmodia* resistant to pyrimethamine, a DHFR inhibitor discovered in 1952. While promising efficacy was obtained in initial *in vitro* experiments ($EC_{50} = 1 \text{ pM}$ against DHFR) and subcutaneously in mice (10 times more active than chloroquine and more than 30 times more active than cycloguanil), its lower absorption by oral administration and especially its relatively short half-life in humans led to deceptive results during clinical evaluations.[147] Thus, the molecule failed to prevent the development of parasitemia in volunteers infected with a multiple-resistant strain of *P. Falciparum* at the dose of 0.84 g/day 11 days after infection.[148] It was also demonstrated by Laing[149] in the Gambia that clociguanil had no advantages over pyrimethamine and/or proguanil and that its rapid excretion precluded single-dose therapy.[150] Therefore, further clinical and biological studies on clociguanil were abandoned.

4.5. WR99210

WR99210 was developed in the 1970s as a clociguanil analog with three chlorine atoms on the phenyl ring and a longer linker between the two cycles (**Scheme 2**). WR99210 attracted the attention of researchers as it showed high activity against *P. Berghei*[151] and *P. Falciparum*[152] that are highly resistant to proguanil and pyrimethamine. Which led WR99210 to enter clinical trials. However, gastrointestinal intolerance, probably derived from a poor bioavailability (oral bioavailability in rats $\leq 1\%$),[153] prevented its development as an antimalarial agent and clinical trials were abandoned in 1973.[154, 155] This poor bioavailability feature is encountered with all cyclic forms of the antifolate biguanides (cycloguanil, chlorcycloguanil, clociguanil and WR99210), thus the use of non-cyclic pro-drugs (like proguanil and chlorproguanil) seems more beneficial for this class of drugs.

4.6. PS-15

Due to the poor bioavailability and gastrointestinal intolerance of WR99210, a biguanide precursor PS-15 was developed (**Scheme 2**).[156] The bioavailability for this compound could be indeed improved compared to WR99210, although it led to a decrease in potency *in vitro*. [157] For instance, WR99210 was strongly more active than PS-15 against CH12 and GA3 parasite strains clones with IC_{50} of 0.05 and 0.02 ng/mL, compared to 326 and 532 ng/mL for PS-15.[155] On the contrary, PS-15 was in 20-86 times more active *in vivo* in monkey models.[154, 158] Furthermore, both WR99210 and PS-15 were tested against another related pathogen *Toxoplasma Gondii* and demonstrated the potential usefulness of this class of compounds for the treatment of toxoplasmosis.[159] Although PS-15 is a very attractive candidate for further development based on its biological activities, it has a significant practical drawback. The logical starting material, 2,4,5-trichlorophenol, has severe constraints on its production and the disposal of all associated wastes, because of stringent legal restrictions regarding pesticides. These make other analogs of PS-15 with similar activity but without such safety or regulatory liability highly desirable.[157]

5. Biguanides as antiseptics and disinfectants

Another area where biguanides established themselves is the market of antiseptics and disinfectants. Antiseptics and disinfectants are extensively used in medicine and health care as both preventive measures and curative treatments. They usually have no specific intracellular target to show a broad spectrum of activity. Since the first long known and commonly accessible hydrogen peroxide and ethanol solutions, different classes of synthetic antiseptics have appeared like anilides, bisphenols, halophenols, diamidines, and biguanides. The antimicrobial activity of biguanides was discovered in the late 1940s when several compounds initially developed by Imperial Chemical Industries as anti-malaria drugs were tested as biocides. After the great success of the first antiseptic biguanide – chlorhexidine – further research toward new antimicrobials based on the biguanide structure led to the discovery of compounds like alexidine, picloxydine, and polymeric biguanides (**Figure 4**).

Those biocides and their mode of action are described in the following section.

5.1. Chlorhexidine

Chlorhexidine is a dicationic bisbiguanine molecule comprising of two moieties analog to proguanil, linked with a hexamethylenediamine spacer (**Figure 4**). In 1946, Imperial Chemical Industries produced several series of biguanides with different linker lengths to evaluate their biological activity. Later, Devis *et al.* found that some of the prepared polyguanides showed a very broad antimicrobial spectrum.[6, 160] Further structure optimization resulted in an agent with very potent bacteriostatic and bactericidal activity: chlorhexidine. Since then, chlorhexidine has established itself as the gold standard of chemical plaque control[161] due to its effectiveness and high safety profile. Along with antidiabetic biguanide metformin, chlorhexidine is included in the World Health Organization's List of Essential Medicines.

5.1.1. Mode of action

Considerable research has been undertaken on the mechanism of the antimicrobial action of chlorhexidine.[162] It shows a broad spectrum of activities which include bacteriostatic, sporostatic,[163] mycobacteriostatic[164], and bactericidal[165] effects. Recent investigations have shown that chlorhexidine also has an effect on yeasts[166] and protozoa,[167, 168] as well as some antiviral activity.[169] At physiological pH, chlorhexidine is a positively-charged bisbiguanide that has two types of activity. First, it inhibits the adherence ability of microorganisms to a surface, thereby preventing the growth and development of

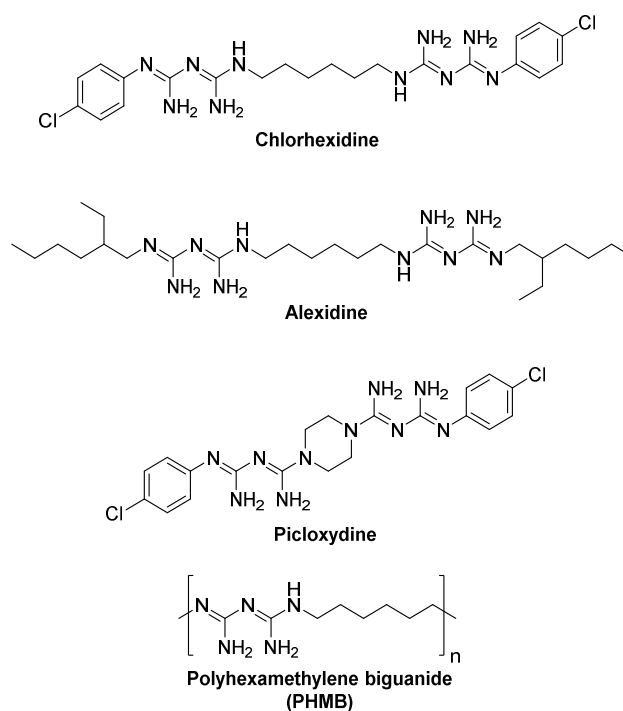


Figure 4. Chemical structure of biguanides with bactericidal activity. Chlorhexidine, alexidine, picloxydine and polyhexamethylenebiguanide (PHMB) are available on the market.

biofilms.[170] This feature makes chlorhexidine an active ingredient of numerous mouthwash solutions designed to reduce dental plaque and oral bacteria. Second, chlorhexidine binds to the negatively charged sites of the cell wall, thereby destabilizing it and interfering with osmosis. After the disruption of the cell wall, the molecule crosses it, presumably by passive diffusion, and subsequently binds to phospholipids in the inner membrane leading to its increased permeability. As a result, damage to this delicate semipermeable membrane is followed by leakage of intracellular constituents, which in turn leads to cell death (**Figure 5**).[171] Uptake of chlorhexidine by bacteria[172] and yeasts[166] was shown to be extremely rapid and pH-dependent, with a maximum effect occurring within 20 seconds. At low concentration (below 10 mg/L), the integrity of the cell walls is damaged[173] but this is insufficient to induce lysis or cell death. The leakage rate is not only correlated to a minimum concentration of chlorhexidine but also presents a maximum efficacy. Indeed, over a certain value of chlorhexidine concentration, the leakage of intracellular components decreases. This is explained by the coagulation of intracellular constituents at high concentrations. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakage.[174] The effects of chlorhexidine in fungi are similar to those described for bacteria. In this case, chlorhexidine spreads into the cell wall, plasma membrane, and cytoplasm of the yeast.[175] Moreover, it shows a similar trend of concentration-lysis dependence, with reduced lysis at higher biguanide concentrations.

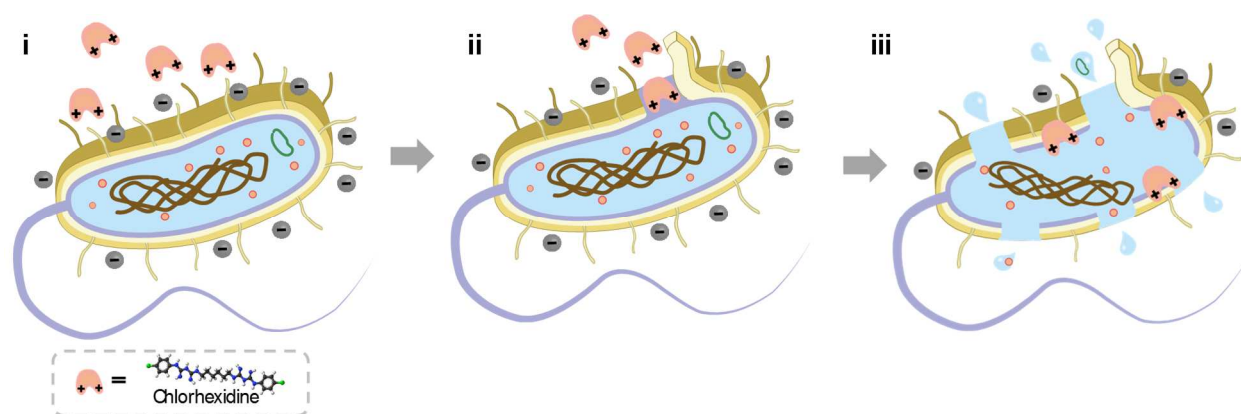


Figure 5. Mechanism of action of bis-biguanide antiseptics. (i) Positively charged chlorhexidine molecules are electrostatically attracted to the negatively charged sites of the bacterial cell wall. (ii) Neutralization of the local charge leads to the loss of cell wall integrity and the appearance of breakages. (iii) Further interaction of chlorhexidine with phospholipids of the membrane leads to increased permeability and leakage of cytoplasmic constituents, resulting in lysis and cell death.

5.2. Alexidine

Another antiseptic in this group is alexidine, which differs from chlorhexidine by the presence of two hydrophobic 2-ethylhexyl groups at the place of the chlorophenyls (**Figure 4**). This structure favors both electrostatic adhesion to the negative sites of cell membranes and hydrophobic penetration into membrane lipids. The first report of the synthesis and pharmacological activity of alexidine dates back to 1966,[176] where alexidine showed antimicrobial activity against gram-positive and gram-negative bacteria. More recently, some tumor cell-specific properties were also reported.[177] However, it is currently only used as a disinfectant in mouthwash and contact lenses solutions.[178] In comparison to chlorhexidine, alexidine shows faster bactericidal activity and produces a significantly faster bacterial permeabilization (estimated by the loss of cytoplasmic ions).[179] In particular, treatments of cell suspensions by equal doses of alexidine and chlorhexidine resulted in the rapid release of potassium ions, completed within 5 min in the case of alexidine,

and that persisted after 60 min for chlorhexidine. Regarding the bactericidal activity of the two biocides, overall activities in the conditions tested (4 µg/ml; time contact = 1 min) were equivalent at the first contact, however higher for alexidine at the second one. This could suggest additional killing mechanisms for alexidine, like the release of autolytic enzymes from the cell or alternative intramolecular interactions on the cell or at the membrane.[179] In addition, compared to chlorhexidine, alexidine has a higher affinity for the virulence factors of bacteria.[180] A study by Barrios *et al.* concluded that alexidine had longer antimicrobial substantively against *Enterococcus faecalis* when compared to chlorhexidine.[181] Moreover, *in vivo* results, indicate that alexidine at 100 mg/L concentration is less toxic than chlorhexidine when applied topically to Chinese hamster cornea.[182] In line with these manifestations of higher efficiency and features, and despite the absence of FDA approval, alexidine has been commercialized using a 510(k) agreement.

5.2.1. Mode of action

Differential thermal analysis (DTA) on phospholipid domains demonstrated that alexidine, unlike chlorhexidine, can produce lipid phase separation and domain formation.[179] Thus, it has been suggested that the nature of the 2-ethylhexyl groups of alexidine confers its ability to produce lipid domains in cell membranes. However, the same authors noted a similar minimal inhibitory concentration (MICs) for alexidine and chlorhexidine (0.7-0.8 µg/L). On the other hand, alexidine proved to be more effective against chlorhexidine-resistant *Pro. stuartii*, PV 67 bacteria, suggesting different interacting sites or mechanisms for alexidine.[179] Furthermore, alexidine alters the permeability of the bacterial cell membrane faster than chlorhexidine as demonstrated by the lysis of spheroplasts. These results are in agreement with previous studies that present greatly faster growth inhibition for alexidine.[179]

Another feature of bis-biguanide antiseptics is their ability to prevent inflammation induced by dead bacteria debris. Zorko and Jerala demonstrated by isothermal calorimetric titration and fluorescence displacement assays the binding to pro-inflammatory components such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA).[180] Alexidine has a higher affinity than chlorhexidine for both LPS and LTA, thus preventing more efficiently the stimulation of TLR2 and TLR4 Toll-like receptors, and minimizing inflammation induced by destroyed bacteria.[180]

Regarding the potential anticancer activity of alexidine, it has been linked to a caspase-dependent apoptosis promotion.[177]

5.3. Picloxydine

Picloxydine is another bis-biguanide compound in which two *para*-chlorophenyl moieties are connected through a rigid piperazine linker (**Figure 4**). It was first synthesized in 1959 by James and Wiggins, as part of a series of N₅-alkyl/-aryl, mono- and bisbiguanides in which the N₁ atom is included in a heterocycle.[183] In 1962, the compound was approved by the British Pharmacopeia with the name picloxydine.[184] Shortly after, James *et al* led a comprehensive study of the structure-antibacterial activity relationships of picloxydine and some derivatives, showing that picloxydine had strong bacteriostatic activity against *Streptococcus pyogenes* Gp.A., *Streptococcus β-haemolyticus*, and *Clostridium septicum* with minimal inhibitory concentrations of 0.76, 0.76, and 0.3 mg/mL, respectively.[7] Later, it was demonstrated that the antibacterial activity of picloxydine affects a panel of gram-positive and gram-negative microorganisms.[185]

In vitro studies have shown that the antibacterial properties of chlorhexidine and picloxydine are similar and that their mode of action on *Streptococcus* mutants is identical.[186] Similarly to chlorhexidine and alexidine, picloxydine also shows a potent antiplaque efficacy.[187] In a double-blind clinical study of chlorhexidine and picloxydine mouthwashes, picloxydine suppressed the oral flora more effectively; however, chlorhexidine inhibited plaque development to a much greater extent.[187] A similar sensitivity of the two bisbiguanides was measured in studies on gram-negative bacteria resistant to antimicrobial agents.[188] An explanation for this similar activity was proposed to be the identical cationic nature of both biguanides.

Recently, picloxidine has also been reported to show some low micromolar inhibitory activity of acetyl- and butylcholinesterase enzymes.[189] Nowadays, picloxydine is mainly used in eye drops for the treatment of keratitis, in the topical therapy of trachoma[190], and as a surface disinfectant.[185]

5.4. Polymeric biguanides

Chlorhexidine, alexidine, and picloxydine are all three bisbiguanide compounds with antimicrobial activity. It was later thought that multiple fragments could be joined together to form polymeric biguanides. The representative and the most studied compound is polyhexamethylenebiguanide (PHMB): an oligomer of hexamethylene biguanide with an average number of biguanide units of 12 (**Figure 4**).[191] Patented in 1968, it has been commercially sold under various brand names (Vantocil, Cosmocil, and Lonzabac) as a disinfecting agent in the food and cosmetic industries as well as for swimming pool cleaning. It should be noted that PHMB is not on the list of FDA-approved drugs but has been marketed in the US using a 510(k) agreement as a disinfectant “substantially equivalent” in safety and effectiveness to chlorhexidine. According to European regulation, PHMB can be used in cosmetic industry as a preservative up to a maximum concentration of 0.3%. [192]

Koburger *et al.* determined for PHMB a minimum inhibitory concentration (MIC) of 0.5 - 4 mg/L and a minimum bactericidal concentration (MBC) of 1 - 32 mg/L against a panel of most representative gram-negative and gram-positive bacteria.[193] In comparison with chlorhexidine, PHMB always showed an efficient improvement, from modest to significant, for both MIC and MBC. In an *in vivo* double-blind randomized controlled clinical setting, PHMB formulations were tested as mouth rinse solutions. They displayed a significant antibacterial effect and *de novo* biofilm formation reduction in the oral cavity, similar to those of chlorhexidine.[194] PHMB also presents interesting clinical data for use as eye antiseptic or as a solution for wound treatment.[195-199] A representative study on 7862 patients derived from a rural-agricultural setting with dirty contaminated wounds revealed that treatment with a 0.04% PHMB solution displayed the lowest post-operative wound infection frequency, compared to other standard antiseptics (povidone-iodine, Ringer’s solution, and hydrogen peroxide).[200] In another study published by Andriessen and Eberlein[198], wounds treated with 0.1% PHMB solution healed significantly faster and with better success rates than Ringer’s solution (97% vs. 89%, mean time to healing 3.31 vs. 4.42 months, 6 months observation period).

However, the most interesting property of PHMB is probably a very high safety profile that classified it as ‘practically non-toxic’. As a representative example, its therapeutic index against *Pseudomonas aeruginosa* is more than 200-fold higher than chlorhexidine. Practically, given its LD₅₀ value (over 500 mg/kg orally in rat) and the fact that it is sold as a diluted solution in water, it is very unlikely to promote intoxication. Chronic oral intake has also proven to be very well tolerated over 2 years.[201, 202] In addition, contrary to its congener

chlorhexidine, PHMB presents a negligible risk of promoting allergy – even if one study reported that very high topical doses of PHMB can trigger a generalized exanthema.[203] For further information on PHMB toxicity and clinical data, the reader can refer to an exhaustive review.[192]

5.4.1. Mode of action

The mechanism of action of PHMB is similar to that of previously described bisbiguanides. Being a cationic polymer, PHMB interacts with negatively charged phospholipids causing domain formation in the bacterial membrane, leading consecutively to increased fluidity, permeability, loss of integrity, and cell death.[204-206] In parallel, neutral lipids remain unaffected, which usually contributes to the explanation of its low toxicity in human cells.[207] PHMB-phospholipids interaction is nonspecific, therefore polymeric biguanides act against a broad spectrum of microbes, including gram-positive and gram-negative bacteria, plaque-forming, and biofilm-building bacteria, sporulating bacteria, and some fungi.[208-210] Moreover, PHMB has been shown to inactivate HIV-1[211] and HSV[212] *in vitro*. It is interesting to note that the activity of PHMB increases with higher levels of oligomerization, which correlates with an enhanced inner membrane perturbation. More information regarding the toxicity, the efficacy, and the clinical applications can be found in a review from Höbner and Kramer.[192]

In conclusion, biguanides presently occupy an important place among the disinfecting and antimicrobial agents based on their broad range of bactericidal activities. Yet, only chlorhexidine has been approved by FDA and can be used for most clinical purposes. Even though alexidine and PHMB exhibited lower MIC and faster action in many cases, they have not been FDA approved. Whereas no clinical trials have been found for alexidine, PHMB is widely studied and one of the more investigated antiseptic substances after chlorhexidine. However, they are available on the market as “substantially equivalent” to the approved drug chlorhexidine in terms of safety and effectiveness. Picloxydine is used in some countries (France and Switzerland) in eye drop solutions, but only limited information regarding its study *in vivo* and *in vitro* models is available. Chlorhexidine remains by far the standard antiseptic around the world for the past 7 decades and is part of the WHO List of Essential Medicines. PHMB appears to be the most promising concurrent, given its combined great and wide antimicrobial efficacy and especially its exceptional low toxicity and tissue compatibility. The number of *in vivo* studies and clinical trials with PHMB is expected to increase over the next few years.

6. Antiviral activity of biguanides

One biguanide compound, moroxydine (**Figure 1**), has found applications as an early antiviral agent which was extensively used in the 1960s and 1970s, before being progressively replaced in the 1980s and 1990s by other more potent antiviral agents. Nowadays, because of the plethora of potent antiviral drugs available,[213] moroxydine has been reclassified as a veterinary drug.

Moroxydine is a simple heterocyclic biguanide compound in which an external nitrogen atom is linked to a diethyleneoxy bridge to form a morpholine ring (**Figure 1**). The compound was developed in the 1950s by the company Kabi, and its antiviral activity was patented in 1957.[214] The first therapeutic use of moroxydine was reported for the treatment against Varicella zoster virus (VZV) in 1959 by Schersten.[215] Over the next 20 years, moroxydine was progressively used for the treatment of different viral diseases. In particular, it was found

to be active against a panel of DNA and RNA viruses such as adenovirus,[216] influenza,[216] herpes,[217] hepatitis,[218] urethritis,[219] meningitis,[218] pneumonia,[218] paramyxoviruses,[220] pityriasis rosea,[221] molluscum contagiosum,[222] dengue[223], and papillomavirus[224]. Moreover, besides its broad antiviral activity, a particularly interesting feature of moroxydine is its surprisingly low toxicity (oral LD₅₀ (mouse): >6250 mg/kg vs. 1450 mg/kg for metformin; subcutaneous LD₅₀ (mouse): 2200 mg/kg vs. 225 mg/kg for metformin).[225]

Despite its broad range of activity, the pharmacopeia has gradually mislaid moroxydine as a drug. The absence of conclusive *in vivo* studies, and clinical trials meeting the modern international standards such as double-blind, randomized, controlled studies, did not allow a normalized use of this compound in humans.[218] Nowadays, moroxydine remains mostly used in veterinary medicine.[226] However, recently, moroxydine regained interest with studies on the inhibition of avian flu and some aquatic viruses affecting fishes.[227][228]

7. Anticancer activity of biguanides

After more than three decades of relative “slumber”, research on biguanides drugs has recently regained interest over the last decade, mainly arising from the repurposing of biguanides as potential tumor treatments.[15, 17, 18] The first mention of anticancer activity of a biguanide compound was reported with metformin after a retrospective epidemiological study of diabetic patients diagnosed with cancer in 2005.[54] Several investigations confirmed later the decreased cancer incidence and cancer-related mortality in diabetic patients using metformin.[15, 229-231] For example, Bowker *et al.*[232] in a study including 10309 diabetic patients, compared the incidence of cancer during treatments with either insulin, metformin, or sulfonylureas for a period of 5 years. The results showed that metformin-treated patients had a significantly lower rate of cancer-related mortality compared to the insulin and sulfonylurea-treated groups. Additional studies examining different types of cancer including breast cancer,[231, 233, 234] colorectal cancer,[235, 236] liver cancer,[237] prostate cancer,[238] melanoma[239-241], and others, also reported a reduced risk of cancer and lower cancer-related mortality in patients receiving metformin. Despite the large number of patients analyzed in these epidemiological studies, most of them lack important information such as metformin dosage, body mass index, or glycemic control. Moreover, some controversial results were later published.[242] Besides, intra- and intratumor metabolic heterogeneity should be taken into consideration when analyzing the anticancer activity of metformin.[243] Nonetheless, this research direction is still in its infancy and more studies are required to decipher both the efficiency and the anticancer modes of action of metformin. In this section, we will report the current knowledge on the anti-cancer effect of metformin - which is by far the most studied and globally accessible biguanide – from its penetration into the cells to the main mechanisms of action. From this analysis, we will discuss the tremendous potential of future biguanides as anticancer drugs. Other reviews on metformin anticancer activity include information on clinical trials[244-246], tumor specific data[247-250], and mechanisms of action[16, 251].

7.1. Metformin transport and organic cation transporters

Prior to excreting its effect on the cell, metformin needs to cross the membrane barrier and penetrate the cytosol. Due to its charge and high hydrophilicity, passive diffusion of metformin is very limited. Thus, to allow intracellular uptake, metformin relies on organic cation transporters (OCTs).[93, 252] OCTs of the

SLC22 gene family are polyspecific transporters expressed on the cell membrane and are critically involved in the uptake, distribution, and elimination of cationic drugs. OCT1, OCT2, and OCT3 are major transporters of metformin, which are highly expressed in the liver, kidneys, and gut.[253] The suggestion that OCTs play an essential role in metformin accumulation in cells was proved using *Oct1*-knockout (*Oct1*^{-/-}) mouse models, which showed a 3.4-fold ($P < 0.0001$) reduced metformin uptake.[254] Accumulation of metformin in the liver as a result of OCT high expression leads to lower rates of gluconeogenesis and facilitation of anticancer activity in liver tissue-derived tumors.[255] It was demonstrated that the expression of OCTs is different depending on tumor tissue.[256] For instance, Schaeffeler *et al.* reported that OCT1 expression is decreased in human hepatocellular carcinoma cells and it correlates with poor prognosis.[257] Patel *et al.* showed that OCT3 is overexpressed in head and neck squamous cell carcinoma cell lines. Moreover, metformin activity was significantly decreased when OCT3 expression was inhibited.[258] It was demonstrated that OCT1, encoded by gene *SLC22A1*, [259] is expressed in different ovarian cancer cell lines and RNA knockdown of OCT1 reduced the sensitivity of cancer cells to metformin.[260] Another key factor that modulates the therapeutic response and activity of metformin is the polymorphism of OCT genes that affects OCT1 function[261] and the regulation of renal clearance of metformin.[262] Therefore, there is a strong correlation between the anticancer activity of metformin and OCTs in cancer cells.

7.2. Modes of action

The great safety profile of metformin allows the administration of this low molecular weight drug at high dosages (millimolar concentrations in cellular assays). Such a concentration inevitably results in pleiotropic effects, *i.e.* drug's actions other than those for which the agent was specifically developed. Consequently, several plausible concomitant mechanisms have been suggested to explain its anti-tumor effects (**Figure 6**). These mechanisms can be divided into two main groups: direct (insulin-independent) and indirect (insulin-dependent).

7.2.1. Indirect mechanism

The indirect mechanism gathers the insulin-dependent effects of metformin, relying on the inhibition of hepatic gluconeogenesis and the stimulation of glucose uptake in muscles, which reduce fasting blood glucose and insulin.[263] It is widely known that cancer cells overexpress insulin as well as insulin-like growth factor (IGF) receptors (IGF-R).[264] For example, Fürstenberger *et al.* found an association between IGF and breast cancer.[265] It was reported that the relative age-adjusted risk of breast cancer for 304 postmenopausal and 76 premenopausal women was 2.33 (95% CI 1.06–5.16; $p=0.08$) and 2.88 (95% CI 1.21–6.85; $p=0.02$) according to the level of IGF1 and IGF3. Moreover, if the analyses were limited by age to 50 years, the respective relative risks were 4.58 (95% CI 1.75–12.0) and 7.28 (95% CI 2.4–2.2) correspondingly. IGF-R, besides its metabolic effect, promotes the proliferation of cancer cells and metastasis.[264] Furthermore, other mechanisms explaining the relationship between high insulin levels and tumor development exist. Hyperinsulinemia results in higher serum levels of insulin-like growth factors (IGF), a faster proliferation of epithelial tissues, as well as the disruption of the adipokines homeostasis. However, adipokines, which are cytokines selectively secreted by adipose tissues, are thought to be implicated in cancer pathogenesis.[266] Actually, metformin treatment has proved to lower serum levels of insulin and IGF-1 and thereby reduce cell growth stimulation.[267] Another insulin-associated effect of metformin is related to reducing glucose levels. It is widely accepted that glucose is

an essential nutrient for fast proliferating cells. Otto Warburg in 1924 observed that cancer cells use much more glucose compared to other cells. In addition, it was observed that glucose was fermented to lactate even in the presence of oxygen.[268, 269] Lately, in 1970s, this effect was called Warburg Effect. In comparison with normal cells, most of the cancer cells rely on aerobic glycolysis to obtain energy and necessary glycolytic intermediates which can be used for protein synthesis and cell division.[270] It results in much higher glucose uptake by cancer cells compared to normal ones. Therefore, treatment with metformin, which reduces glycemia, also decreases growth advantages associated with cancer cells.

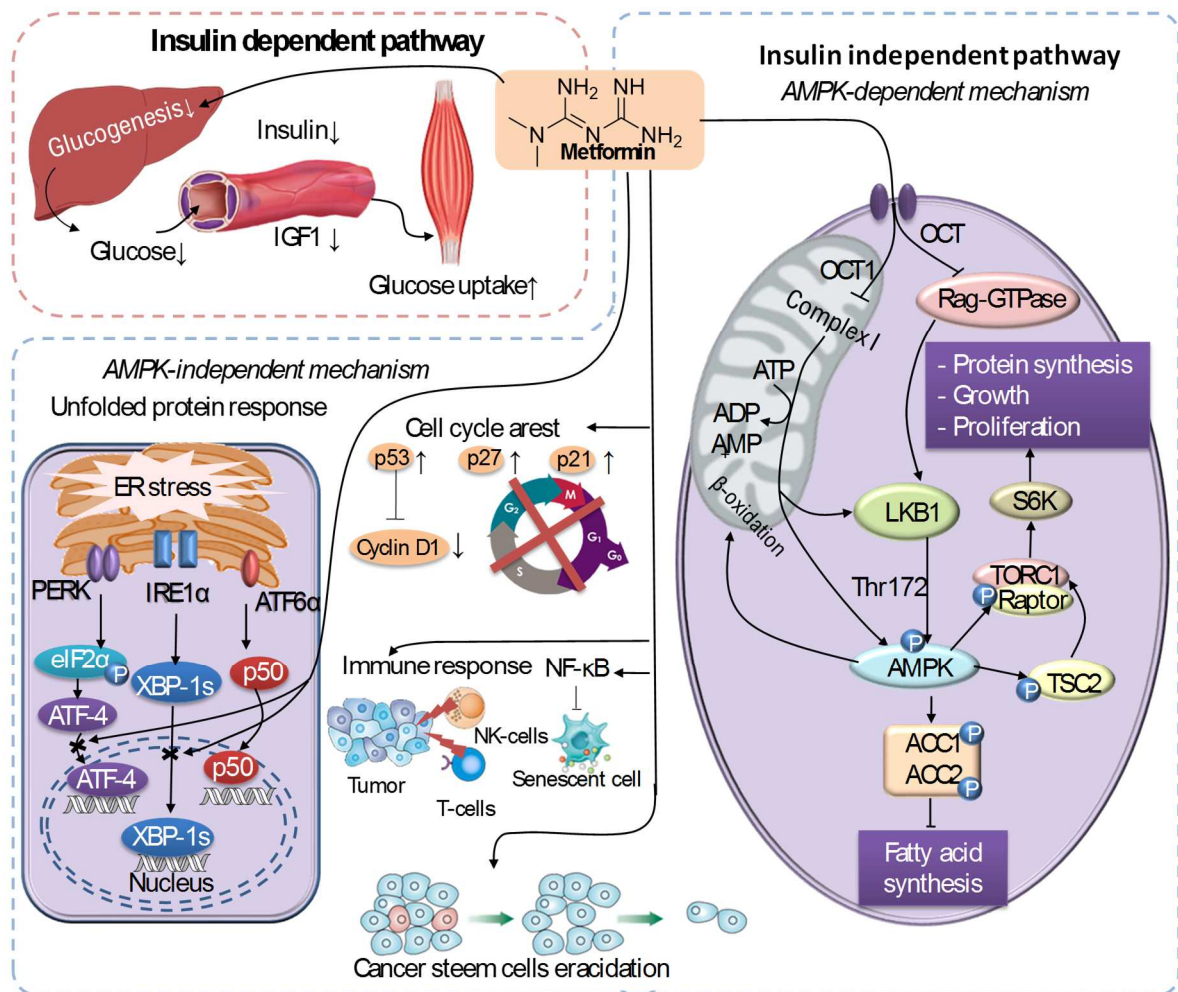


Figure 6: Mechanisms of action of metformin. *Indirect (insulin-dependent) pathway:* metformin downregulates glycogenesis and reduces hepatic glucose production, thus lowering serum levels of insulin and IGF-1. Metformin increases glucose uptake in skeletal muscles. *Direct (insulin-independent) pathway:* divided into AMPK dependent mechanisms where metformin inhibits the complex I and target-of-rapamycin complex-1 (mTORC1) in an AMPK-dependent manner and AMPK independent mechanisms that include unfolded protein response, cell cycle arrest, immune response, eradication of cancer stem cells, and inhibition of NF-κB pathway.

7.2.2. Direct mechanism

The direct (insulin-independent) effects of metformin are attributed to several mechanisms and can be divided into two main groups: AMPK dependent and AMPK independent. These mechanisms are further discussed in detail. For more exhaustive information reader can refer to other reviews written on the topic of the mechanism of action of metformin.[15, 231, 271, 272]

7.2.2.1. AMPK dependent

A part of the anticancer activity of metformin can be explained by using a simplified model focused on cellular metabolism in which metformin activates AMPK (**Figure 6: Insulin independent pathway**), leading to a reduction in mammalian target of rapamycin (mTOR) signaling and inhibition of cellular protein synthesis and cancer cell growth.[273, 274] Activation of AMPK starts with the inhibition of the complex I of the mitochondrial respiratory chain, which results in the interruption of mitochondrial respiration, followed by a decrease in proton-driven ATP production, a reduction in the ATP/AMP ratio, and energetic stress.[96, 98] In turn, increased levels of AMP activate AMPK by three separate mechanisms:[275] i) activation by specific phosphorylation of the α catalytic subunit on residue Thr172 by the upstream kinase LKB1, ii) allosteric activation, and iii) interference with dephosphorylation of AMPK Thr172 by protein phosphatases. Once activated, LKB1 phosphorylates AMPK resulting in the initiation of the energy stress that hinders cell growth.[276] Downstream, AMPK phosphorylates tuberous sclerosis complex-2 (TSC-2)[277] that in turn, inhibits the activity of mTOR, a signaling pathway that plays a central role in malignant cell growth and cancer pathogenesis. AMPK is also able to directly phosphorylate various mediators such as TSC-2 and the regulatory associated protein of mTOR (RAPTOR) which will bind and inhibit mTOR.[276] As mentioned above, AMPK activation also induces the inhibition of anabolic processes and the activation of catabolic processes. Furthermore, plays a crucial role in vascular cell proliferation, migration, and angiogenesis. AMPK signaling pathway activation in vascular cells was critical for angiogenesis in hypoxic conditions.[278] It was reported that metformin-induced adenylate-activated protein kinase-dependent signaling pathways limits protein kinase B (AKT) activation and phosphorylation, thereby regulating cell growth, proliferation, and circulatory homeostasis.[279]

For example, phosphorylation of other downstream targets than mTOR results in fatty acid β -oxidation and glycolysis. This also involves the suppression of many processes dependent on an ample cellular ATP supply, including gluconeogenesis, protein and fatty acid synthesis, and cholesterol biosynthesis.[280, 281] Apart from the metabolic stresses, AMPK also induces autophagy.[282] This inhibition of lipogenesis and AMPK-related autophagy are described in detail below.

7.2.2.1.1. Inhibition of lipogenesis

It is widely accepted that fast-growing cancer cells require more energy and resources than normal cells to support their rapid divisions. This energy is mainly provided by *de novo* synthesized lipids and as a result, cancer cells show high rates of lipogenesis.[283] Metformin was shown to inhibit lipid accumulation, lower plasma total cholesterol, and triglyceride levels[284-286], and stimulated fatty acid oxidation[103, 287]. It also was found to stimulate AMPK-activity in the liver and skeletal muscles.[288]. Clinical studies of metformin and salicylate were shown to synergistically activate AMPK *in vitro* and *in vivo*, resulting in reduced liver lipogenesis, lower liver lipid levels, and improved insulin sensitivity in mice.[289]

AMPK phosphorylates and inhibits acetyl CoA carboxylase (ACC) and HMG-CoA reductase, which are necessary enzymes for *de novo* synthesis of fatty acids and cholesterol, respectively.[290] HMG-CoA reductase

is the rate-limiting enzyme of the mevalonate pathway, the metabolic pathway that produces cholesterol and other isoprenoids. It was shown that inhibitors of HMG-CoA reductase could be used to prevent cancer in experimental models as well as in humans.[291, 292] Furthermore, Graaf *et al.* reported that using statins for more than 6 months was associated with a 20% reduced risk of cancer.[293] Another evidence was presented by Blais *et al.* in a nested case-control study of 6721 participants older than 65 years which showed 28% lower cancer risk in the group of patients who used statins compared with treatment by bile acid-binding resin.[294] Another important enzyme involved in lipogenesis and long-chain fatty acid production is fatty acid synthase (FAS). FAS plays an important role in cancer metabolism and is overexpressed in many human cancers.[295, 296] ACC and FAS are indirectly regulated by the transcription factors sterol regulatory element-binding protein 1 (SREBP-1) and sterol regulatory element-binding protein 2 (SREBP-2).[297] It appears that AMPK indeed inhibits SREBP1, although *via* a not yet fully elucidated mechanism.[101] Moreover, the phosphorylation by AMPK of the isoform SREBP-1c proved to result in reduced FAS and ACC gene translocation and expression and reduced related lipogenesis.[298]

7.2.2.1.2. AMPK induced autophagy

Besides the activation of catabolic processes to save energy, AMPK also plays a key role in autophagy induction.[299, 300] Autophagy is the main survival mechanism, which is characterized by the degradation of the own components of a cell, in order to redistribute nutrients from unnecessary processes to more essential ones. In oncology, this recycling mechanism can play a dual role in pro-survival or pro-death events.[301-303] Usually, autophagy is promoted by conditions of nutrients deprivation, allowing cells to degrade organelles and macromolecules, to provide a source of precursors such as fatty acids, amino acids, and nucleotides. In this sense, autophagy serves a protective role, allowing cells to survive during nutrients deprivation. Moreover, this mechanism seems essential for the cell, as the prevention of autophagy has been shown to trigger apoptosis in several cases.[304, 305] In cancer therapy, autophagy plays an additional protective role by cleaning and recycling the cell constituents damaged by chemotherapeutic agents. It was reported by Buzzai *et al.* that metformin selectively induces autophagy in colon cancer cell lines HCT116 p53 (+/+) and not in HCT116 p53 (-/-) which could trigger p53-dependent protective autophagy by phosphorylation of AMPK at Thr172 and further inactivation of mTOR.[306] Recently, Moon *et al.* demonstrated that metformin induces autophagy in ovarian cancer cells *via* PERK/eIF2a pathway as a pro-survival mechanism to protect themselves from metformin-induced apoptosis. Moreover, it was shown that inhibition of autophagy enhanced growth inhibition and apoptotic effects of metformin.[307] In contrast, the loss of key autophagy genes such as BCL1 is frequently found in many human cancers such as human breast, ovarian, and prostate among others.[308, 309] Recent studies show that AMPK directly triggers autophagy by phosphorylation and activation of the ULK1 protein kinase [310-312] and by inactivation of mTORC1.[313] Metformin in combination with doxorubicin enhances the tumor cells response in murine lymphoma models *via* activation of pro-death autophagy.[314] Takahashi *et al.* reported that metformin suppresses endometrial cancer cell growth *via* cell cycle arrest, autophagy, and apoptosis. The authors showed that inhibition of autophagy by Beclin1 knockdown or by 3-methyladenine-mediated inhibition of caspase-3/7 decreases the anti-proliferative effects of metformin.[315] In our lab, we have demonstrated that metformin induces cell cycle arrest, autophagy and apoptosis of melanoma after 24, 72 and 96 hours of treatment, respectively. The important role of autophagy in inducing apoptosis was

proved by knocking down of LC3 or ATG5 which results in apoptosis delay.[239] It was also reported that metformin inhibits a retinoblastoma cell lines growth *in vitro* via inducing autophagy.[316] These results show that autophagy plays a dual pro-death and pro-survival role in anticancer activity of metformin.

7.2.2.1.3. Downstream effects of the mTORC1 inhibition

Serine/threonine kinase mTOR is the core component of two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).[317] Numerous studies have demonstrated that mTORC1 activation plays an important role in tumor growth, angiogenesis, and metastasis.[318] In one study authors showed that metformin exposure leads to increased phosphorylation of IRS-1 at Ser and decreases AKT activation, thereby regulating cell growth, proliferation, and circulatory homeostasis.[279] It was shown that metformin indeed inhibits mTORC1 and consequently affects the activity of various downstream targets of mTORC1.[319, 320] For instance, mTORC1 is implicated in protein synthesis and cell growth *via* phosphorylation of S6 kinase 1 (S6K1).[321] Moreover, it was demonstrated that S6K1 is overexpressed in ovary and breast cancers and it is correlated with poor prognosis of kidney, breast, and hepatocellular carcinomas.[322-326] Additionally, it was shown that mTORC1 kinase regulates the protein synthesis by phosphorylation and increases Cap-dependent translation initiation by the eIF4E-binding proteins (4EBPs).[327] Eukaryotic translation initiation factor 4E (eIF4E) is a family of proteins that binds 5',7-methylguanosine Cap structure of mRNAs and deliver these mRNAs to the eIF4F translation initiation complex. The eIF4F complex regulates the Cap-dependent mRNA translation process and is composed of eukaryotic translation initiation factor 4G (eIF4G), eIF4E and the eukaryotic initiation factor-4A (eIF4A). eIF4F complex unwinds the secondary structure of mRNA in order to disclose the translation of an initiation codon and initiates translation. Normally, eIF4F complex assembly is blocked because eIF4E is bound to 4E binding proteins (4EBP) and therefore not available. Activation of the mTOR pathway leads to 4EBP phosphorylation, decomplexing eIF4E from 4EBP, enabling the formation of the eIF4F complex and resulting in an increase of translation and protein synthesis.[328] These findings indicate that *via* mTORC1 inhibition, metformin affects different downstream targets which results in cell growth, proliferation and angiogenesis downregulation (**Figure 7**).

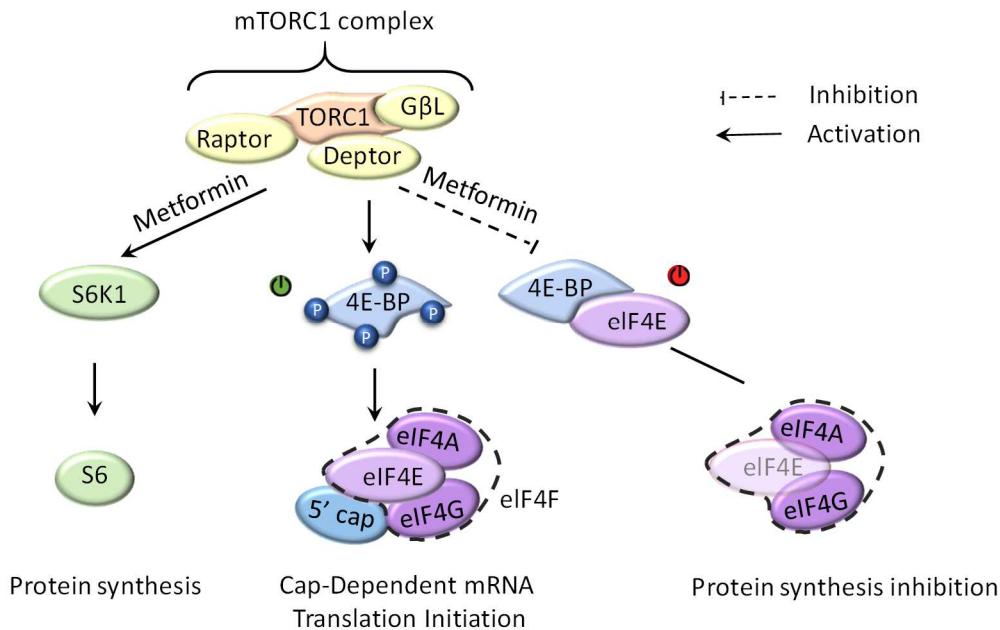


Figure 7. General scheme of mTORC1 complex inhibition by metformin. Metformin inhibits mTORC1 complex, which leads 1/ to a dose-dependent specific decrease in Cap-dependent translation *via* disruption of 4E-BP/eIF4E interaction, and 2/ to an inhibition of protein synthesis *via* decreased phosphorylation of S6K1.

7.2.2.1.4. Inhibition of NF- κ B pathway

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is a protein complex that controls DNA transcription, production of cytokines and regulates cell proliferation and apoptosis.[329] NF- κ B plays an important role in the initiation and progression of many human cancers.[330] Inhibition of NF- κ B leads cancer cells to death by proliferation blockade and increase of their sensitivity to apoptosis-inducing stimuli or chemotherapeutic agents.[331, 332] As a result, NF- κ B is considered as an important biological target in cancer therapy.[333-335] Recently, it was shown that metformin exerts an antiproliferative effect on human epidermoid carcinoma xenograft in nu/nu mice by inhibition of NF- κ B and mTOR.[336, 337] By activating AMPK, metformin also inhibits mTOR through downregulation of NF- κ B and cAMP response element-binding protein CREB.[338] CREB is a transcription factor that regulates diverse cellular responses, including proliferation and survival. CREB can transactivate the expression of Ras homolog enriched in brain (RHEB), a direct key activator of mTORC1. Thus, the downregulation of CREB by metformin results in mTOR inhibition.[339] Zheng *et al.* reported that metformin-activated AMPK inhibits NF- κ B in human hepatocellular carcinoma by reducing I κ B α degradation.[340] I κ B α is a critical regulator of the transcription factor NF κ B. I κ B α masks the nuclear localization signals (NLS) of NF- κ B proteins and keeps them sequestered in an inactive state in the cytoplasm.[341] Additionally, I κ B α inhibits the ability of NF- κ B transcription factors to bind to DNA.[342] Thus, by decreasing I κ B α degradation, metformin inhibits NF- κ B. In addition, it was shown that this metformin-induced NF- κ B inhibition resulted in reduced secretion of pro-inflammatory cytokines by senescent cells.[343]

7.2.2.1.5. Inhibition of cancer cell invasion and migration

Cancer cells can change their position within the tissues due to their ability to undergo migration and invasion. Thereby transforming a local metastasis into a life-threatening disease. On several occasions, metformin was found to limit migration of thyroid cancer cells[344], pancreatic ductal carcinoma[345], ovarian cancer

cells[346]. Metformin, activated TIP30, a cancer suppressor via AMPK in liver cancer cells, promoting metastasis inhibition and sorafenib effects[347]. Consequently, it was reported that metformin also blocked melanoma invasion and metastasis in an AMPK/p53-dependent manner. Recent research suggests that metformin activates LKB1/AMPK signaling pathway. This in turn leads to aerobic glycolysis inhibition in cells containing a functional LKB1/AMPK pathway. AMPK-activation leads to high energy stress. Additionally, down-regulation of mTOR inhibits cell growth after translocation. Metformin may also inhibit invasion and migration through epithelial-mesenchymal transition (EMT) signaling pathways and migration of siRNAs in EMTsignaling. A more detailed overview of mechanisms by which metformin inhibits cell invasion and migration are reviewed by Chen *et al.*[348]

7.2.2.2. AMPK independent

If AMPK plays a key role in metformin's action, there are also pieces of evidence of AMPK-independent effects of metformin (**Figure 7: Insulin independent pathway**). This includes i) a cancer cell cycle arrest by suppression of the genes involved in cell division;[349] ii) the inhibition of the unfolded protein response (UPR) which forces the cancer cells to enter apoptosis;[350] iii) an improved immune response by increasing the number of CD8⁺ T cells;[351] iv) the targeting of cancer stem cells (CSC) in various tumors;[352] v) an AMPK-independent mTOR inhibition through NF- κ B pathway inhibition;[336, 337] and vi) the reduction of insulin and IGF, described in details in the dedicated section, which leads to reduced growth stimuli of cancer cells.[353] These first six main mechanisms of the AMPK-independent mode of action are presented next in detail.

7.2.2.2.1. Cell cycle arrest

The cell cycle is regulated mostly by cyclin-dependent kinases (CDK).[354] The importance of cell cycle regulation in many human cancers has been extensively demonstrated.[355] It was shown that metformin induces cell cycle arrest in G0/G1 and G2/M phases in different cancer types, including breast and esophageal squamous cancer cells.[356] The molecular mechanisms particularly involve the upregulation of CDK inhibitors, such as p21 or p27 proteins.[357] P27 is an atypical tumor suppressor which regulates G0 to S phase transitions by binding to CDK and regulating their activity.[358, 359] In most human cancers, p27 protein levels are reduced and this is associated with poor disease prognosis.[360] The CDK inhibitor p21 promotes p53-dependent G1 growth cell cycle arrest in response to many different stimuli.[361, 362] Different types of human cancers such as colorectal, small-cell lung cancers, cervical and head, and neck, are also associated with reduced p21 expression.[363] Treatment with metformin results in the reduction of cyclin D1 levels and the increase of Bax, p21, p53, and cyclin E expression.[364] The cyclin D1 proto-oncogene is an important regulator of G1 to S phase progression, that plays a role in the development and progression of many cancers such as breast, esophagus, bladder, and lung.[365-369] Moreover, overexpression of cyclin D1 has also been connected to the development of endocrine resistance in breast cancer cells.[370] Similar results were reported by Cai *et al.* in esophageal squamous cells carcinoma xenograft models treated with metformin: the authors observed the downregulation of cyclin D1 and an upregulation of p53, p21 and p27.[371] Furthermore, metformin selectively decreases cyclin D levels in prostate cancer cells where they induce cell cycle arrest in G0/G1 phase.[349] Buzzai *et al.* demonstrated that metformin selectively inhibits p53-deficient cell growth and induces autophagy

in vitro and *in vivo* in colon cancer xenografts.[306] It was also shown that metformin affects the activity of CDK1, CDK2, CDK4, CDK6 in MCF7 cells in head and neck squamous cell carcinoma.[372, 373] Taken together, these studies suggest that the suppression of tumor growth by metformin is partially dependent on cell cycle arrest.

7.2.2.2.2. Inhibition of unfolded protein response (UPR)

The endoplasmic reticulum (ER) is the organelle of eukaryotic cells responsible for protein folding, and transportation of the synthesized proteins to the Golgi apparatus.[374] In addition, ER possesses a quality control system aimed at repairing folding defects before releasing the proteins, or sending them to the proteasome for degradation if not repairable. However, several factors may destabilize the balance within the ER and lead to the accumulation of misfolded proteins such as glucose deprivation, changes in redox conditions, or poor regulation of calcium concentration.[375] This accumulation of misfolded proteins is toxic to the cell at high concentrations and is called ER stress. In cancer cells, protein synthesis is overactivated to support the growth rate and intense cell division required for rapid cell proliferation.[376] This overactivation is associated with an increase in the probability and the number of folding abnormalities, and it results in an inherent ER stress of the cancer cells. Stressed cells are able to trigger a protective mechanism called Unfolded Protein Response (UPR) which aims to eliminate the excess of malformed proteins and restore the homeostasis of the ER.[377] UPR consists of a series of adaptive mechanisms organized in three axes: 1/ attenuation of protein translation in order to slow down the production of proteins, 2/ recruitment of new chaperones to support the global folding effort and decrease the stock of misfolded proteins, and 3/ evacuation of waste for their destruction by the proteasome.[378, 379] Moreover, if the stress is prolonged and cannot be resolved in due time, UPR initiates apoptosis of the stressed cell.[380, 381] UPR is orchestrated by a pivotal enzyme: 78kDa-Glucose-Related Protein GRP78 (also named BiP or HSPA5), which simultaneously plays the role of folding chaperone and acts as the main ER stress sensor. The three major downstream effectors of GRP78 are protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor-6 (ATF6).[382] Once activated, PERK inhibits protein synthesis by phosphorylating eukaryotic initiation factor 2 α (ϵ IF2 α), resulting in the suppression of mRNA translation, with the exception of ATF4 mRNA.[383] ATF4 is a transcription factor that plays a crucial role in the adaptation to stress conditions by transcription of genes for amino acid metabolism and redox reactions to restore ER homeostasis.[350] IRE1 is an ER transmembrane sensor that defines the splicing of a 26-nucleotide-long intron from the mRNA encoding the transcription factor X box-binding protein 1 (XBP1).[384] XBP1 also regulates the expression of genes important in the cellular stress response[385] and has been demonstrated both to play an important role in cancer progression and to be overexpressed in many human cancers such as breast cancer, hepatocellular carcinoma, and pancreatic adenocarcinoma.[386] Metformin has been found to inhibit transcription of XBP1 and ATF4 and as a result to induce massive cell death.[350] In addition, Leclerc *et al.* recently showed that metformin could downregulate UPR which led to UPR-mediated cell death in acute lymphoblastic leukemia.[387]

7.2.2.2.3. Immune response

Metformin can show anticancer activity by increasing levels of immune CD8⁺ T cells, which play an important role in different tumors.[351, 388-390] After recognition of the antigen, CD8⁺ T cells become activated and initiate their differentiation into effector cytotoxic T lymphocytes (CTLs). After clearance of the antigen, most CTLs die, and only a fraction of those who survive as long-lived memory cells (35-50 years in humans).[388, 391] However, chronic activation by cancer cells leads to immune exhaustion because it makes effector or activated T cells unable to produce cytokines or promote their apoptosis. It was demonstrated that this immune exhaustion plays an important role in cancer development and progression.[392] Recently, it was shown that metformin can increase the number of CD8⁺ tumor-infiltrating lymphocytes (TILs) and protect them from exhaustion and apoptosis. Metformin also shifts CD8⁺TILs from central memory T cells (TCM, inactive against tumor cells) to effector memory T cells (TEM, active against tumor cells), an event that correlates with regression of tumor cells.[351] Moreover, Pearce *et al.* published that treatment with metformin increased the fraction of CD8⁺ long-lived memory cells.[389] There has been a growing body of evidence suggesting that metformin may play a role of an immune modulator in the tumor microenvironment of ovarian cancer.[393] Recently, the role of metformin in metabolic reprogramming of tumor-specific T cells was reviewed, and it was concluded that AMPK inhibitors such as metformin may increase the efficacy of cancer immunotherapy.[394]

7.2.2.2.4. Eradication of cancer stem cells

Cancer stem cells (CSCs) are a group of malignant cells that possess the ability to regenerate all cell types of the tumor. CSCs are often resistant to chemotherapeutic agents and are believed to be one of the main reasons for the recurrence of cancer.[395-397] The presence of CSCs has been identified in most solid tumors including breast, pancreas, colon, lung, brain, melanoma, and prostate.[398-402] Metformin, was shown to reduce the CSC population and inhibit the proliferation of neoplastic cells and angiogenesis in ovarian cancer.[272, 403] The exact mechanism by which metformin kills or sensitizes cancer stem cells has not been fully elucidated yet. For instance, Vazquez-Martin *et al.* reported in a basal-like breast cancer model that metformin suppresses epithelial-mesenchymal transition (EMT) status which plays an important role in the acquisition of malignant properties including stemness.[404] Furthermore, Bao *et al.* showed in gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer cells, that metformin can inhibit CSC self-renewal capacity by re-expression of miRNAs and down expression of CSC markers.[405] Despite the unclear mechanism of action, there are many pieces of evidence proving that treatment with metformin effectively kills CSCs as monotherapy or as an adjuvant drug. It was shown that CSCs become more sensitive to other drugs such as carboplatin,[406] phenethylisothiocyanate,[407] cisplatin,[408] doxorubicin[409] among others after metformin treatment, suggesting that metformin could be used not only alone but also as a sensitizer in combination with other drugs for the treatment of different types of cancer. It was demonstrated that metformin effectively targets breast CSCs and increases the effectiveness of chemotherapy in breast cancer. Moreover, Song *et al.* showed that 48 hours of treatment with 1 and 5 mM of metformin reduces a fraction of breast CSCs from 2.78% to 2.20% and 1.29% correspondingly.[352] Hirsch *et al.* demonstrated that metformin not only targeted breast CSCs in four different cell lines but also promoted prolonged remission *in vivo* in a xenograft mouse model.[395] Furthermore, metformin effectively reduced the proliferation of CSCs in an Akt-dependent manner in glioblastoma cell lines.[410] Zhang *et al.* showed that colon CSCs were significantly reduced by treatment with metformin with a synergistic antiproliferative effect of 5-fluorouracil in comparison to 5-

fluorouracil single treatment (15.13 ± 3.43 vs 21.30 ± 1.40 ; $p=0.027$, respectively), resulting in growth inhibition of resistant tumors.[411] In pancreatic cancer, metformin has been shown to significantly reduce the expression of CSC markers such as ALDH1, CD44, CD133, reducing cell viability, clonogenicity, wound-healing and sphere-forming capacity in pancreatic CSCs.[412, 413]

7.2.2.3. Mechanisms via other pathways

Besides these five main AMPK-independent modes of action of metformin, several other actions have been reported. For instance, Liu *et al.* demonstrated that metformin shows anticancer activity on glioblastoma cells through AMPK-independent inhibition of mTOR by enhancing PRAS40's association with RAPTOR.[336, 337] Another possible mechanism of AMPK-independent mTOR inhibition was recently demonstrated by Memmott *et al.* who observed inhibition of mTOR in lung tumor tissues, by inhibition of the phosphorylation of IGF-IR/IR, Akt, and ERK.[414] Kalender *et al.* described another evidence of AMPK-independent inhibition of the mTORC1 *via* Rag GTPase.[319] Metformin also inhibits the proliferation in human hepatic carcinoma cells by downregulation of human-oxygenase-1 in an AMPK-independent manner possibly by attenuation of Raf-ERK-Nrf2 signaling.[415]

Recently the relationship between metformin and miRNA expression in human cells has received increasing attention[272]. miRNAs are short RNA sequences that were reported to regulate angiogenesis by affecting the proliferation, migration, and apoptosis of vascular cells[416]. Among them, miR-21 is known for the regulation of vascular cell proliferation, migration, apoptosis, and angiogenesis. Several studies reported downregulation in the expression of miRNA under metformin treatment. For example, metformin treatment resulted in a significant dose- and time-dependent decrease in miR-21 expression in human umbilical vein endothelial cells.[417] Indeed, metformin inhibits TGF- β and thereby activates miR-21 expression in endothelial cells. This leads to increased mRNA and protein levels (PTEN and Smad7), which in turn decreases phosphorylation of AKT, ERK1/2, and Smad2.[272] These changes ultimately inhibit the proliferation, migration, and tubule formation of endothelial cells, suggesting that metformin can regulate angiogenesis. Other studies suggested that metformin-associated upregulation of miRNA34a in heme oxygenase-1 renal cancer cells could lead to cell growth inhibition and cell cycle arrest.[418] It is evident that further studies into the role of metformin influence on miRNA expression are required to assemble a complete picture of the role of metformin on the cell cycle and proliferation.

The diversity of mechanisms of action proposed for metformin is still debated today. But its pleiotropic action is self-evident. The respective contribution of each mode of action, and the extent for each specific cancer is currently under investigation by several groups around the world. Additional research will be necessary in any case in the future to dissociate its complex action in cancer cells. Besides, the anticancer potential of other biguanide-based drugs has been very little studied so far.

7.3. Past and recent clinical studies

While the exact mechanism of action of metformin remains debatable, numerous clinical studies indicate its efficacy in various cancer models. A comprehensive summary of clinical studies on metformin could be found in contemporary reviews[68, 246, 419, 420]. Here we chose to highlight some past and most recent clinical data reported at the moment of publication.

Originally systematic meta-analysis of the diabetic patients revealed that patients taking metformin were at lower risk of developing cancer.[231, 421] Those data together with preclinical, empirical evidences[422-424] suggesting that metformin inhibits carcinogenesis promoted further clinical studies of metformin as an anticancer treatment for a broad group of patients.

For example, a significant body of work suggested that metformin intervention may prevent and reduce the risk of colorectal cancer at various stages. A study on diabetic patients conducted by Sehdev *et al.*[425] reported a 12% risk reduction in patients with colorectal cancer. Later multicenter phase 3 trial revealed that low dose metformin treatment reduced the prevalence and number of metachronous adenomas, thereby proving the antitumor potential of metformin against colorectal cancer.[426] Similarly, a meta-analysis of diabetic patients revealed lower incidents of ovarian cancer in the patients taking metformin.[427] A recent study observed that metformin use in postmenopausal women may prevent age-associated ovarian fibrosis, decreasing the risk of ovarian cancer.[428] Randomized clinical trials carried out to assess the impact of metformin on endometrial cancer risk showed trends toward positive effects on endometrial cancer risk-related serum markers and body composition.[429] Phase II clinical trial of metformin as a cancer stem cell-targeting agent in ovarian cancer proved that metformin therapy was associated with better-than-expected overall survival, supporting the use of the biguanide in phase III studies.[430]

Recent evidence suggests that metformin could be a potential candidate for breast cancer treatment as it was shown to inhibit breast cancer cell proliferation through the pathway miR-483-3p/METTL3/m⁶A/p21[431]. It was shown that mitochondrial metabolism can be exploited by targeting BACH1 to sensitize breast cancer to electron transport chain inhibitors such as metformin.[432] Another clinical study pointed at metformin anticancer effect through integrated measurement of transcriptomics and metabolomics.[433] Potential benefits of metformin in breast cancer treatment are further supported by other pre-clinical and clinical evidence.[434]

Metformin was found to decrease the growth and mobility of melanoma cells[435], inhibited proliferation, and induced apoptosis in the oral squamous cell.[436] Phase 1b/2 clinical studies are underway for evaluating the effect of metformin and chloroquine on solid tumors including glioma.[437] In glioma models, the use of metformin in combination with five other repurposed drugs (itraconazole, naproxen, pirfenidone, rifampin, and quetiapine; known as EMT inhibiting sextet (EIS)) is shown to inhibit glioblastoma cells proliferation, invasion, chemoresistance, and metastatic activities which further blocked the epithelial to mesenchymal transition (EMT).[438] Another area where metformin therapy where recent clinical data are available is lung cancer. A systematic review of diabetic patients with lung cancer showed that metformin use was related to a lower lung cancer risk in diabetic patients compared to non-users.[439] Later in clinical trials it was established that metformin in combination with immune checkpoint inhibitors improves clinical outcomes in non-small-cell lung cancer.[440]

While various clinical trials report improved outcomes for non-diabetic cancer patients, those results are somewhat limited and further comprehensive investigation into the clinical application of metformin as an anticancer drug (mono and/or combination therapy) is necessary. Furthermore, the immunomodulation effect of metformin should be closely investigated and taken into account to improve clinical usage.

8. Conclusions and Outlook

The biguanide class of compounds remains under-investigated despite their very attractive pharmacological properties, with a comparatively small number of bioactive analogs synthesized. At the same time, the number of successful biguanide drugs is very significant in comparison to the explored chemical space (**Figure 1**). Indeed, ten biguanide drugs are commercially available, and products like metformin or chlorhexidine are among the top-selling drugs in their therapeutic indication: type II diabetes and antibacterial agents. Other important biguanides include proguanil, which constitutes a standard treatment against malaria, and PHMB which could one day compete with chlorhexidine, given its exceptionally broad therapeutic index.

The current renewed interest in biguanide research stems mainly from promising reports as potential tumor treatments. Indeed, since the first observation of the anticancer effect of metformin by Evans in 2005, growing attention has been given to the use of this compound as a monotherapy or adjuvant anticancer drug. Owing to its great safety profile, about 350 clinical trials involving metformin are currently underway, with around 90% of those related to cancer treatment (**Table1**).^[441] Contrarily, other biguanide drugs have been poorly investigated for their anticancer potential yet. Furthermore, a recent pandemic linked to SARS CoV-2 triggered the further exploration of a possible therapeutic potential of biguanides and metformin for those infected with the virus.^[442]

Table 1. Summary of the main biguanide drugs used for therapeutic applications.[†]

Drugs (date of discovery)	Mode of action	Clinical trials in total (ongoing)	FDA approval status	Status
Metformin (1922)	Antidiabetic ^[343] Anticancer ^[317]	2196 (348)	Approved	Commercially available as first-line treatment of type II diabetes. ^[320] Present on the WHO List of Essential Medicines
Proguanil (1945)	Antimalarial ^[5, 130]	54 (11)	Approved	Commercially available. Present on the WHO List of Essential Medicines
Chlorhexidine (1946)	Antimicrobial ^[443] Sporostatic ^[163] Mycobacteriostatic ^[322] Bactericidal ^[165] Antiviral ^[169] Antiprotozoal ^[444] Antifungal ^[445]	787 (288)	Approved	Commercially available as a standard antiseptic. Present on the WHO List of Essential Medicines
Chlorproguanil	Antimalarial ^[138]	14 (0)	Unapproved	Commercially available

(1950)				
Moroxydine (1957)	Antiviral[218]	No clinical trials reported (tested in humans)	Unapproved	Drug progressively abandoned due to lack of efficacy compared to newer antivirals in modern clinical evaluation settings[218] Today mostly used for veterinary purposes. Commercially available in China.
Phenformin (1958)	Antidiabetic[340, 446] Anticancer[447, 448]	4 (1)	Unapproved	Withdrawn from most drug markets in 1978 for safety reasons[119]
Buformin (1958)	Antidiabetic[449] Anticancer[450]	5 (1)	Unapproved	Withdrawn from most drug markets in 1978 for safety reasons[119]
Picloxydine (1959)	Antiplatelet[187] Antibacterial[185]	No clinical trials reported (tested in humans)	Unapproved	Commercially available in Switzerland and France
Clociguanil (1965)	Antimalarial[149]	No clinical trials reported (tested in humans)	Unapproved	Drug development abandoned in 1974 because of a lack of clinical improvement
Alexidine (1966)	Bactericidal[179] Anticancer[177] Antimicrobial[451]	No clinical trials reported (tested in humans)	Unapproved but with 510(k) agreement	Commercially available
Polyhexamethylenebiguanide (1968)	Antimicrobial[208-210]	26 (7)	Unapproved but with 510(k) agreement	Commercially available
WR99210 (1973)	Antimalarial[155]	No clinical trials reported (tested in humans)	Not approved	Drug development abandoned in 1973 for safety reasons[452]
PS-15 (1993)	Antimalarial[155]	No clinical trials reported	Not approved	Drug development progressively abandoned for safety reasons[157]

†Data from clinicaltrials.gov

One of the reasons for the enthusiasm for metformin is the fact that it has recently proved to affect cancer cells resistant to both antibodies[453, 454] and small molecule drugs.[455, 456] This action is believed to arise from an inhibition of the multidrug resistance MDR1 gene transcription and P-glycoprotein expression,[338] but also from its inherent broad mechanism of action affecting multiple cellular targets. However, while metformin appears at first glance as an attractive alternative to current anticancer treatments subject to resistance phenomena, this broad mode of action could become a double-edged sword in a long run.

Indeed, pleiotropic action of metformin arises mainly from the high doses administered - so in other terms from its lack of efficacy. At the millimolar concentrations used in many *in vitro* assays, it is not surprising to observe the modulation of many biological processes and signaling pathways. On the other side, *in vivo*, drugs with multiple targets are more likely to interfere with other treatments and compounds, which in turn could lead to unexpected side effects. Thus, the high metformin doses required to reach an anticancer effect render the deciphering of its main mode of action particularly complex, and to which extent the action of metformin is rather due to one or another specific mechanism of action is still unknown. Thus, although the emphasis has logically been given to the simplest biguanide derivative so far, oncology research would certainly have more to gain from developing more effective and targeted biguanide molecules, rather than relying on an old pleiotropic drug. Indeed, the design of new more specific and therapeutically useful metformin analogs will not only permit to better understand the primary mode of action of this family of molecules, but also provide clinical research with new potential drug candidates with optimized efficiency and pharmacological features.

Biguanide is a small chemical group with outstanding properties for drug design. Such molecular structure favors interaction probability with biomolecules, along with displaying reasonable flexibility. In addition, its high polarity promotes its aqueous solubility. Overall, those features render biguanides a particularly valuable pharmacophore. In addition, this motif may allow the compound to be taken over by OCT transporters, and promote its penetration into cells. Furthermore, the representatives of the biguanide drug family are characterized by a common absence of major toxicity and a particularly high safety profile. Thus, it would be interesting to verify the antitumor properties of certain other biguanide derivatives like proguanil, chlorproguanil, clociguanil, or moroxydine, to study the possibilities of repositioning of compounds devoid of primary toxicity.

More broadly, new drugs based on biguanide would certainly have a very bright future in medicinal chemistry.[457-460] However numerous challenges remain such as identification of the right target for biguanides. Investigation into metformin mode of action revealed that this simple drug acts on many cellular pathways, while new anticancer drugs should be more specific. Whether the new molecules should target mTOR, cell cycle proteins, or miRNA synthesis is yet unknown. We believe that some of those avenues are worth perusing and the medicinal chemist will be rewarded with new highly active compounds.

Above all, the past successes of old biguanide drugs, which are structurally simple and sparsely developed, are very encouraging for the creation of new dedicated, modern, more effective biguanides with anticancer activity (**Figure 9**). Those should present more pronounced drug-like features with higher molecular weight and more complex structure, to allow better efficiency, lower dosages, and less marked pleiotropic character. Current modern rational drug design, using tools such as virtual drug design, QSAR, molecular modeling, fragment-based screening will undoubtedly succeed in meeting this challenge and lead to both to an improvement in our understanding of the pharmacological effects of this family of drugs, but also to obtaining very interesting potential anticancer agents.

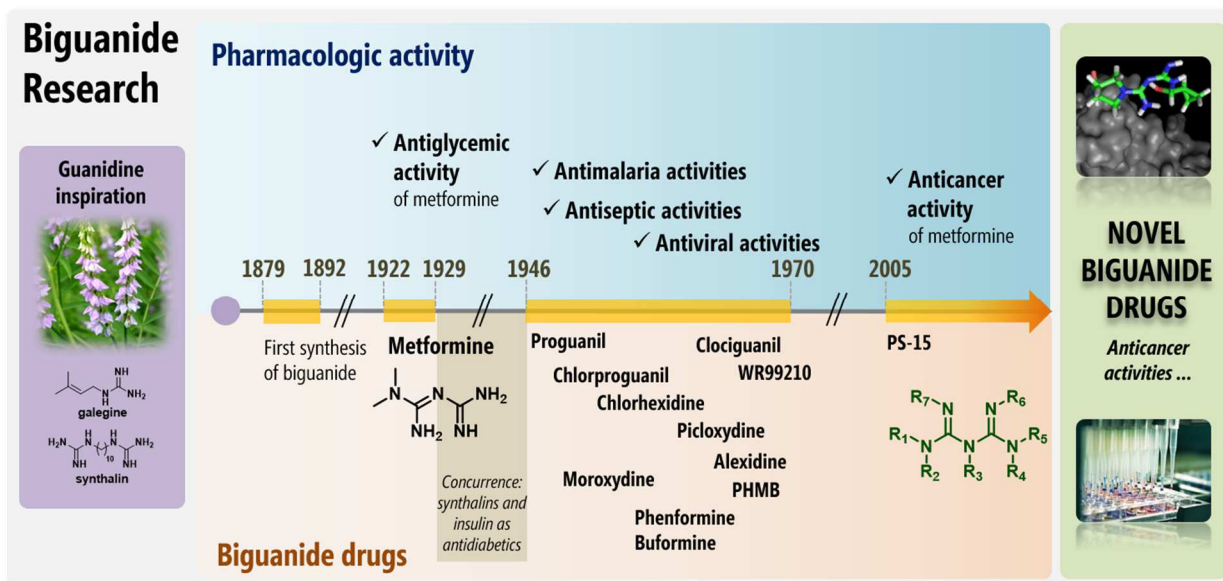


Figure 9. Biguanides research: pharmacological activities and clinically used drugs.

In conclusion, the overview of these successful examples of applications of biguanides as drugs demonstrates the value of this class of compounds in various fields of medicinal chemistry. In fact, drug discovery would greatly benefit from new biguanide derivatives, particularly in the field of oncology. Thus, biological and pharmaceutical research could, in parallel or rather than focusing on the efficacy(ies) of metformin, start to develop new biguanide-based drugs. This implies an increased curiosity of medicinal chemists to incorporate the biguanide motif into the structure of biological modulators. Therefore, this review aims to highlight the unexploited biomedical potential of this class of molecules, particularly as targeted anticancer agents, and to inspire further research into synthetic routes and biomedical evaluation of new biguanide analogs.

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