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Review Article

Glucose-6-phosphatase inhibitors

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Abstract

Glucose-6-phosphatase (G6Pase) is a multifunctional enzyme system that catalyzes the final step in both the glycogenolytic and the gluconeogenic pathways, cleaving phosphate from glucose-6-phosphate to liberate free glucose into the circulation. It is thus uniquely situated to regulate both circulating concentrations of glucose and the storage of excess glucose as glycogen. G6Pase activity is elevated in animal models of type 2 diabetes mellitus and appears to contribute to the excessive hepatic glucose production, and hence to the hyperglycemia, that characterizes this disease. As a consequence, the identification of inhibitors of G6Pase that could be used therapeutically in the treatment of diabetes has been the focus of considerable pharmacological and pharmaceutical research. This review describes a number of natural products and synthetic compounds that have been found to inhibit components of the G6Pase enzyme complex. The inhibitory activity of these agents and their efficacy in cell-based assays and *in vivo* are discussed, as are the potential negative consequences of pharmacological intervention at this key site of metabolic regulation.

Introduction

Our understanding of the role of glucose-6-phosphatase (G6Pase) in glucose homeostasis in health and in type 2 diabetes mellitus has advanced considerably in recent years. G6Pase catalyzes the final step in both the glycogenolytic and the gluconeogenic pathways, cleaving phosphate from glucose-6-phosphate to liberate free glucose into the circulation. It is thus uniquely situated to contribute to the regulation of glycemia. G6Pase activity has been shown to be elevated in animal models of diabetes and has been postulated to contribute to the excessive hepatic glucose production that characterizes this disease. As a consequence, a number of pharmaceutical companies have attempted to identify inhibitors of G6Pase that could be used therapeutically in the treatment of diabetic hyperglycemia. This review describes a number of natural products and synthetic compounds that inhibit G6Pase and discusses their efficacy and potential liabilities as agents for the treatment of diabetes mellitus.

The glucose-6-phosphatase complex

Glucose-6-phosphatase (D-glucose-6-phosphatase phosphohydrolase, E.C. 3.1.3.9, G6Pase) is a multifunctional enzyme system that catalyzes the breakdown of glucose-6-phosphate (G6P) to glucose and inorganic phosphate. Two hypotheses have been advanced to describe its structure-function relationship: the combined conformational flexibility-substrate transport model, which describes G6Pase as a multifunctional enzyme with both catalytic and transport activates embedded within the membrane of the endoplasmic reticulum, and the substrate transport-catalytic unit mode, which assigns catalytic and transport activities to separate components of the enzyme complex (1). The latter hypothesis, which is the more widely accepted, is the basis for the model that has generally been utilized in the discovery and design of G6Pase inhibitors and its validity is supported by the cloning of individual components of the complex (2, 3). It is the model that will be used in this review and is represented schematically in Figure 1. According to this model, the G6Pase enzyme complex is an integral constituent of the membrane of the endoplasmic reticulum with its active catalytic site directed towards the lumen (4, 5). The

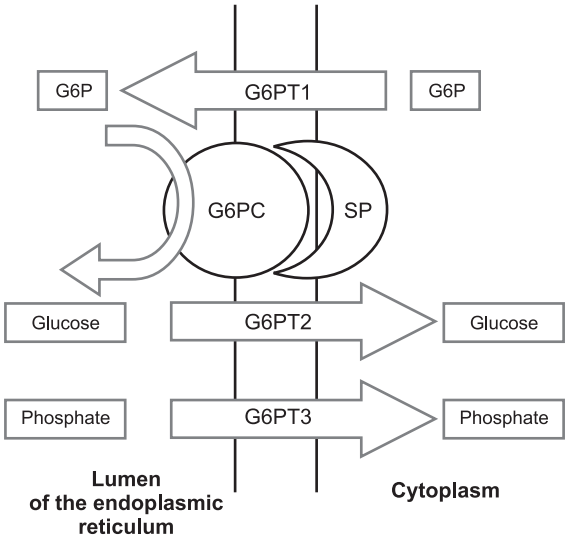


Fig. 1. Schematic representation of the glucose-6 phosphatase enzyme complex. G6PC, catalytic subunit; G6PT1, glucose-6-phosphate translocase; G6PT2, glucose transporter; G6PT3, phosphate transporter; SP, stabilizing protein.

complex comprises: (i) a transport protein, G6PT1, which facilitates the entry of glucose-6-phosphate (G6P) into the endoplasmic reticulum; (ii) a catalytic subunit, G6PC, which cleaves the substrate G6P; (iii) two additional transporters, G6PT2 and G6PT3, that return the products, phosphate and glucose, respectively, to the cytoplasm; and (iv) a stabilizing protein, SP, that appears to function as a regulatory calcium binding protein (6). The multicomponent structure of G6Pase provides several sites at which regulation, either by physiological effectors or by pharmacological intervention, could occur.

Tissue distribution of the G6Pase complex

G6Pase is expressed primarily in the liver, but also to a lesser extent in the kidney (7), the pancreatic islets (8) and the mucosa of the small intestine (9, 10). In the liver and the kidney its primary role is to release free glucose from G6P produced as a result of glycogenolysis or gluconeogenesis, but in the kidney it is also hypothesized to play a role in tubular reabsorption (11). In the intestinal mucosa it has been hypothesized to be involved in glucose transport and absorption (11), although here too, it appears to have a gluconeogenic function (12). In the β-cell, it appears to be involved in the mechanisms that coordinate glucose-dependent initiation of insulin release (13). While G6Pase expressed in the liver is the primary target of therapies designed to modulate glycemia, the presence of G6Pase in other tissues, and its participation in functions other than glycemic control, must be borne in mind when evaluating the consequences of G6Pase inhibition.

G6Pase and the regulation of hepatic glucose output

Euglycemia is the result of a hormonally controlled balance of glucose utilization by the tissues, intestinal absorption of dietary fuels and hepatic glucose output. The liver plays a central role in the maintenance of glucose homeostasis, storing excess glucose in the form of glycogen following dietary intake and releasing it into the circulation during fasting conditions. In a normal, nondiabetic human the circulating concentration of glucose is maintained at approximately 5 mM with little variation, regardless of prandial state. In subjects suffering from type 2 diabetes, hepatic glucose production is increased under postabsorptive conditions, contributing to fasting hyperglycemia (14-18).

The two metabolic pathways by which the liver can produce glucose are gluconeogenesis and glycogenolysis. Glucose production from glycogen and via gluconeogenesis also occurs in the kidney, however, the magnitude of the contribution to whole body glucose production remains a matter of debate (19, 20). When glycogenolysis is active, stored glycogen is broken down by the enzyme glycogen phosphorylase to produce glucose-1-phosphate, which is then converted to G6P. Gluconeogenesis permits the *de novo* synthesis of G6P from 3-carbon precursors such as pyruvate and lactate. A single enzyme, G6Pase, catalyzes the final step in both glycogenolysis and gluconeogenesis, removing the phosphate from G6P to liberate free glucose. Estimates of the relative importance of the contributions of these two processes to hepatic glucose production in the fed and the fasting state, and in normal health and in diabetes mellitus, vary (21-25). However, since G6Pase catalyzes the final step of both pathways (26, 27), it follows that inhibition of G6Pase should reduce hepatic glucose production via both the glycogenolytic and the gluconeogenic pathways, regardless of their relative contributions to net hepatic glucose output and, hence, to glycemia.

There is evidence to indicate that in diabetic animal models, in which hepatic glucose output is excessive, hepatic G6Pase activity is elevated (28-31). Furthermore, overexpression of G6PC in normal animals can replicate many of the metabolic abnormalities that characterize type 2 diabetes mellitus (31). These findings point to a key role for elevated G6Pase in the maintenance of inappropriately high rates of hepatic glucose output and, hence, of diabetic hyperglycemia, and suggest the possibility that reducing G6Pase would tend to reduce glycemia. As a consequence, considerable effort has been invested in attempting to identify G6Pase inhibitors that would be suitable for the treatment of diabetes.

Inhibitors of G6Pase

Known inhibitors of G6Pase fall into 2 main classes: inhibitors of the catalytic subunit, G6PC, and inhibitors of the translocase G6PT1, which facilitates the entry of G6P into the endoplasmic reticulum. In general, an inhibitor

that reduces activity in preparations of microsomes that have been disrupted or solubilized as well as in preparations of intact microsomes is considered to act on G6PC, while an inhibitor that is active only in intact microsomes is deemed to inhibit G6PT1. Expression of G6PC protein is increased in diabetes, and probably contributes to the elevated G6Pase activity and, hence, to the excess hepatic glucose production that characterizes the disease. Inhibition of G6PC is, therefore, a reasonable approach to reducing hepatic glucose output. However, since G6PT1 catalyzes the rate-limiting step in G6P breakdown, it is also a logical target for pharmacological intervention.

Inhibitors of G6PT1

Inhibitors of G6PT1 are numerous and structurally diverse (Table I). They include phloretin, the aglycone metabolite of the natural product phlorizin (32), pyridoxal phosphate (33), taurocholate and sulfhydryl reagents (34), diazobenzene sulfonate (35), mercaptopicolinic acid (36) and its analogues (37), *N*-bromoacetyl-ethanolamine phosphate (38) and 2-hydroxy-5-nitrobenzaldehyde (39). While many of these agents have proved to be useful tools for the study of G6Pase function *in vitro*, none has a high degree of specificity for G6PT1. For example, phlorizin has been widely used as an inhibitor of Na^+-glucose cotransporter activity, mercaptopicolinic acid is a more potent inhibitor of phosphoenolpyruvate carboxykinase than of G6Pase and hydroxynitrobenzaldehyde binds to both G6PT1 and G6PT2 (39) and also inhibits pyrophosphatase.

Natural products have been proven to be a rich source of starting material for the design of more potent, specific G6PT1 inhibitors. The active principle in *Bauhinia megalandra* leaves, used as a treatment for diabetes in Venezuelan traditional medicine, is reported to inhibit G6Pase in intact, but not disrupted microsomes, consistent with an inhibition of G6PT1, although the mixed noncompetitive inhibition kinetics implies inhibition of G6PT2 and G6PT3 as well (40). A series of thielavin G6Pase inhibitors with micromolar potency have been isolated from the fungi *Chaetomium carinthiacum* (41) and *Chloridium* sp. (42), and were shown to inhibit glucose output from isolated rat hepatocytes. The kodaistatins are T1 inhibitors isolated from extracts of *Aspergillus terreus* (43). Kodaistatin A and kodaistatin C, with IC_{50} values of 80 nM and 130 nM, respectively for the inhibition of G6Pase activity in intact hepatic microsomes, are among the more potent of the G6PT1 inhibitors identified thus far (43, 44). Still more potent is the microbial product, mumbaistatin, with an IC_{50} value of 5 nM (45)

The plant constituent chlorogenic acid has been used as the starting point for the design of many of the novel G6PT1 inhibitors synthesized in recent years. Chlorogenic acid itself is a weak, although selective, inhibitor of G6PT1 with an IC_{50} of 230 μM (46). Systematic modification of the molecule by combinatorial chemistry and rational drug design yielded a series of specific G6PT1 inhibitors with IC_{50} values in the nanomolar range (39, 46, 47) exemplified by S-3438, a competitive G6PT1-specific inhibitor with IC_{50} and K_i values of 210 nM and 190 nM, respectively, in intact microsomes prepared from rat liver (47), and its more potent analogue S-4048 (IC_{50} = 2 nM) (46).

Inhibitors of G6PC

Glucose mimetics and metabolic intermediates were among the earliest inhibitors of G6PC to be identified (48). Most are too weak to be of any physiological relevance as modulators of metabolic flux, although fructose-1-phosphate (K_i = 3.8 mM) and dihydroxyacetone phosphate (IC_{50} = 2 nM) (49) may have physiologically significant roles. Other small-molecular-weight inhibitors include tungstate, which competes at the catalytic site of G6PC (50), peroxyvanadium compounds (51, 52) and aluminum fluoride (53). All of these agents lack specificity for G6PC, which limits their therapeutic potential, although they have been proven useful and informative as research tools.

Some more selective inhibitors of G6PC are shown in Table II. Ilicicolinic acid, a fungal product, has been reported to inhibit G6Pase with micromolar potency (54), and the fact that this inhibition is present in disrupted microsomes suggests that its site of action is G6PC. Synthetic chemistry has yielded a series of tetrahydrothienyl pyridines that have been demonstrated to be G6PC inhibitors (55). These are structurally novel and more potent with IC_{50} values as low as 140 nM, but no data on their selectivity for G6Pase relative to other phosphatases have been published. More recently, a series of *N*,*N*-dibenzyl-*N*-benzylidenehydrazines have been described that are selective for G6PC relative to other phosphatases and have IC_{50} values as low as 170 nM (56).

Biological effects of G6Pase inhibitors

Effects of G6PT1 inhibitors

Inhibiting the G6PT1 component of the G6Pase complex avoids a potential liability of targeting the G6PC, namely the possibility that agents that inhibit G6PC activity are likely to inhibit other phosphatases with undesirable consequences. This may be one reason why much of the effort to identify pharmacological agents that reduce flux through G6Pase has until recently been directed toward G6PT1 rather than G6PC.

The most thoroughly characterized inhibitors of G6PT1 are chlorogenic acid derivatives (Table I). Chlorogenic acid itself is a specific, reversible, competitive inhibitor of G6PT1 (39, 46) that is without effect on either G6PC or inorganic pyrophosphatase activity (39). In isolated perfused rat livers, it effectively and dose-dependently reduced glycogenolysis and gluconeogenesis (46).

Table I: Inhibitors of G6PT1.

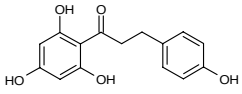
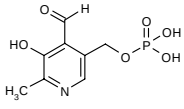
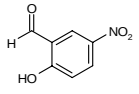
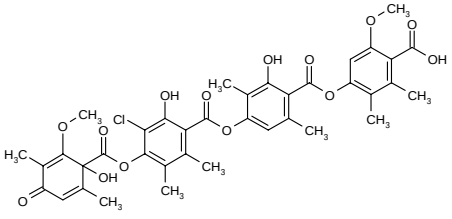
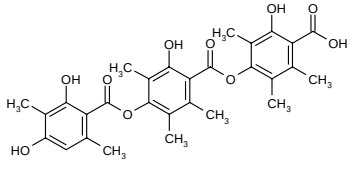
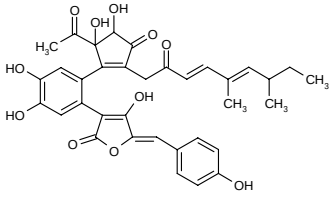
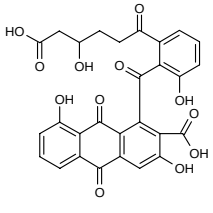
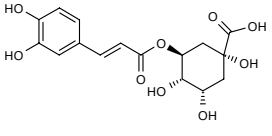
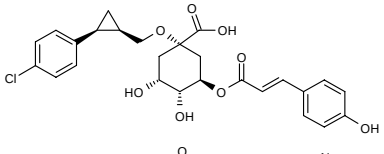
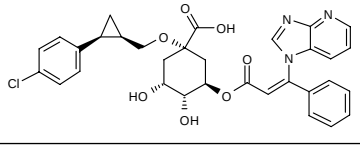
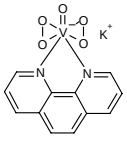
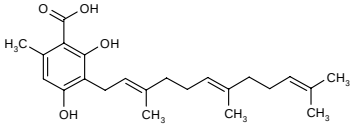
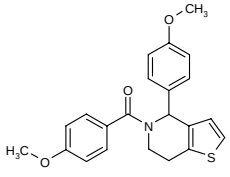
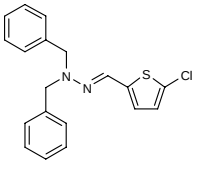
Compound	Structure	IC_{50} (μM)	Ref.
Phloretin		340	32, 54
Pyridoxal phosphate		446	33, 54
2-Hydroxy-5-nitrobenzaldehyde		338	39, 54
CJ-21164		1.6	42
Thielavin A		4.6	41
Kodaistatin A		0.08	43
Mumbaistatin		0.005	45
Chlorogenic acid		230	46
S-3438		0.2	47, 57
S-4048		0.002	46, 58

Table II: Inhibitors of G6PC.

Compound	Structure	IC_{50} (μM)	Ref.
Oxodiperoxo(1,10-phenanthrolin)vanadate		0.96	52, 66
Ilicicolinic acid B		7.7	54
Tetrahydrothienopyridine series (4-Methoxyphenyl)[4-(4-methoxyphenyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-5-yl]methanone NNC 60-0452		0.61	55, 67
N,N-dibenzyl-*N*'-benzylidenehydrazine series 5-Chlorothiophene-2-carbaldehyde dibenzylhydrazone		0.17	56

The chlorogenic acid analog S-3438, which was approximately 100-fold more potent than chlorogenic acid itself as an inhibitor of G6PT1 (47, 57), reduced the circulating plasma glucose concentration when administered intravenously to glucagon-infused fed rats (57). Liver glycogen and G6P levels were increased in these animals, consistent with the inhibitory mechanism reducing both glycogen breakdown and gluconeogenesis. Comparable effects were observed with the more potent chlorogenic acid derivative S-4048, which dose-dependently reduced blood glucose levels in both fed and fasted normal rats, while increasing glycogen and G6P in liver and kidneys, and elevating plasma free fatty acids and hepatic triglycerides (58). Insulin levels were also reduced in treated animals, probably secondary to the treatment-induced reduction in plasma glucose concentration. A study using radiolabeled gluconeogenic precursors and S-4048 in isolated rat hepatocytes and in fasted rats demonstrated that acute inhibition of G6PT1 did not affect net gluconeogenic flux to G6P, but that the G6P produced was redirected from glucose production into glycogen synthesis, resulting in a very substantial increase in glycogen accumulation (59). In a diabetic rodent model, the *ob/ob* mouse, S-4048 administered in conjunction with an oral glucose tolerance test reduced glucose levels without increasing plasma insulin (60). An increase in hepatic glycogen accumulation was also noted in this study.

Effects of G6PC inhibitors

Since the levels of G6PC protein and hence net hepatic glucose output are increased in diabetes (61, 62), inhibition of its activity ought to contribute to normalization of glycemia. Studies with inhibitors of G6PC support this premise. Tungstate has been shown to reduce plasma glucose in streptozotocin-induced diabetic rats, while leaving the glucose levels of healthy controls unchanged (63). With chronic treatment it also served to normalize activity levels of other enzymes involved in hepatic glucose metabolism (63), leading the authors to propose that tungstate could be a useful adjunct to the treatment of human diabetes. However, the effects on blood glucose may be attributable to improvements in pancreatic function (64) and/or insulin signal transduction (65) as much as to normalization of hepatic metabolism. Peroxyvanadium compounds can reverse the rise in plasma glucose levels caused by glucagon (52). However, since peroxyvanadium compounds are known to have insulin-mimetic activity (66), it is not possible to know how much of the effect of peroxyvanadium administration is attributable to G6Pase inhibition. One of the thienopyridine series of inhibitors, (4-methoxyphenyl)[4-(4-methoxyphenyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-5-yl]methanone, has been shown to reduce glucose output from isolated rat hepatocytes under basal and glucagon-stimulated conditions (67). There are as yet no published data on the biological effects of the *N,N*-dibenzyl-*N*-benzylidenehydrazines beyond enzyme inhibition.

Therapeutic potential and possible liabilities of G6Pase inhibitors

Proof of concept studies using potent inhibitors of one or more components of the G6Pase enzyme complex support the contention that hepatic glucose output, and consequently plasma glucose levels, can be reduced acutely by this mechanism. Since inhibition of G6Pase inhibits glucose production via both glycogenolysis and gluconeogenesis, a G6Pase inhibitor is likely to be more effective in reducing hepatic glucose output than an inhibitor of either process alone. Indeed, since there are reports that suggest that gluconeogenesis and glycogenolysis are coordinately regulated (68, 69), inhibition at a site common to both processes may be not just the most efficient, but possibly the only effective way of reducing total hepatic glucose production (70). However, this potential for robust glucose-lowering efficacy comes with the associated risk of hypoglycemia. S-4048 at single doses up to 100 mg/kg markedly reduced plasma glucose levels in overnight-fasted animals, in the case of fasted mice to below 2 mM (< 36 mg/dl) (60). Hypoglycemia was transient and returned to normal levels by 3 h postdose, which may be attributable to counter-regulatory mechanisms, although the short duration of action of S-4048 may also have served to limit its hypoglycemic potential (60, 71).

Another potential consequence of lowering plasma glucose levels by this mechanism is the possibility of elevating circulating lactate concentrations to unacceptable levels, and there is certainly evidence that inhibition of G6PT1 does increase lactate concentrations both in the liver (60) and in the general circulation (58). In these studies, the hyperlactemia observed was relatively mild and did not approach lactic acidosis; however, as noted above, these studies were conducted with a short-acting inhibitor.

Studies with the G6PT1 inhibitor S-4048 have also documented a variety of alterations in carbohydrate and lipid intermediary metabolism. Treatment of rodents with this agent increased hepatic concentrations of glucose-6-phosphate (58, 60), with increased hepatic (58-60) and renal (58) glycogen content, and increased triglycerides, cholesterol and uric acid (58). The reported increase in hepatic glucose-6-phosphate levels is unsurprising, given that the means of transporting G6P into the endoplasmic reticulum for breakdown to glucose is inhibited. The increase in glycogen accumulation could be a simple mass-action effect consequent upon the increased levels of glucose-6-phosphate. However, it is also possible that the synthesis of glycogen is promoted by a direct activation of glycogen synthase by G6P (72). These studies were too short in duration to reveal whether the increase in glycogen accumulation was self-limiting or if continued treatment would result in a condition analogous to a glycogen storage disease. The observed increases in cholesterol and triglycerides in S-4048-treated rats were interpreted to be due to resterification of increased levels of plasma free fatty acids, which occurred secondary to the reduction of blood glucose levels (58). However, there is evidence to suggest that S-4048 directly increases *de novo* hepatic lipogenesis due to increased glycolytic flux and flux through hepatic acetyl-CoA precursor pools leading to development of hepatic steatosis (73).

Defects in various components of the G6Pase complex have been implicated in a number of types of glycogen storage diseases (4). A deficiency in G6PT1 manifests as glycogen storage disease type 1b (74), in which patients present with hypoglycemia, lactic acidemia, hyperlipidemia and hyperuricemia as well as abnormal storage of glycogen. It appears that administration of G6PT1 inhibitors produces perhaps a milder, but essentially comparable metabolic profile. The physiological consequences of an absence of G6PC activity (glycogen storage disease type 1a) are even more severe, and before the implementation of more aggressive clinical management often resulted in death in early childhood (75). While pharmacological intervention rarely achieves effects comparable to those resulting from a loss-of-function gene mutation, it would certainly be appropriate to monitor plasma levels of lactate, cholesterol, triglycerides and uric acid as well as clinical markers of hepatic safety in any clinical studies using agents with this mechanism.

It is also possible that chronic treatment with a G6Pase inhibitor might not achieve the robust glucose-lowering efficacy demonstrated in acute studies. There is evidence that treatment of normal rats with an inhibitor of G6PT1 (S-3438) can quickly elicit a significant upregulation of G6Pase gene expression (76). If this effect were a consequence of inhibitor-induced changes in metabolism, it would tend to limit the glucose-lowering potential of this mechanism.

At present no inhibitors of the G6Pase enzyme complex have advanced to clinical trials. Possibly this is a consequence of poor biopharmaceutical properties rather than of insufficient potency, efficacy or safety. It is noteworthy that in all of the *in vivo* preclinical studies using G6PT1 inhibitors that have been published thus far, the inhibitors were either infused intravenously (57, 58) or injected intraperitoneally (60) because systemic exposure after oral dosing was extremely low. There are as yet no published reports of the *in vivo* administration of inhibitors of G6PC activity, so it remains to be seen whether the biopharmaceutical properties of these classes of compounds are superior to those of G6PT1 inhibitors.

Conclusions

A body of published data supports the contention that inhibition of the G6Pase enzyme complex could potentially be used as a treatment for diabetes mellitus. Animal studies with G6PT1 inhibitors have demonstrated robust glucose-lowering efficacy, and in recent years potent selective inhibitors of G6PC have been synthesized. The *in vivo* efficacy of these latter inhibitors has yet to be evaluated. At present it appears that the main obstacle to further preclinical or clinical development may be

identification of an agent that combines *in vitro* potency with pharmacokinetic and biopharmaceutical characteristics consistent with oral activity. Given the metabolic consequences of administration of G6Pase inhibitors in preclinical studies, and the metabolic profile known to be associated with the glycogen storage diseases, monitoring of plasma lipids, lactate, uric acid and hepatic function during any clinical study is indicated.

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