Review Article

Glutaminolysis-ammonia-urea Cycle Axis, Non-alcoholic Fatty Liver Disease Progression and Development of Novel Therapies

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Abstract

The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing worldwide, reflecting the current epidemics of obesity, insulin resistance, type 2 diabetes mellitus, and metabolic syndrome. NAFLD is characterized by the accumulation of fat in the liver, and is known to be a cause of cirrhosis. Although many pathways have been proposed, the cause of NAFLD-linked fibrosis progression is still unclear, which posed challenges for the development of new therapies to prevent NASH-related cirrhosis and hepatocellular carcinoma. Cirrhosis is associated with activation of hepatic stellate cells (HSC) and accumulation of excess extracellular matrix proteins, and inhibiting the activation of HSCs would be expected to slow the progression of NAFLD-cirrhosis. Multiple molecular signals and pathways such as oxidative stress and glutaminolysis have been reported to promote HSC activation. Both mechanisms are plausible antifibrotic targets in NASH, as the activation of HSCs the proliferation of myofibroblasts depend on those processes. This review summarizes the role of the glutaminolysis-ammonia-urea cycle axis in the context of NAFLD progression, and shows how the axis could be a novel therapeutic target.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a serious public health problem affecting more than half a billion people worldwide,[1] being a leading cause of liver transplantation and hepatocellular carcinoma (HCC).[2,3] It is defined as a liver disease with evidence of lipid accumulation by hepatocytes (hepatic steatosis) confirmed by histology, imaging, or noninvasive biochemical tests in the absence of significant alcohol intake, defined as 30 g/day for women and 40 g/day for men, long-term use of steatogenic medication, or monogenic hereditary disorders.[4]

In most patients, NAFLD is associated with metabolic comorbidities like overweight or obesity, dyslipidemia, arterial hypertension, and type 2 diabetes mellitus (T2DM). Approximately 90% of patients with NAFLD have a history of least one metabolic syndrome, which means that NAFLD comprises a group of conditions and is the reason that underlies estimates that the worldwide prevalence will continue to increase. In that setting, a group of experts has recently proposed a change in name to MAFLD (metabolic-associated fatty liver disease) and a diagnosis based on the presence of metabolic dysfunction and not on the absence of other conditions.[5] The pathogenesis of NAFLD is thus multifactorial, and changes in several physiological systemics have been implicated. Insulin resistance is one of most relevant mechanisms, and is ultimately associated with lipotoxicity, endoplasmic reticulum stress, and autophagy. Also, a complex interaction of environmental factors, changes in the microbiota, and predisposing genetic variants contribute to NAFLD.[6] The histological spectrum of NAFLD ranges from simple steatosis with accumulation of triglycerides by hepatocytes to non-alcoholic steatohepatitis (NASH), characterized by apoptosis, necrosis, inflammatory cell infiltration, ballooning, and fibrosis against
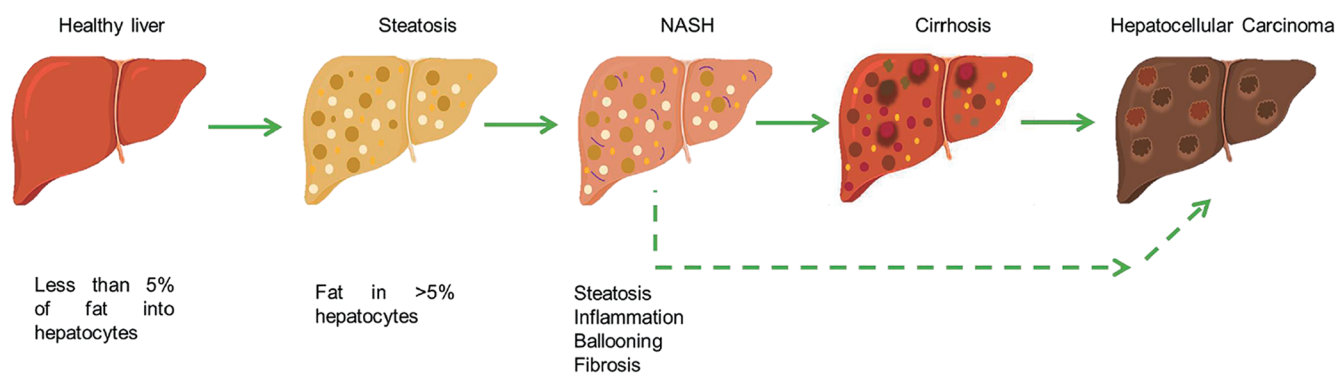
Fig. 1. Spectrum of non-alcoholic fatty liver disease (NAFLD).

a steatotic background.[7] NASH is considered the most harmful part of the spectrum of NAFLD, being an important risk factor for cirrhosis and liver cancer development as well as extrahepatic events.[8] About 20–30% of NAFLD patients progress to NASH, which is more likely to progress to advanced fibrosis, cirrhosis, and HCC.[9] Notably, HCC can also arise in the NASH stage before cirrhosis occurs (Fig. 1).[10]

Management of NASH should focus on the restitution of metabolic derangements and halting inflammatory, fibrogenic, and carcinogenic pathways.[11] Lifestyle intervention, based on the Mediterranean diet and increasing physical activity, is the first-line therapy.[12] Several drugs are under investigation in phase II and III clinical trials in patients with unsuccessful lifestyle interventions. However, there are currently no drugs approved for the management of this entity. The causes and metabolic reprogramming underlying the pathogenesis of NAFLD-driven cirrhosis and HCC remain to be discovered. In this review, we summarize the role of the glutaminolysis-ammonia-urea cycle in the pathogenesis of NAFLD and its role as a potential novel therapeutic target.

Glutaminolysis and the urea cycle

Previous studies have reported a strong associations between NAFLD and the glutamine pathway and urea cycle[13,14] that are involved in intracellular fatty acid accumulation and fibrogenesis. L-glutamine is the most abundant free amino acid in the human body. Its hepatic metabolism has previously been implicated in regulating cellular redox reactions and energy balance as glutamine can be used as a substrate in the tricarboxylic acid cycle (TCA) to support mitochondrial metabolism.[15] Glutamine metabolism pathways and the several of the required enzymes are shown in Figure 2. Glutaminase catalyzes the first step of glutamine catabolism in the liver by converting glutamine to glutamate and ammonia, and resulting in two phosphate-activated glutaminase isoforms, GLS2 and GLS. The *GLS2* gene, located in chromosome 12, encodes two splice variants with low activity and allosteric regulation, liver-type glutaminase (LGA, short transcript isoform), and glutaminase B (GAB, long transcript isoform), which are highly expressed in the normal adult liver.[16] Likewise, the *GLS* gene, located in chromosome 2, encodes two splice variants with high activity and low substrate affinity, kidney-type glutaminase (KGA, long transcript isoform) and glutaminase C (GAC, short transcript isoform), which are mainly expressed in the kidney under healthy conditions.[17] Previous studies have shown that GLS is upregulated in cells with increased proliferation rates and accounts for most glutaminase activity in some human tumor cells, whereas GLS2 expression is associated with resting or quiescent cell states.[18,19]

Ammonia is a gut-derived nitrogen compound produced by hydrolysis of urea by bacterial organisms, and by glutamine catabolism.[20] Changes in the microbiota and the gut–liver axis affect the metabolism of lipids and carbohydrates, impact the balance of inflammatory mediators, and cause metabolic deregulation that promote NAFLD progression. The microbiota and its metabolites, such as ammonia, play direct and indirect roles in gut barrier function and fibrosis development, but further studies are needed.[21] In normal conditions, ammonia is eliminated by the liver through the hepatic urea cycle, a process known as ureagenesis. The main role of this pathway is to nitrogen balance in the whole body and requires five urea cycle enzymes, carbamoyl phosphate synthase I (CPS1), ornithine transcarbamylase (OCT), argininosuccinate synthase (ASS), argininosuccinate lyase (ASAL), and arginase-1 (ARG1). If the urea cycle is disrupted, the concentration of ammonia in the liver increases, and glutamine synthetase (GS) acts to promote glutamine synthesis. Newly synthesized glutamine is released into the blood, enters the liver and is metabolized by GLS or GLS2 to glutamate and ammonia.

Glutamine, glutaminase activity, and ammonia metabolism have been described in liver diseases, mainly in acute liver failure and cirrhosis,[22] hepatic encephalopathy[23,24] and HCC.[25,26] However, its role in the progression of liver diseases like NAFLD has not been described. Glutaminolysis, mainly GLS activity, has been associated with (a) the accumulation of intracellular lipids by hepatocytes,[13] (b) HSC activation and fibrosis progression,[14,27] and (c) metabolic reprogramming that is needed to fuel cancer cell growth.[28]

Glutamine metabolism and NAFLD

The detailed mechanism of NAFLD progression to NASH remains as a challenge. It is well known that insulin resistance and steatosis promote NASH progression.[29] In addition, the accumulation of excess lipids in the liver promotes oxidative stress, increases fatty acid oxidation, and releases proinflammatory cytokines ultimately responsible for mitochondrial and hepatocyte damage, inflammation, and activation of fibrogenesis pathways.[30] The evidence suggests that NASH is a multifactorial process involving numerous conditions, including aberrations of lipid metabolism, oxidative and endoplasmic reticulum stress, insulin resistance, microbiota imbalance, immune responses, genetic susceptibility, and genomic instability.[31] The processes appear to modify some signaling pathways in which the glutaminolysis-ammonia-urea cycle is included. There are several hypotheses about the nitrogen imbalance in NAFLD. Although further studies are needed for confirmation, they include (a) intestinal dysbiosis or microbiota changes;[21] (b) increased glutaminolysis,[13] (c) urea cycle dysfunction,[32] and (d) decrease of glutamine synthesize activity.[33]
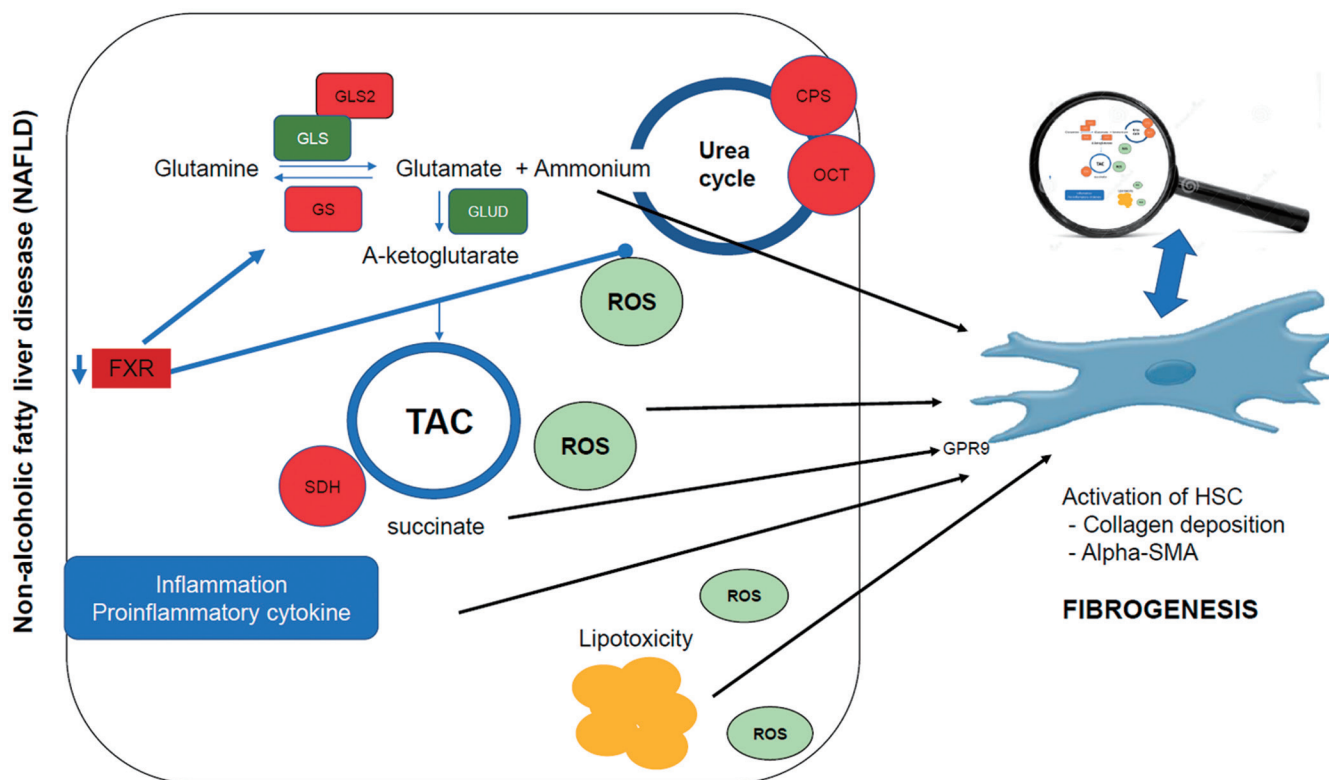
Fig. 2. Glutamine metabolism pathway and its association with NASH-fibrogenesis. Upregulated pathways are shown in red. CPS, carbamoyl phosphate dehydrogenase; FXR, farnesoid X receptor; GLS, glutaminase; GLUD, glutamate dehydrogenase; GS, glutamine synthetase; NASH, non-alcoholic steatohepatitis; OCT, ornithine transcarbamoylase; SDH, succinate dehydrogenase.

Steatosis and the glutamine-ammonia-urea cycle axis

Recently, Simon *et al*.[13] showed that GLS was overexpressed in the livers of NASH patients and in mice in diet-induced NASH models, providing evidence that glutamine metabolism is a potential therapeutic target in NASH progression. It is well known that dysfunction of very-low-density lipoprotein (VLDL) synthesis and secretion occurs in NASH.[34] Mice feed a choline-deficient diet (MCD), the main component of VLDL membranes, results in fat liver accumulation.[35] Li *et al*.[36] showed in an animal model that a methionine and choline-deficient diet resulted in significant changes in serum glutamine levels, and other studies reported a switch from the GLS2 to the GLS isoform in the end stages of chronic liver disease.[18,27] Both mRNA and protein expression of GLS were significantly upregulated, whereas GLS2 expression decreased in genetic Mat1a$^{-/-}$ mice fed a 0.1% methionine- and choline-deficient diet (MCDD) for 6 weeks. The evidence the hepatic GLS was increased in preclinical NASH models, led to studies in liver tissue from NASH patients. Hepatic GLS levels were increased in NASH patients compared with healthy controls. In addition, if *Gls* was silenced in mice fed a 0.1% MCDD and choline-deficient high-fat diet (CD-HFD) then the fat content of the liver decreased because of the restoration of VLDL synthesis pathways and reduction of oxidative metabolism.[13]

Liver fibrosis and the glutamine-ammonia-urea cycle axis

Recent reports have described the involvement of glutaminolysis in liver disease, mainly in the fibrogenesis pathway

and HCC development.[27,37] Activation of HSCs is a key step in liver fibrogenesis and carcinogenesis. Multiple molecular signals and pathways have been reported to modulate HSC activation, including oxidative stress[38] and cell metabolism.[39] Induction of glutaminolysis is a key component of fibrogenesis progression and is necessary for cancer cell growth.[27,40] Indeed, some cancer cells are glutamine dependent, and the inhibition of glutaminolysis has been proposed as a potential therapy for some cancers.

Hepatic mitochondrial dysfunction has a major role in NASH progression. Several vicious cycles involving TNF-α, reactive oxygen species (ROS), and chemically reactive lipid peroxidation products alter mitochondrial respiratory chain polypeptides and mitochondrial DNA in hepatocytes containing fat vacuoles. In this setting, mitochondrial ROS formation results in biologically active lipid peroxidation products and cytokine release, which combine to trigger hepatic damage.[41] ROS originating from an impaired TCA cycle, fatty acid oxidation, oxidative phosphorylation, lipotoxicity.[38,39] and infiltrating inflammatory cells during liver injury induces transdifferentiation of HSCs to MF-HSCs and the consequent synthesis of extracellular matrix.[42]

Recently, Chiara *et al*.[33] reported increased ammonia and progressively decreased GS concentrations in NASH patients compared with those with simple steatosis because of a reduction in the expression of several urea cycle genes. In addition, chronic exposure to high ammonia levels was found to have profound effects on HSC activation and fibrogenesis.[14] Du *et al*.[27] reported increased bioenergetic and biosynthetic demands by activated HSCs that were supported by an increase in glutaminolysis.[43] In addition, activated HSCs in culture and *in vivo* were shown to have increased utilization of glutamine and expression of genes involved in

glutamine metabolism, such as *GLS*, glutamic-oxaloacetic transaminase, and glutamate dehydrogenase 1 (*GLUD1*).[44] In the same way, succinate, which is converted to fumarate in the TCA cycle by succinate dehydrogenase, is involved in HSCs activation binding to and activating its cognate G protein-coupled receptor 91 (GPR91). The induction of GPR91 promoted the upregulation of fibrogenic markers such as alpha-smooth muscle actin (α-SMA), transforming growth factor-beta (TGF-β), and collagen type I, suggesting that the succinate-GPR91 pathway may also be a potential therapeutic target in liver fibrosis.[45,46] The available results confirm that glutamine metabolism is increased in NASH, and related to steatosis, oxidative stress, and fibrogenic pathways, which makes it a potential therapeutic target of NASH treatment.

Role of drugs on the glutaminolysis axis in NAFLD

No drugs have been licensed and approved for treating NAFLD but the effects of many drugs on a number of pathways are under investigation.[47] In this scenario, administering an ammonia-lowering drug in NAFLD could reduce disease progression by scavenging non-urea ammonia and thereby reducing the activation of HSCs.[14] Despite increasing of the role of the glutaminolysis-ammonia-urea cycle axis in the pathogenesis of NAFLD, no drug is being developed to specifically target this mechanism. The roles of some glutaminase inhibitors in NAFLD, such as BPTES and CB-839, remain unclear. Instead, ammonia-lowering drugs, such as L-ornithine L-aspartate (LOLA) or ornithine phenylacetate (OP), initially indicated for other diseases (e.g., hepatic encephalopathy)[48] are being tested for NAFLD. In addition, some drugs initially developed for NAFLD have off-target effects on the glutaminolysis-ammonia-urea cycle axis.

Specific NAFLD-targeted drugs

Obeticholic acid is a Farnesoid X receptor (FXR) agonist that has shown efficacy in primary biliary cholangitis[49] and promising results for NAFLD in Phase II and III clinical trials.[50] FXR, in addition to having an essential role in carbohydrate and lipid metabolism, exerts effects on some genes that regulate enzymes in the urea cycle and acts as a transcriptional regulator of ammonia detoxification via ureagenesis and glutamine synthesis in the liver.[51] Inactivation of FXR leads to the hepatic accumulation of some urea cycle precursors, and activation results in an increase in the expression of glutamine synthetase GS) and urea cycle-related genes that promotes glutamine synthesis and urea production. Obeticholic acid induces the expression of *Glul* via activation of FXR, which is an alternative way to dispose of excess nitrogen because of the conversion of glutamate into glutamine in pericentral hepatocytes.[52]

Repurposing nonspecific NAFLD drugs

Metformin is a biguanide used to treat T2DM. No data supports its use for NAFLD despite initial enthusiasm related to its mechanism of action, because of a lack of efficacy in reducing fibrosis.[53] However, metformin appears to be effective as a chemopreventive agent for HCC and other tumors.[54] A previous study has reported partial inhibition of glutaminase activity (about 20%), both in chemical and cell assays compared with controls.[55] On the other hand, pioglitazone, a peroxisome proliferator-activated receptor (PPAR)-γ agonist, is another antidiabetic drug that could interferes with the glutaminolysis-ammonia-urea cycle axis by restricting the conversion of glutamine to glutamate by reducing the expression of GLS1, the rate-limiting enzyme.[56] Thus, in a personalized medicine scenario, some antidiabetic drugs are effective for NAFLD patients in whom the glutaminolysis-ammonia-urea cycle axis is the predominant pathogenic insult of liver disease.

LOLA is a well-known drug to treat hyperammonemia in cirrhosis,[57] acute liver failure, and hepatic encephalopathy[58] by synthesizing urea and glutamine via the enzyme GS. In the setting of NAFLD, a significant improvement in ALT levels has been observed, together with a significant decrease of triglycerides, in a randomized controlled trial including 72 patients receiving LOLA for 12 weeks.[59] In another trial, LOLA also improved hepatic microcirculation in 78 patients with NASH.[60] The evidence supports the benefits of LOLA for patients with NAFLD.

OP is an ammonia-lowering agent that promotes glutamine synthesis from ammonia in skeletal muscle and excretes the ornithine-related glutamine from the kidneys.[61] OP is a safe and effective ammonia scavenger for treating hyperammonemia in conditions including cirrhosis,[62] acute-on-chronic and acute liver failure.[63] OP was shown to reduce plasma ammonia, tissue markers of HSC activation, and portal pressure in bile duct-ligated rats with advanced fibrosis and hyperammonemia.[64] Regarding the NAFLD scenario, OP appears to prevent hepatocyte death and to reduce the development of fibrosis, hepatic ammonia, and inflammation in an animal model, by restoring of urea cycle enzyme activity and function.[14]

Preclinical discovery of glutaminolytic ammonia-targeting therapies

Drug discovery is an intensive process that requires extensive preclinical and clinical studies (Fig. 3).[65] Successful drug discovery requires in-depth knowledge of a disease, the significant elements in pathogenesis. In NAFLD, may be a relevant enzyme because of its role in ammonia production (Fig. 4). Essential initial steps are the development of prototypes or "hits" against the predetermined target (e.g., GLS has been demonstrated as a validated target)[13] and optimization to improve potency and reduce side effects, or "leads". Once a lead compound is found, the candidate has to be assessed in the preclinical stage to establish the safety and efficacy, including information about properties such as absorption, distribution, metabolization, and excretion, as well as the best dosage and administration route, and side effects/adverse events. Preclinical trials require *in vivo*, *in vitro*, and *ex vivo* assays in living organisms or cells in animals and humans, or using nonliving organisms or tissue extracts. In silico assays based on computer simulations can accelerate the process.

Conclusions

NAFLD is a complex, multifaceted condition the molecular and cellular levels, which is reflected by the difficulty of developing new therapeutics, but the need to develop preventive treatments that slow the progression of or even reverse fibrosis is clear. In that setting, the glutaminolysis-ammonia-urea cycle axis emerges as a fundamental pathogenic process in NAFLD. Future studies should focus on it to bring personalized medicine closer.
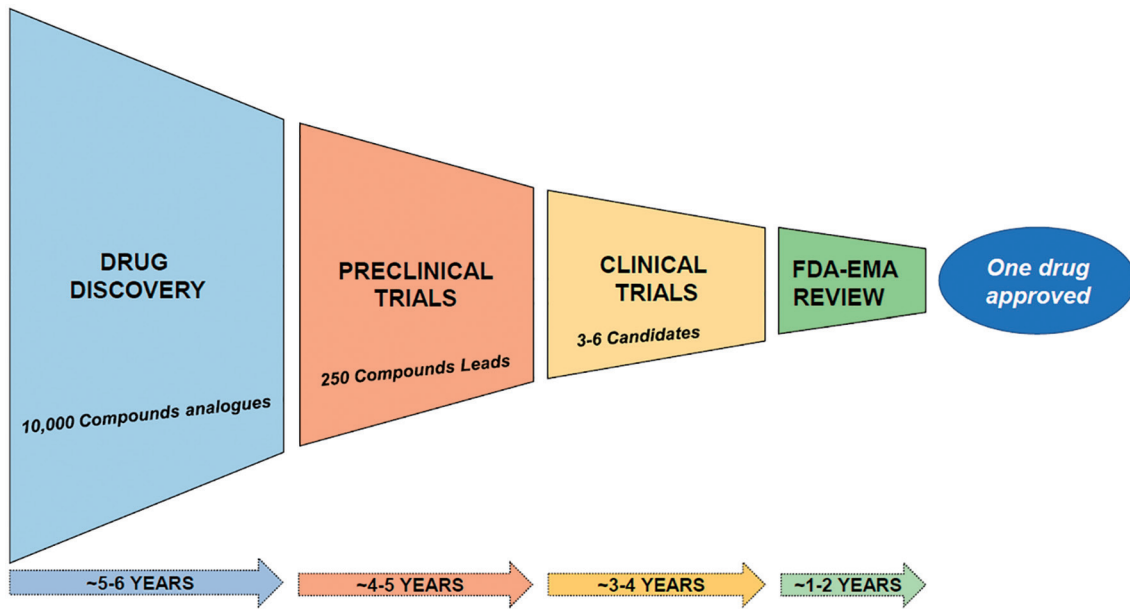
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Fig. 3. Drug discovery process.
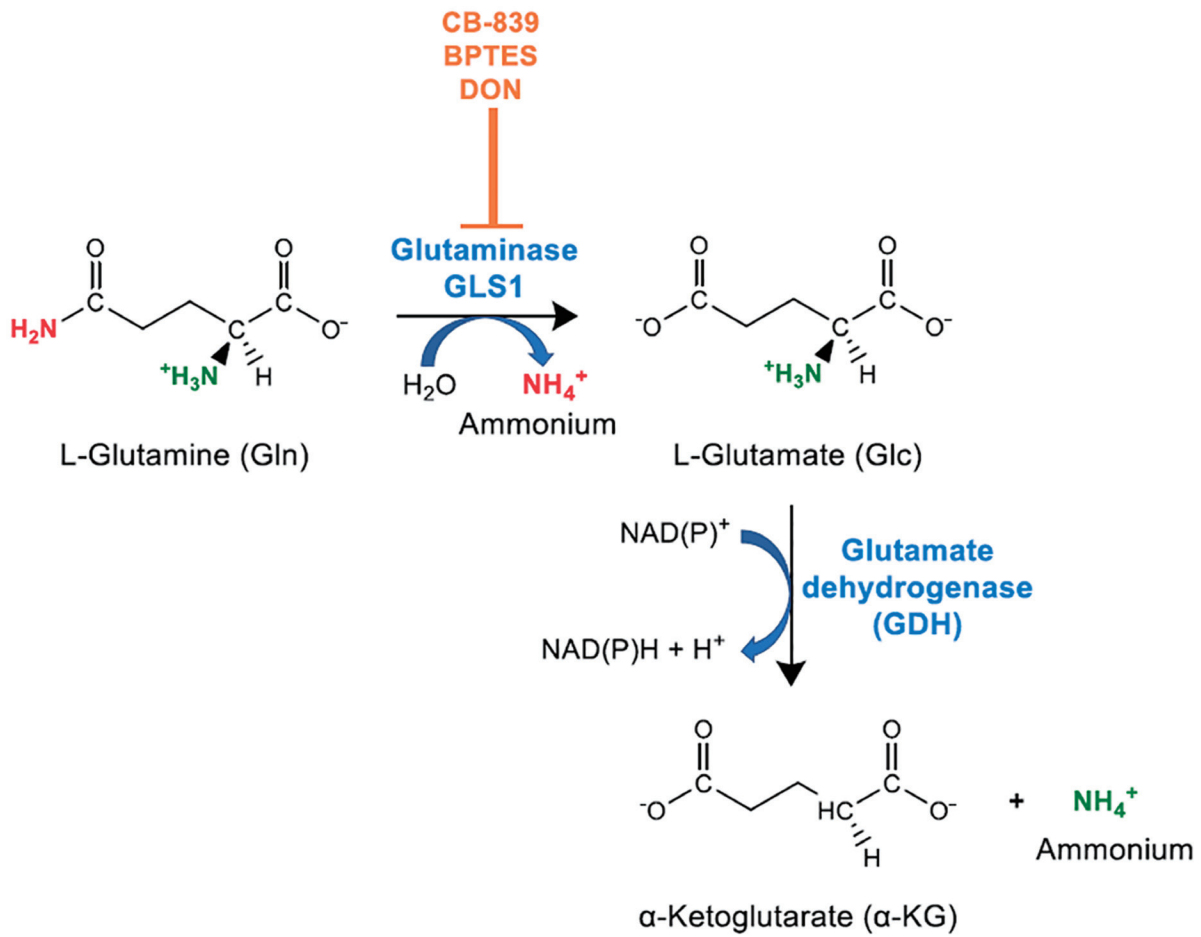
Fig. 4. Targeting glutaminolysis: examples of selected glutaminase inhibitors.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Guarantor of the article (JA), drafting the manuscript (AR, MGL, AG, MRG, JA), critical review of the manuscript (all authors). All authors approved the final version of the article.

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