Published in final edited form as:

Expert Opin Drug Metab Toxicol. 2017 April ; 13(4): 439–448. doi:10.1080/17425255.2017.1262843.

An update on the use of benzoate, phenylacetate and phenylbutyrate ammonia scavengers for interrogating and modifying liver nitrogen metabolism and its implications in urea cycle disorders and liver disease

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Abstract

Introduction—Ammonia-scavenging drugs, benzoate and phenylacetate (PA)/phenylbutyrate (PB), modulate hepatic nitrogen metabolism mainly by providing alternative pathways for nitrogen disposal.

Areas Covered—We review the major findings and potential novel applications of ammonia-scavenging drugs, focusing on urea cycle disorders and liver disease.

Expert Opinion—For over 40 years, ammonia-scavenging drugs have been used in the treatment of urea cycle disorders. Recently, the use of these compounds has been advocated in acute liver failure and cirrhosis for reducing hyperammonemic-induced hepatic encephalopathy. The efficacy and mechanisms underlying the antitumor effects of these ammonia-scavenging drugs in liver cancer are more controversial and are discussed in the review. Overall, as ammonia-scavenging drugs are usually safe and well tolerated among cancer patients, further studies should be instigated to explore the role of these drugs in liver cancer. Considering the relevance of glutamine metabolism to the progression and resolution of liver disease, we propose that ammonia-scavenging drugs might also be used to non-invasively probe liver glutamine metabolism *in vivo*. Finally, novel derivatives of classical ammonia-scavenging drugs with fewer and less severe adverse effects are currently being developed and used in clinical trials for the treatment of acute liver failure and cirrhosis.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

Keywords

Glutamine; urea cycle disorders; acute liver failure; cirrhosis; hepatocellular carcinoma; phenylacetate; phenylbutyrate; benzoate

1. Introduction

Reduced nitrogen enters the human body as dietary free amino acids derived from proteins or as nitrogen-containing compounds produced by intestinal tract bacteria [1]. The liver is the gatekeeper for the nitrogen balance, playing an important role in redirecting the use of amino acids for tissue protein synthesis, heme formation, pyrimidine and purine synthesis (nucleotide precursors), and for the *de novo* synthesis of ketone bodies and carbohydrates. On the other hand, nitrogen excretion in the form of urea takes place primarily in the liver via the urea cycle. Disruptions of nitrogen homeostasis, leading to the accumulation of the toxic waste nitrogen-containing product, ammonia (NH₃), characterize urea cycle disorders (UCDs), a group of genetic inborn errors of metabolism, and liver disease. The main areas covered by this review are the mechanisms of action in the liver, adverse effects, and future prospects for the different ammonia-scavenging drugs used in the management of UCDs and liver disease.

2. Overview of liver nitrogen metabolism

The principal point of entry of nitrogen into liver metabolism is the non-essential amino acid glutamine. While glutamine released by the muscle accounts for about 50–70% of whole-body glutamine rate of appearance [2, 3], this amino acid is also released, in far smaller amounts, by the lung and the brain. In theory, the liver would be capable of relevant glutamine synthesis by expressing high levels of glutamine synthetase, the enzyme that catalyzes the synthesis of glutamine from glutamate (Fig. 1). However, under normal conditions, the liver plays a more regulatory role in glutamine metabolism taking up large amounts of glutamine derived from extrahepatic tissues.

On this basis, the role of the liver in the regulation of glutamine homeostasis is almost exclusively mediated through changes in the activity of glutaminase, the enzyme that deaminates glutamine to glutamate via hydrolysis. Liver-specific glutaminase 2 is the main isoenzyme of glutaminase present in the liver [4]. Other isoenzymes of glutaminase are only expressed in the liver under specific conditions, as we will address later. Through the conversion of glutamine to glutamate and the deamination of the later to alpha-ketoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle, the glutamine carbon skeleton can be used as a hepatic gluconeogenic precursor, resulting in the *de novo* generation of carbohydrates from amino acid sources [2, 5, 6].

The conversion of glutamine to glutamate by glutaminase or the direct deamination of glutamate results in the release of ammonia. Ammonia is also released as the result of increased transsulfuration pathway, a metabolic pathway that can be viewed as part of the methionine and folates metabolism, involving the interconversion of cysteine and homocysteine through the intermediate cystathionine [7]. Ammonia is usually eliminated

through its conversion to urea by a series of biochemical reactions, known as the urea cycle (Fig. 1) (see [8] for a complete review of enzyme regulation of the urea cycle). An alternative route of nitrogen clearance is through the conjugation of the nitrogen-containing amino acid L-glycine to benzoate to form hippurate, later excreted in the urine. Overall, a tight metabolic regulation between amino acid metabolism, transsulfuration pathway, gluconeogenesis, urea and TCA cycles is crucial for nitrogen homeostasis.

3. Ammonia-scavenging drugs

Disruptions of nitrogen homeostasis lead to an excessive and noxious accumulation of ammonia. Elevated ammonia levels ($>100 \mu\text{mol/L}$) are extremely toxic to the central nervous system. Indeed, prognosis is considered very poor if hyperammonemic coma has lasted more than 3 days, intracranial pressure is markedly elevated or if ammonia has peaked at $>1000 \mu\text{mol/L}$ [9, 10]. In recent decades, interest has grown in the use of drugs that are able to lower ammonia levels, acting as ammonia scavengers, such as benzoate and phenylacetate (PA)/phenylbutyrate (PB).

3.1. A historical perspective

Benzoate, a monocarboxylate usually used as a food preservative, was the first described ammonia-scavenging drug. The use of benzoate was proposed in 1914, when Lewis and colleagues described for the first time a correlation between the decrease in urinary urea and the appearance of the nitrogen-containing compound hippurate after ingestion of sodium benzoate in healthy individuals [11]. Five years later, other authors demonstrated the elimination of nitrogen in humans after treatment with PA that conjugates to glutamine to form phenylacetylglutamine (PAGN) [12]. Based on these two reports, Brusilow and colleagues decided to give sodium benzoate as a supplement to 26 children with defective ureagenic pathways. Under these circumstances, formation of hippurate from benzoate accounted for up to almost 60 per cent of the total excretion of nitrogen [13], highlighting that benzoate could be used to control defects in nitrogen excretion. Later, the same authors showed that administration of sodium phenylacetate also causes ammonia elimination by its conjugation to glutamine and PAGN excretion in UCD patients [14].

These studies led to the approval by the US Food and Drug Administration (FDA) of the combined therapy with sodium benzoate and sodium phenylacetate to treat hyperammonemia in UCDs in the early 1980s. In 1983, a further amendment permitted the replacement of PA by PB, a metabolic precursor of PA with a less disagreeable odor, and finally, in the early 1990s, the use of the first PB derivative salt, sodium phenylbutyrate, to replace the combined therapy [13], was approved as a safe, well-tolerated monotherapy orphan drug for the treatment of UCDs [15].

3.2. Hepatic drug metabolism

Liver and kidney are the major sites for the metabolization of drugs. In the case of benzoate, its liver metabolism occurs exclusively by conjugation with L-glycine to form the nitrogen-enriched and rapidly excretable urinary compound, hippurate. The first step of this pathway is activation by benzoyl-CoA ligase to form benzoyl-CoA which then conjugates to glycine

[16]. Another readily excretable non-urea metabolite is the amino acid acylation product, PAGN, that results from the conjugation of PA to glutamine [17]. In the human liver, the pro-drug PB is rapidly oxidized to PA [17]. The conjugation of PA to glutamine proceeds via phenylacetyl-CoA catalyzed by an acyl-CoA:L-glutamine *N*-acyltransferase [18]. The total amount of either PA or PAGN excreted in urine is as low as 50% of the PB dose administered, suggesting that other secondary metabolites are formed during this process [19]. Indeed, other urinary metabolites derived from PB metabolism have been described in humans including phenylbutyrylglutamine (PBGN) presumably formed from the reaction of phenylbutyrate-CoA with glutamine [20], and β -hydroxy-phenylbutyrate (PHB) formed via partial β -oxidation of PB to phenylbutyrate-CoA [19]. In spite of the wide variety of PA/PB-derivative metabolites found in urine, urinary PAGN was shown to be a useful non-invasive biomarker for monitoring adherence and the effects of therapy after PA/PB administration [21].

In the following paragraphs, we provide a comprehensive and integrated review of the therapeutic and diagnostic applications of ammonia-scavenging drugs in UCDs and liver disease.

3.3. Ammonia-scavenging drugs in UCDs

Urea cycle disorders are a group of genetic conditions characterized by deficiencies of the enzymes and transporters involved in the urea cycle. These include deficiencies of carbamoyl phosphate synthetase 1 (CPS1) (MIM #237300) [22], ornithine transcarbamylase (OTC) (MIM #311250) [23], argininosuccinate synthetase (ASS1) (NIM #215700) [24], argininosuccinate lyase (ASL) (NIM #207900) [25] and arginase (ARG) (NIM #207800) [26] (Fig. 1). Except for OTC deficiency, which is X-linked, these disorders have autosomal recessive inheritance [27, 28]. The incidence of OCDs is estimated to 1:8,000–1:44,000 births [29, 30], though these numbers may actually underestimate the prevalence, due to unreliable newborn screening and underdiagnosis of the disorders in fatal cases.

When presenting in the neonatal period, typically after a variable symptom-free interval, UCDs are characterized by overwhelming illness that rapidly progresses from poor feeding, vomiting, lethargy and/or irritability to coma and/or death [9, 31, 32]. On the other hand, the majority of these disorders have a late-onset form, presenting in childhood and/or adulthood, and these are usually related to partial rather than complete enzyme deficiencies. In these cases, the symptoms are usually less severe and more variable, comprising poor developmental progress, behavioral problems, hepatomegaly and gastrointestinal symptoms. Children and adults with UCDs frequently present chronic neurological illness that is characterized by variable behavioral problems, confusion, irritability and episodic vomiting. In patients with UCDs, acute episodes of hyperammonemia are recurrent and can be triggered by metabolic stress in response to infection, trauma, surgery, and pregnancy or the postpartum, among other conditions [9, 31, 32].

Prompt treatment, mainly relying on a timely diagnosis, is crucial for the management of UCDs. Overall, the aim of treatment is to improve the control of the biochemical disorder, while ensuring that all nutritional needs are met. Hence, the long-term management of UCDs is based on a diet that is low in protein, but contains all essential amino acids, and

vitamin and mineral supplementation, complemented with medications to increase ammonia excretion [33]. On this basis, benzoate and PA/PB are often included in treatment regimens to successfully activate the synthesis of either hippurate or PAGN and provide an alternative pathway for nitrogen excretion. On the one hand, this avoids the usage of an already compromised urea cycle while generating a reserve urea synthetic capacity to further support nitrogen homeostasis when required [29, 34, 35].

3.4. Ammonia-scavenging drugs in acute liver failure

Acute liver failure (ALF) is characterized by complications that indicate the liver has suffered severe damage. Common causes of ALF are hepatitis virus infections (hepatitis A, B and E) and intentional or unintentional drug hepatotoxicity [36]. Hyperammonemia is a feature of ALF [37]. Therefore, ammonia-scavenging drugs may provide a valuable tool to control metabolic decompensations in ALF. Indeed, preliminary studies indicate that combined therapy with L-ornithine and PA, as L-ornithine phenylacetate, can successfully attenuate hyperammonemia in animal models of ALF [37]. L-ornithine phenylacetate acts at two levels: (1) L-ornithine being used as a substrate for glutamine synthesis from ammonia in skeletal muscle; and (2) PA excreting the ornithine-related glutamine as PAGN in the kidneys. Further *in vivo* studies and clinical trials should be conducted to evaluate the hepatic effects of ammonia-scavenging drugs in ALF.

3.5. Ammonia-scavenging drugs in chronic liver disease

Nonalcoholic fatty liver disease (NAFLD) refers to a group of conditions characterized by the accumulation of excess fat in the liver, a condition currently affecting approximately one-third of the population in the USA [38]. In NAFLD patients, deregulated liver methionine metabolism drives homocysteine elevation [39], a precursor of ammonia through the transsulfuration pathway, suggesting that ammonia-lowering drugs may be helpful in preventing the progression of NAFLD. NAFLD is closely associated with insulin resistance and an elevated risk of chronic liver disease, a process that involves the progressive destruction and regeneration of the liver parenchyma leading to fibrosis and eventually cirrhosis, its clinical endpoint. Hepatic encephalopathy is a common neuropsychiatric abnormality, which further complicates the clinical course of cirrhotic patients; however, the exact pathophysiological mechanisms underlying this condition in such patients are unclear. Hyperammonemia, systemic inflammation (including sepsis, bacterial translocation, and insulin resistance) and oxidative stress are potential key players [40]. Importantly, blood ammonia levels can identify patients at higher risk of suffering hepatic encephalopathy-related morbidity, suggesting direct involvement of ammonia in the pathogenesis of hepatic encephalopathy [41].

The main sources of blood ammonia in cirrhotic patients are breakdown of glutamine in enterocytes and the kidneys [42], and defective nitrogen metabolism in the liver [43]. In this regard, variations in the promoter region of the hepatic glutaminase gene, coding for the enzyme that converts glutamine to glutamate and ammonium formation, were found to be associated with the exacerbation of hepatic encephalopathy in cirrhotic patients [44]. Based on this evidence, it has been recently proposed that cirrhotic patients may benefit from treatment with ammonia-lowering drugs. Indeed, preliminary studies show that treatments

with glycerol phenylbutyrate, sodium benzoate or L-ornithine phenylacetate reduce hepatic encephalopathy events in cirrhotic patients by lowering blood ammonia levels [45–47].

Further research and clinical trials should be undertaken in the near future to evaluate the beneficial actions of the ammonia-lowering drugs in preventing both NAFLD progression and hepatic encephalopathy in cirrhotic patients.

3.6. Ammonia-scavenging drugs in liver cancer

Previous *in vitro* findings have shown that treatment with the ammonia-scavenging drug, PA, arrests growth of cancer cells in renal cancer and in promyelocytic leukemia cell lines [48, 49] whereas PB induces apoptosis in prostate cancer cells, medulloblastoma cells and colon cancer cells [50, 51]. In spite of these promising results *in vitro*, clinical trials addressing the anti-cancerous potential of these agents are more questionable. In the past, both PB [52–54] and PA [55] were used in clinical trials to treat recurrent malignant gliomas. Even though these drugs were usually well tolerated by cancer patients, there was little antitumor activity, at least at the dose schedule used. An *a posteriori* study concluded that the pharmacokinetics and pharmacodynamics of PB in glioblastoma patients is affected by concomitant administration of P450-inducing anticonvulsants [52], it being plausible that the limited concentrations of PB achievable in the cells *in vivo* partly explain the lack of efficiency shown in the earlier clinical trials. In agreement with this, a very recent study has assessed PB-treatment in different glioblastoma cell lines, concluding that the effects of PB seem to be cell-type-specific in a dose-dependent manner [56].

Liver cancer, or more specifically its most common primary form, hepatocellular carcinoma (HCC), is the fifth most common cancer and the third leading cause of death due to cancer worldwide [57]. To date, the biological heterogeneity of HCC makes prognosis difficult and, more importantly, limits therapeutic approaches. In previous studies, PB-based treatments were shown to induce apoptosis in hepatoma-derived cells [58] and to promote the regression of tumors derived from hepatocarcinoma cells in rats [59, 60]. Other encouraging studies have shown the radiosensitizing effects of a PB-derivative in human hepatoma cell lines [61]. In spite of these promising *in vitro* findings, the exact mechanism underlying the liver antitumor effects of ammonia-scavenging drugs is less clear.

Histone acetylation is associated with gene activation, while deacetylation, mediated by histone deacetylases, is associated with gene silencing, explaining why histone deacetylases are considered a powerful drug target in cancer [62]. PB is considered to be a first generation histone deacetylase inhibitor [63]. In addition, numerous studies have clarified the relationship between endoplasmic reticulum (ER) stress and cancer, and particularly the involvement of the unfolded protein response (UPR) (see review [64]). Phenylbutyrate is known to act like a chemical chaperone attenuating activation of the UPR, an important player in mediating ER stress and in cellular transformation and proliferation in several types of cells (e.g., adipogenesis [65, 66], fibroblast differentiation [67], and prostate cancer cell proliferation [68], among others). Notably, the liver is composed of different cell types that in a coordinated manner undergo transformation driving HCC progression. Hepatocytes, due to their role as metabolic hubs, are enriched in both smooth and rough ER. Moreover, it is also well documented that the UPR plays an important role in immunity and

inflammation, this being particularly relevant considering that tumor-associated macrophages play a pivotal role in the progression of HCC [69]. In relation to this, a recent study has shown that the transmission of ER stress from hepatocytes to macrophages drives an alternative macrophage polarization that can be shut off by PB treatment [70], raising the question about which types of cells are targeted by PB treatment *in vivo*.

Even though metabolic alterations may not be initiating events in liver cancer, they definitely play an important role in the progression of the disease, and hence are an attractive therapeutic target. Indeed, it has been described that in addition to the abnormal dependency on glycolysis for energy production [71], cancer cells show other atypical metabolic characteristics such as glutamine-addiction [72] and concomitant elevated rates of glutamine catabolism through glutaminase [73]. As mentioned earlier, glutaminase has tissue-specific isoenzymes: the *GLS2* gene, located on chromosome 12, encoding two isoforms with low activity and allosteric regulation, liver-type glutaminase (LGA, short transcript isoform), and glutaminase B (GAB, long transcript isoform), both highly expressed in normal adult liver [74]; on the other hand, the *GLS1* gene, located on chromosome 2, encodes two isoforms with high activity and low K_m , the kidney-type glutaminase (KGA, long transcript isoform) and the glutaminase C (GAC, short transcript isoform), both mainly expressed in the kidney under normal healthy conditions. Notably, recent evidence highlights that a metabolic switch from the GLS2 to the high activity GLS1 isoenzyme occurs in liver disease [75, 76], consistent with the increased utilization of hepatic glutamine under these circumstances. Other authors have recently described that glutamine seems to provide acid resistance to *Escherichia coli* through the enzymatic release of basic ammonium ions, allowing survival in a highly acidic environment [77]. This evidence seems to further implicate glutamine catabolism in tumor cell growth in that it would provide tumor cells with acid resistance.

In our laboratory, we have preliminary data showing that the expression of the highly active glutaminase isoenzyme, GLS1, is specifically enriched in tumors of animal models that spontaneously develop HCC, such as prohibitin-1 (*Phb-1*) [78] and glycine-*N*-methyltransferase (*Gnmt*) [79] deficient mice (Fig. 2). While *Phb-1*-KO mice develop HCC in a mechanism mediated by mitochondrial dysfunction aberrant methylation patterns account for HCC development in the *Gnmt*-KO mouse. Our unpublished findings showing increased GLS1 expression in HCC mouse tumors of different etiologies are in agreement with other evidence showing that glutamine uptake is aberrant in human hepatoma cells, when compared to uptake in normal human hepatocytes [80]. Further, the observed decrease in free glutamine levels in serum, liver, and tumors of rats with fast-growing tumors does not take place in rats with slow-growing hepatomas [81]. Moreover, glutamine is known to be essential for the proliferation of macrophages [82], inflammatory cells that express glutaminase and glutamine synthetase [83]. Importantly, hepatoma cells are very sensitive to glutamine depletion [84] suggesting that glutamine-deprivation or glutamine-depletion based therapies could be used in the treatment of liver cancer. In addition, in glioblastoma, compensatory glutamine metabolism has been reported to play a critical role in multi-drug resistance, the main mechanism underlying cancer resistance to antitumor drugs used in chemotherapy and other systemic therapies [85].

Through conjugation to glutamine, PA/PB are able to target liver nitrogen metabolism both by offering an alternative pathway for nitrogen disposal through urinary excretion of PAGN, and secondly, by inducing hepatic glutamine depletion by acting as a “glutamine trap”. Indeed, both in children with inborn errors of urea cycle metabolism, who have high plasma levels of glutamine, and also in healthy adults, with normal plasma glutamine levels, administration of PA/PB causes an abrupt decrease in plasma glutamine [15]. It seems therefore that PA/PB-based treatments provide means for hepatic glutamine depletion and therefore there is growing interest in their potential use to treat liver cancer. To date, the extent to which glutamine depletion induced by ammonia-scavengers could contribute to the antitumor or drug-resistance actions remains questionable. Further experiments are needed to explore the mechanism and the target-cells underlying the antitumor actions of PA/PB in liver cancer and these should be followed by clinical trials.

3.7. Ammonia-scavenging drugs as non-invasive probes of liver intermediary metabolism

Disrupted regulation of liver intermediary metabolism is a hallmark of liver disease. Metabolic flux analysis is useful firstly to improve our understanding of the disease, and secondly to monitor pharmaceutical and lifestyle interventions. In recent years, liver intermediary carbohydrate metabolism fluxes have been non-invasively evaluated using chemical agents that are excreted in urine, such as acetaminophen [86, 87], menthol [88], and also PA/PB and their excretable urinary by-product, PAGN [89, 90]. For example, Burgess and colleagues assessed the ^{13}C -labeling pattern of PAGN to evaluate hepatic TCA cycle fluxes assuming that the labeling pattern of PAGN glutamine moiety reflects that of the liver TCA cycle intermediate, alpha-ketoglutarate. With this methodology, the authors showed that hepatic mitochondrial oxidative and anaplerotic TCA cycle activities are elevated in NAFLD patients [91].

Glutamine metabolism is considered a crucial step in cancer metabolism and thereby a potential diagnostic and therapeutic target in cancer. Previously, the fate of ^{13}C -labeled glutamine has been tracked in cancer cells [92] and in mouse tumors [93]. Assessment of liver glutamine metabolism *in vivo* is currently far more challenging. Even though hyperpolarized ^{13}C magnetic resonance spectroscopy can provide insight to glutamine metabolism *in vivo* [94, 95], it is a rather costly technique requiring equipment that is expensive and not currently available in all hospitals. As an alternative, we propose that the ratio between the ^{13}C -enrichments of the glutamine moiety of urinary PAGN and blood glutamine after ingestion of ^{13}C -labeled glutamine and PA/PB could be used to assess liver glutaminase activity *in vivo*. Indeed, PB was previously used to assess the contribution of proteolytic and metabolic sources of hepatic glutamine, inferred from the ^2H nuclear magnetic resonance-based analysis of the different urinary PAGN ^2H -positional enrichments from deuterated water ($^2\text{H}_2\text{O}$) [96]. Finally, a metabolomics-based study has recently shown that the analysis of hippurate by ^1H nuclear magnetic resonance spectroscopy of urine samples can provide some insight into liver toxicity and therefore could be used to predict or screen for drug-induced liver injury [97].

3.8. Common adverse effects of ammonia-scavenging drugs

Commonly reported adverse effects of the combined therapy with sodium benzoate/sodium phenylacetate include infections, disorders affecting the respiratory system, blood and lymphatic system, nervous system or metabolism and nutrition, and vomiting [98]. Specifically, at the recommended doses for the treatment of UCDs (250–500 mg/kg/day, maximum 12 g/day), sodium phenylbutyrate has been shown to decrease appetite, disturb taste and cause disagreeable body odor in approximately 5% of patients [34, 99]. Further, chronic PB treatment causes menstrual dysfunction/amenorrhea in about 25% of postpubertal females [99]. Even though PA/PB administration is well tolerated in most cases, hepatotoxicity side effects associated with interactions between these drugs and liver cytochrome P450 enzyme have been described [100]. Lastly, treatment with PA/PB has also been described to cause depletion of branched chain amino acids (BCAAs), which may increase the risk of endogenous protein catabolism [101, 102].

In recent years, a great effort has been made by the scientific community to develop new derivatives of ammonia-scavenging drugs with fewer adverse effects. For example, the use of the PB-derivative glycerol phenylbutyrate has been recently recommended to replace sodium phenylbutyrate, as treatment with sodium phenylbutyrate has palatability issues and other adverse effects associated with high sodium content and high pill burden. Moreover, the maximum approved daily dose of sodium phenylbutyrate (20 g) contains $\approx 2,400$ mg of sodium, which exceeds the daily allowance of 2,300 mg/day for the general population and 1,500 mg/day for individuals with hypertension and certain other sodium-retaining states, as recommended by the US Department of Health and Human Services in the 2005 Dietary Guidelines for Americans [103]. Glycerol phenylbutyrate is composed of three molecules of PB esterified to a glycerol backbone. The metabolically active agent PA is generated through multiple metabolic steps including hydrolysis in the small intestine by pancreatic triglyceride lipases. Its pharmacokinetic pattern is characterized by a slower release of the active metabolite than unconjugated PB, which helps to improve control of blood ammonia and consequently reduce the rate of hyperammonemia events and associated complications. In agreement with this, glycerol phenylbutyrate is well tolerated with fewer gastrointestinal complications compared with sodium benzoate or sodium phenylbutyrate [104].

4. Expert opinion

Even though ammonia-scavenging drugs started to be used about 40 years ago, benzoate and PA/PB are still included in the therapeutic regimens for acute and chronic treatment of UCDs. These drugs prevent the accumulation of ammonia providing, on the one hand, an alternative pathway for nitrogen disposal and, on the other, relieving the urea cycle by diverting its substrates. Despite the reasonably successful outcome associated with the treatment of UCDs with benzoate and PA/PB, at the moment, a large clinical study is addressing the safety and efficacy of the novel derivative glycerol phenylbutyrate in UCDs. Another issue related to the use of ammonia-scavenging drugs concerns the fact that in patients with severe UCDs, metabolic decompensation with hyperammonemia resulting in severe neurological damage or death typically occurs during the first days of life. Even though therapies based on alternative pathways to the urea cycle for nitrogen disposal are

usually started soon after birth, prenatal administration might improve metabolic stability. Indeed, prenatal benzoate treatment by benzoate infusion of the mother shortly before birth was proven to be safe and resulted in therapeutic levels of benzoate in umbilical cord blood [105]. Based on this evidence, prenatal infusion of ammonia-scavenging drugs to the mother may reduce postnatal complications due to hyperammonemia and provide a valuable prenatal therapeutic approach in the treatment of UCDs.

Over the last decade, it has been proposed that ammonia-scavenging drugs could be used in the treatment of liver disorders. In fact, treatment with these ammonia-lowering drugs has been shown to reduce hepatic encephalopathy events in cirrhotic patients. Other authors have reported beneficial effects of a PB derivative-based treatment in controlling arterial ammonia levels in animal models of ALF. In addition, PA/PB-mediated treatment provides means for glutamine-depletion and therefore its potential application to treat cancer and promote the death of glutamine-addicted liver cancer cells is of growing and renewed interest. Though previous reports have demonstrated the antitumor effects of PA/PB *in vitro*, to date, the results of clinical trials with these drugs in glioblastoma have not been promising. Recent studies describing interactions with other drugs used in the treatment of glioblastoma patients and the dose and cell-dependent effect of PA/PB may partially account for the reduced bioavailability of these drugs and the rather poor outcomes. Further studies and clinical trials should be undertaken exploring the role of PA/PB in liver cancer, paying special attention to the doses used and possible interactions with other therapeutic agents that might reduce overall bioavailability of these ammonia-scavenging drugs. Moreover, as some of the beneficial mechanisms underlying PA/PB activity as an antitumor agent are not exclusively related to its ability to affect liver nitrogen metabolism, the extent to which glutamine depletion or even the gain in ammonia-induced acid resistance produced by PA/PB contributes to this outcome remains questionable and further experiments are necessary to investigate this issue. Overall, considering the poor prognosis of liver cancer patients with the current therapeutic approaches and the fact that ammonia-scavenging drugs are well tolerated by cancer patients, clinical trials should be initiated including these drugs in therapeutic regimens for liver cancer.

Very recently, the use of ammonia-scavenging drugs has been extended to other diseases. Maple syrup urine disease (MSUD), a classical inborn error of amino acid metabolism, is caused by deficiency of the mitochondrial branched-chain keto acid dehydrogenase complex, resulting in an accumulation of BCAAs (isoleucine, leucine and valine) and their corresponding branched-chain α -keto acids in tissues and plasma [106]. As previously mentioned, therapy with sodium phenylbutyrate in UCDs has been associated with a selective reduction in BCAAs in spite of adequate dietary protein intake [102], an adverse effect that could be exploited for the treatment of MSUD. Indeed, a very recent study has shown that PB treatment is able to reduce the plasma levels of neurotoxic BCAAs and their corresponding α -keto acids in a subset of MSUD patients [107]. Another ongoing clinical trial is exploring the role of glycerol phenylbutyrate in cystic fibrosis therapy based on early findings highlighting the capacity of sodium phenylbutyrate to restore the function of the cystic fibrosis transmembrane conductance regulator both *in vitro* and *in vivo* [108].

Nowadays, the optimization and development of novel pharmacological derivatives of benzoate and PA/PB is also a hot topic in research. In relation to this, a novel analogue of PB, the glycerol phenylbutyrate, has been highly recommended in recent years to replace sodium phenylbutyrate, at least for the treatment of hypertensive cirrhotic patients, in whom the high sodium content after ingestion of sodium phenylbutyrate has highly adverse effects. Moreover, glycerol phenylbutyrate is well tolerated, producing fewer gastrointestinal complications than sodium benzoate or sodium phenylbutyrate. Another PA derivative, L-ornithine phenylacetate, is currently being used in clinical trials for both ALF and cirrhosis. In addition, due to the palatability issues associated with sodium phenylbutyrate, in recent years, the scientific community has been interested in the development of taste-masked granules of this pediatric drug which will be very useful to improve treatment adherence [109]. To date, two clinical trials have shown that the recently developed taste-masked formulation of sodium phenylbutyrate granules improves quality of life in patients with UCDs [110, 111].

Some of the early applications of ammonia-scavenging drugs as non-invasive probes of liver intermediary metabolism further suggest their potential use to non-invasively analyze liver glutamine metabolism. This is very interesting considering the potential role of altered glutamine metabolism in liver disease progression and in cancer development and aggressiveness as well as response to chemotherapy. An understanding of changes of glutamine metabolism underlying liver disease is important as a prognostic tool and to monitor therapeutic and lifestyle interventions.

To summarize, even though the ammonia-scavenging drugs benzoate and PA/PB have been used over the last 40 years for the treatment of UCDs, in the last decade there has been a surge in interest in these drugs for new applications, especially in the field of liver disease and other conditions, such as MSUD and cystic fibrosis. In addition, there is renewed interest in the potential application of these drugs as antitumor agents, especially in glutamine-addicted liver cancer cells, after recent pharmacodynamic studies, which can partially explain the disappointing results in the treatment of glioblastoma in early clinical trials. On the other hand, taking advantage of the urinary excreted conjugation products of benzoate and PA/PB that reflect liver metabolism, we believe that novel non-invasive diagnostic methods may emerge in the near future to monitor liver glutamine metabolism. Finally, the greater scientific efforts in recent years have led to the identification of novel ammonia-scavenging derivatives that show improved actions, exhibiting overall fewer and less severe adverse effects.

Acknowledgments

Funding

This work was supported by grants from NIH (US Department of Health and Human services)- CA172086, MINECO:SAF2014-54658-R integrado en el Plan Estatal de Investigación Científica y Técnica y Innovación 2013–2016 cofinanciado con Fondos FEDER to M.L.M.-C, EITB BIO15/CA/014 (M.L.M.-C.) y Asociación Española contra el Cáncer (M.L.M.-C. and T.C.D.), Ciberehd_ISCIII_MINECO is funded by the Instituto de Salud Carlos III.

This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Article highlights box

- Ammonia-scavenging drugs target liver nitrogen metabolism in urea cycle disorders by offering an alternative pathway for nitrogen disposal through the urinary excretion of hippurate and phenylacetylglutamine.
- Treatment with ammonia-scavenging drugs has been shown to reduce hepatic encephalopathy events by lowering blood ammonia both in cirrhosis and in mouse models of acute liver failure.
- Phenylacetate and phenylbutyrate-based treatment provides a means for hepatic glutamine depletion in liver cancer highlighting its therapeutic potential to target glutamine-addicted cancer cells.
- Phenylacetate and phenylbutyrate-based therapy is able to selectively reduce branched-chain amino acids and is currently being used in clinical trials on patients with maple syrup urine disease, a classical inborn error of amino acid metabolism.
- Benzoate, phenylacetate and phenylbutyrate and their excretable urinary by-products, hippurate and phenylacetylglutamine can be used to non-invasively assess intermediary metabolism of carbohydrate in the liver and potentially in vivo liver glutamine metabolism.
- There is currently great interest in the development of more efficient ammonia-scavenging pharmacological derivatives with fewer side effects and greater therapeutic potential.

Figure 1. Hepatic nitrogen metabolism

A tight metabolic regulation of glutamine catabolism, gluconeogenesis, folate/methionine cycles and transsulfuration pathway, and the urea and tricarboxylic acid (TCA) cycle is crucial for glutamine and nitrogen homeostasis. The pathway involved in the conjugation of phenylbutyrate (PA), a product of phenylbutyrate (PB) metabolism, to glutamine originating phenylacetylglutamine (PAGN) as well as the conjugation of benzoate to L-glycine is shown.

Figure 2. Expression of the high activity isoenzyme of glutaminase, GLS1, in liver tumors detected by immunohistochemical analysis

Prohibitin-1 (*Phb1*) and Glycine N-methyltransferase (*Gnmt*) deficient mouse models were compared to their wild-type littermates.