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The Rise of Glutaminase in End-Stage Liver Diseases

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1. Introduction

Ammonia plays a major role in the pathogenesis of hepatic encephalopathy (HE). Systemic hyperammonemia has been largely found in patients with HE with underlying cirrhosis and acute liver failure. HE is associated with a poor prognosis of both acute and chronic liver disease (Bustamante, Rimola et al. 1999; Hui, Chan et al. 2002). Cirrhosis is the major cause of chronic liver dysfunction and affects 6.5 million people worldwide (Eurostat. 2010, as cited in Albrecht, 2010). HE is the hallmark for acute liver failure (ALF), a syndrome with very high mortality in which liver transplant is the often the only effective treatment.

The HE pathophysiological mechanisms include alterations of blood brain barrier function cytokine production, ammonia-induced changes in neurotransmitter synthesis and release, neuronal oxidative stress, impaired mitochondrial function and osmotic disturbances resulting from astrocytic metabolism. However, none of the metabolic discoveries have yet lead to a therapy which improves prognosis. In the clinic, liver transplantation remains the only curative therapeutic option. Cytotoxic brain edema and intracranial hypertension occurring in encephalopathic ALF patients account for a large number of deaths owing to cerebral herniation. It has been shown that in chronic liver failure there is a low grade brain edema (Andrade, Lucena et al. 2005) that is resolved after transplantation. In comatose patients, moderate hypothermia using cooling blankets to depress energy consumption in the brain seems to be the only relatively effective palliative therapy, but it is expensive, difficult to implement, and not routinely available (Stravitz, Lee et al. 2008; Albrecht 2010).

Studies focusing on inter-organ ammonia metabolism in patients with cirrhosis indicate that the liver, muscles, kidney and the small bowel are important in regulating the circulating levels of ammonia. Historically it was thought that the majority of ammonia was produced by gut bacteria, and treatment regimens including non-absorbable antibiotics and enemas have been extensively used. Contrary to popular belief, it has now been shown that at least 50-60% of total gut ammonia is derived from uptake of glutamine, which is metabolized to glutamate and ammonia by the enzyme glutaminase (GA) (Olde Damink, Jalan et al. 2002; Romero-Gomez, Ramos-Guerrero et al. 2004). Ammonia that would normally be converted

to urea by a healthy liver increases to toxic levels. In this situation, the enzyme glutamine synthetase (GS) plays a pivotal role in ammonia detoxification, effectively removing ammonia during the conversion of glutamate to glutamine (Rose, Michalak et al. 1999). Glutamine deamidation by intestinal GA seems to be the main source of ammonia in patients with cirrhosis (Olde Damink, Jalan et al. 2002), and hyperammonaemia and hepatic encephalopathy can appear without the participation of gut bacteria (Weber and Veach 1979).

The following data support the hypothesis that a genetic factor is implicated in the development of overt hepatic encephalopathy: Glutaminase activity has been linked to hepatic encephalopathy and ammonia production; 40% of persons with cirrhosis and minimal hepatic encephalopathy do not develop overt hepatic encephalopathy in long-term follow-up (Romero-Gomez, Boza et al. 2001); patients with cirrhosis who have the same degree of liver dysfunction and the same precipitating factor (for example, variceal bleeding) may or may not develop overt hepatic encephalopathy; and at least 2 different polymorphisms in the promoter region of the glutaminase gene influence protein activity by increasing or decreasing glutaminase activity (Taylor L 2001).

In this chapter we analyze the studies supporting the inhibition of glutaminase based in the identification of mutations in the glutaminase gene to facilitate selection of patients for close monitoring and evaluation for expedited transplantation. Firstly, we have identified a variant in the promoter region of the glutaminase gene that increases glutaminase activity and is associated with the development of HE. Following a simple blood test to identify these patients with the variant, it would be possible to offer a treatment with glutaminase inhibitors. Based on these and another studies we have developed a new molecule, THDP17 that inhibits glutaminase in CACO-2 cell cultures (intestinal cells).

An alternative treatment to HE is currently being investigated: Ornithine phenylacetate (OP). OP is a novel drug that is targeted at reducing ammonia concentration in patients with liver disease and therefore a potential treatment for HE (Jalan, Wright et al. 2007). The mechanism by which OP directly reduces ammonia levels in cirrhosis is by normalization of gut glutaminase activity and concomitant increasing muscle glutamine synthesis activity, subsequently trapping the increased glutamine with phenylacetate,and increasing ammonia excretion as phenylacetylglutamine in the urine.

These studies support glutaminase such as focus for new treatments of HE and the identification of a genetic variation greatly facilitates the selection of patients for close monitoring and evaluation for expedited transplantation.

2. Glutaminase genetic study

It has been described that there are two forms of mitochondrial glutaminase in the body, liver type glutaminase (L-GA) and kidney type glutaminase (K-GA) or extrahepatic (located in other organs such as the intestine). In humans, increased glutaminase activity is localized to the duodenum. There are indirect data suggesting that glutaminase activity in the enterocyte is increased in cirrhotic patients, as demonstrated by the fact that after oral administration of glutamine there is a rapid increase in blood ammonia level in cirrhotic individuals but not in healthy controls (Romero-Gomez, Grande et al. 2002).

Hyperammonemia is noticably marked in patients with liver cirrhosis with poor liver function, but this increase in ammonia production following a glutamine challenge returns to normal after liver transplantation and normalization of liver function. The specific activity of glutaminase in the enterocyte is a crucial point in the stability of nitrogen metabolism in patients with liver cirrhosis. It has been shown that glutaminase activity is increased in cirrhotic subjects compared to controls and that this activity is related to the presence of encephalopathy and the degree of hepatic dysfunction (Romero-Gomez, Ramos-Guerrero et al. 2004). Thus, also the accumulation of glutamine in the astrocyte is responsible, in large part on the toxicity induced by ammonia (Albrecht and Norenberg 2006).

The cDNA of the human renal-type glutaminase (HK-GA) was cloned in 1998 and subsequently further cDNAs have been isolated encoding for three isoforms of HK-GA, which were designated as K-GA (which is predominantly expressed in kidney, intestine and brain but not liver), M-GA (which is expressed only in cardiac and skeletal muscle), and C-GA (which is expressed primarily in cardiac muscle and pancreas but not in brain or liver). The K-GA isoform is localized to the kidney and has 669 amino acids, C-GA, a protein of 598 amino acids and differs from the K-GA in the carboxyl terminus and the M-GA is a protein of 169 amino acids, which is identical to C-GAP up to amino acid 161 only varying for the c-terminal 8residues. After sequencing the genomic DNA, it was proposed that the three isoforms were the product of alternative splicings of the same gene. Recently described two haplotypes of this gene: TACG and CACG, which place a lower activity of glutaminase, which results in lower intestinal production of ammonia and improved liver function and lower risk of developing hepatic encephalopathy (Romero-Gomez 2005).

We assessed whether alterations in the glutaminase gene could explain, at least in part, the risk for overt hepatic encephalopathy in patients with cirrhosis. We have described a variation in the promoter region of the glutaminase gene that is associated with development of HE in patients with cirrhosis (Romero-Gomez, Jover et al. 2010).

This study included subjects from outpatient clinics from 6 Spanish hospitals: 109 consecutive patients with cirrhosis in the estimation cohort, 177 patients in the validation cohort, and 107 healthy control participants. Patients were followed every 3 or 6 months until the development of hepatic encephalopathy or liver transplantation, death, or the end of the study.

The genetic analyses showed that glutaminase TACC and CACC haplotypes were linked to the risk for overt hepatic encephalopathy. Mutation scanning of the glutaminase gene identified a section in the promoter region where base pairs were repeated (a microsatellite). Over a mean follow-up of 29.6 months, hepatic encephalopathy occurred in 28 patients (25.7%) in the estimation cohort. Multivariable Cox models were used to determine the following independent predictors: Child–Turcotte–Pugh stage (hazard ratio [HR], 1.6 [95% CI, 1.29 to 1.98]; P=0.001), minimal hepatic encephalopathy (HR, 3.17 [CI, 1.42 to 7.09]; P=0.006), and having 2 long alleles of the microsatellite (HR, 3.12 [CI, 1.39 to 7.02]; P=0.006). The association between 2 long alleles of the microsatellite and overt hepatic encephalopathy was confirmed in a validation cohort (HR, 2.1 [CI, 1.17 to 3.79]; P=0.012). Functional studies showed higher luciferase activity in cells transfected with the long form of the microsatellite, which suggests that the long microsatellite enhances glutaminase

transcriptional activity. In Figure 1 is shown that patients with long microsatellite showed higher risk of overt hepatic encephalopathy.

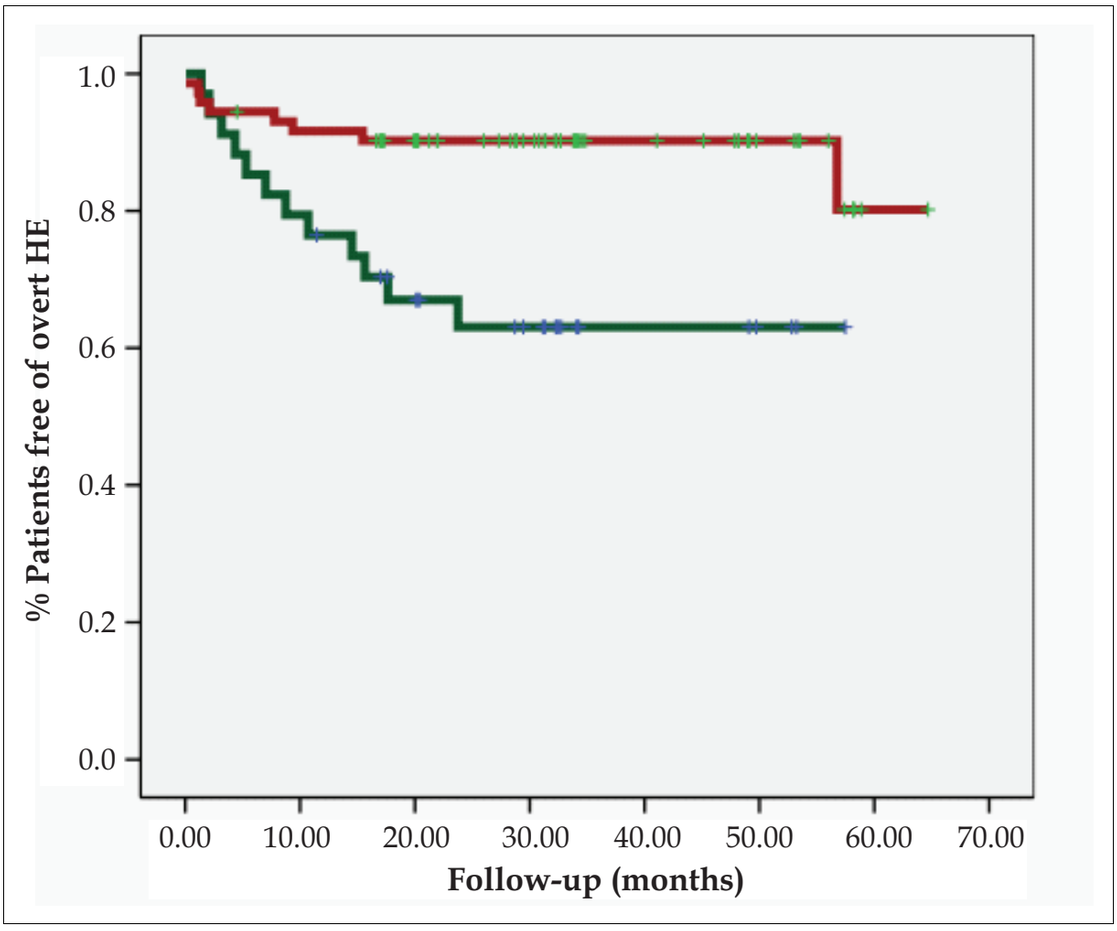

Fig. 1. Actuarial curve showing patients free of bouts of overt hepatic encephalopathy according to the microsatellite in the promoter region. Patients with long microsatellite showed higher risk of overt hepatic encephalopathy (log-rank: 7.74; $p<0.01$)

3. Effects of Ornithinephenylacetate on glutaminase activity in bile duct rats: Inflammation and ammonia

In cirrhotic patients it has been shown that the effects of hyperammonemia are synergistic with inflammation (Shawcross, Davies et al. 2004). The effects on cell swelling by cytokines in ammonia-sensitized cultured astrocytes has also been shown (Rama Rao, Jayakumar et al. 2010). However, the mechanisms by which ammonia produces brain swelling are still subject of much investigation. Although the effects on inflammatory processes have been found to contribute to the formation of cerebral edema, it is not clear whether ammonia promotes inflammation or both are independent factors. Inflammatory pathways identified as contributing to the edema include cyclo-oxygenase, nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling and cytokine release (Andrade, Lucena et al. 2005; Montoliu, Piedrafita et al. 2009; Montoliu, Rodrigo et al. 2010).

Hyperammonemia could increase blood-brain-barrier permeability to systemic cytokines. It is also possible that several factors associated with the systemic inflammatory response syndrome could modulate brain dysfunction induced by hyperammonaemia. These processes, together with a genetic factor, may help to explain the differences that sometimes exist between lower ammonia levels and observed brain impairment in some patients. It has been shown that the presence of HE grade 3/4 correlates better with inflammation than with ammonia plasma levels (Shawcross, Sharifi et al. 2010), though extracellular brain ammonia levels may be significantly higher. One recent study showed that in a cirrhosis animal model in which plasma and brain cytokines were markedly elevated following administration of lipopolysaccharide (LPS), pre-treatment with OP prevented increased levels of TNFα and IL-6 (trend) in plasma and in brain observed in the control group (Wright G 2010). Moreover, OP reduced LPS induced development of pre-coma/coma and worsening of brain edema. It is well-known that the transcription of NFkB directly increases pro-inflammatory cytokines and leads to induction of nitric oxide synthase. (Li and Verma 2002). OP reduced iNOS and NFkB expressions in the cortical brain region of cirrhotic animals, indicating that ammonia reduction may modulate neuroinflammation. In cirrhosis a paradox exists between reduced intrahepatic NO generation and excess NO in the splanchnic circulation. Splanchnic vasodilatation leads to vasoconstriction of numerous vascular beds, including the liver, kidneys, and has significant effects on the brain. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of eNOS (endothelial nitric oxide synthase), the levels of which are increased in liver failure (Leiper, Nandi et al. 2007; Mookerjee, Malaki et al. 2007). It has been shown that treatment of cirrhotic rats with OP resulted in restoration of the NOS pathways which may have a direct effect on cerebral perfusion (Balasubramaniyan, Wright et al. 2011).

Physical symptoms of HE and minimal HE have been detected by motor-evoked potentials (MEP) which examines the function of signal transmission along the nerve, which is perturbed by low grade brain edema. Similar disturbances have been found in patients with cirrhosis using magnetic resonance (MR), with signs compatible with low-grade edema along the corticospinal tract. These abnormalities were related to functional impairment detected by transcranial magnetic stimulation and were found to be reversed after liver transplantation. Recently the assessment of MEP in awake rats has been validated to monitor HE in animal models of liver failure (induced by portocaval anastomosis-PCA) and precipitated HE (simulated gastrointestinal bleed-GB). These models have been utilized to test the efficacy of OP, demonstrating that OP treatment prevents the neurophysiological abnormalities induced by a GB insult in PCA animals. Administration of OP over differing time periods (between 3 hours and 3 days) as a pretreatment prevents the decrease in the amplitude and increase in MEP latency at 6 hours post GB (Oria M, Romero-Gimenez J et al. 2011).

In preliminary studies, it has been shown that the combination of ornithine with phenylacetate to treat hyperammonaemia in cirrhosis is effective in animal models. Administration of OP results in increased conversion of glutamate to glutamine by stimulation of GS activity in the muscle with the subsequent excretion of phenylacetylglutamine in the urine. GA has been found to contribute to hyperammonaemia in cirrhosis and in hyperammonaemia animal models (Romero-Gomez, Grande et al. 2004; Romero-Gomez, Jover et al. 2006).

In this novel approach to target the altered interorgan ammonia metabolism in liver failure, OP utilizes the activity of GS to trap ammonia as glutamine, then phenylacetate facilitates its excretion as phenylacetylglutamine (Davies, Wright et al. 2009; Ytrebo, Kristiansen et al. 2009). The effectiveness of this approach with OP has been confirmed in animal models of cirrhosis and ALF. The reduction (≈50%) of plasma ammonia was associated with (a) an improvement in grade of HE in cirrhotic patients and (b) a reduction in ICP in acute liver failure. OP treatment significantly reduced ammonia concentrations, which was associated with a reduction in brain water and an increase in myoinositol levels, indicating an improvement in brain metabolism (Jalan, Wright et al. 2007; Davies 2009; Ytrebo LM 2009).

In a devascularised pig model of ALF, the rise in arterial ammonia was attenuated with OP which was accompanied by a significant decrease in extracellular brain ammonia and prevention of intracranial hypertension (Ytrebo, Sen et al. 2006).

In our studies we included twenty-five male Sprague-Dawley rats: 4 sham operated, and 21 BDL. 5 BDL's received OP (5 days, IP 0.6 g/kg), 5 BDL's received ornithine (5 days, IP 0.6 g/kg), 5 BDL's received phenylacetate (5 days, IP 0.6 g/kg) and 6 received saline (IP). We measured plasma levels for: ammonia and standard biochemical markers. Expressions of GS, GA and ornithine amino transferase (OAT) were determined by Western-blot (expressed as a % of sham values) and enzyme activity was determined by end-point methods in liver, kidney, gut, muscle and lung. We found that plasma ammonia was decreased in BDL-OP rats vs. BDL-saline (58.97±6.02 vs. 106.2±20.56 μmol/L;$P<0.05$). BDL-OP rats showed increased GS expression in liver (66% BDL-OP vs. 55% BDL-saline; $P < 0.01$) and showed further increased levels in the muscle (153% BDL-OP vs.142% BDL-saline). OP ameliorates the BDL related increases in glutaminase expression (124%vs.163%; $P<0.05$) and activity (0.45±0.16mIU/mg protein BDL-OP vs. 1.14±0.046mIU/mg protein BDL-saline; $P<0.01$) in gut. We demonstrated that this prevention is due to effect of ornithine in glutaminase activity (0.46±0.17mIU/mg protein BDL-O vs. BDL-saline; $P<0.05$) and not to phenylacetate. OP treatment in BDL rats increased the conversion of glutamate to glutamine by stimulation of GS in the muscle and also resulted in normalization of glutaminase expression and activity in the gut, indicating that OP effectively restricts the production of *in vivo* ammonia in a cirrhotic. Mechanism of action of OP on the metabolism of ammonia is shown in the Figure 2.

4. THDP-17: Glutaminase inhibitor in CACO-2 cultures

It has been described as a glutaminase inhibitors Mersalyl, *N*-ethylmaleimide and 6-diazo-5-oxo-L-norleucine (DON). DON has been used in the inhibition of glutaminase in cell cultures of astrocytes in studies demonstrating the importance of GA activity in cell damage induced by ammonia. Also, in rats subjected to portocaval shunt, neomycin inhibits intestinal glutaminase activity (Hawkins, Jessy et al. 1994), although the mechanisms by which neomycin may inhibit glutaminase activity are not described. GA activity is increased in patients with high levels of nitric oxide, glucagon, or tumor necrosis factor, and in a wide number of cancer types as in liver, breast and leukemia as glutamine is implied as a factor in in cell proliferation (Perez-Gomez C 2005; Gao P 2009).

So based on the above and in the previous studies of assosiation of the presence of long-long microsatellite in the glutaminase gene and the mechanism of action of OP in gut

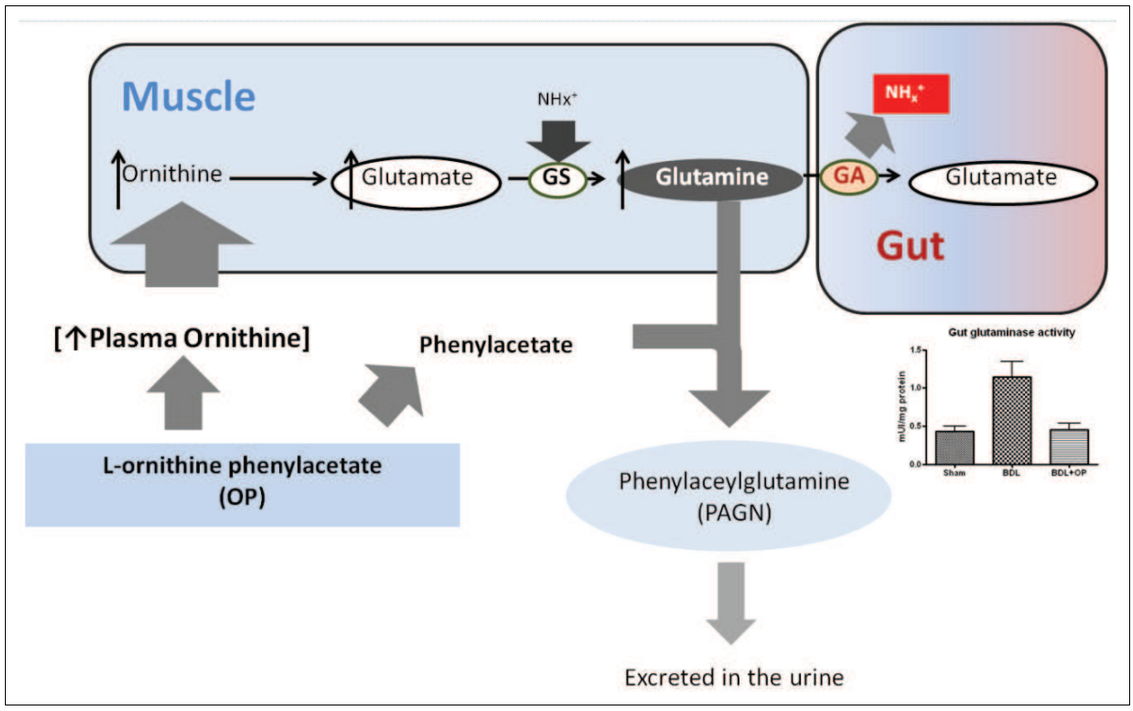

Fig. 2. Mechanism of action of OP in glutamine synthetase (GS) and glutaminase (GA) enzymes. GS is stimulated in muscle by glutamate increased levels. At the same time PAGN is formed and excreted in the urine. In adition, GA is restored to normal levels in the gut

glutaminase, the inhibitition of glutaminase activity is a therapeutic target in the management of hepatic encephalopathy. It is very important to investigate new molecules for the purpose of decreasing the intestinal production of ammonia, since it determines a high risk of encephalopathy and decreased survival. However, when developing these inhibitory molecules is necessary to note that complete inhibition of the enzymatic activity of glutaminase could severely damage the normal function of the enterocytes, so that molecules inhibiting the activity of glutaminase should cause a reduction in activity ie, a partial inhibition of enzyme activity, without significantly affecting the functionality of the enterocyte.

Colon carcinoma cell cultures (CACO2) have been used to test this compound. 50,000 cells per well were used in a 12-well plate with 1.2ml DMEM medium supplemented with 2mM L-Glutamine, 15% FBS, 1X antibiotic/antifungal solution, 1X non-essential amino acids (PAA Laboratories GmbH, Linz, Germany). Plates were incubated at 37°C and 5% CO_2 for 24 and 48h. THDP-17 and 6-diazo-5-oxo-norleucina (DON) (Sigma, St. Louis, EE.UU.) were assayed in duplicates at 0, 5, 20 and 100µM. The glutaminase activity was assayed using the protocol described by Heini (Heini Hans G. 1987).

Using both THDP-17 and DON (positive control) 100µM, glutaminase activity was inhibited after 48h. The product THDP-17 reduces 42% of the initial glutaminase activity, while DON reduces 46% of the initial activity. Therefore THDP-17 is considered to have potential to act as a therapeutic option for the hepatic encephalopathy as a glutaminase partial inhibitor that is able to cross the cellular and mitochondrial membranes. Further studies to evaluate toxicity and *in vivo* experiments to as certain efficacy need to be done in the future. In Figure

3 it can be seen that glutaminase activity is inhibited at 48 hours with differing concentrations of THDP-17 and DON (0,5,20 and 100μM).

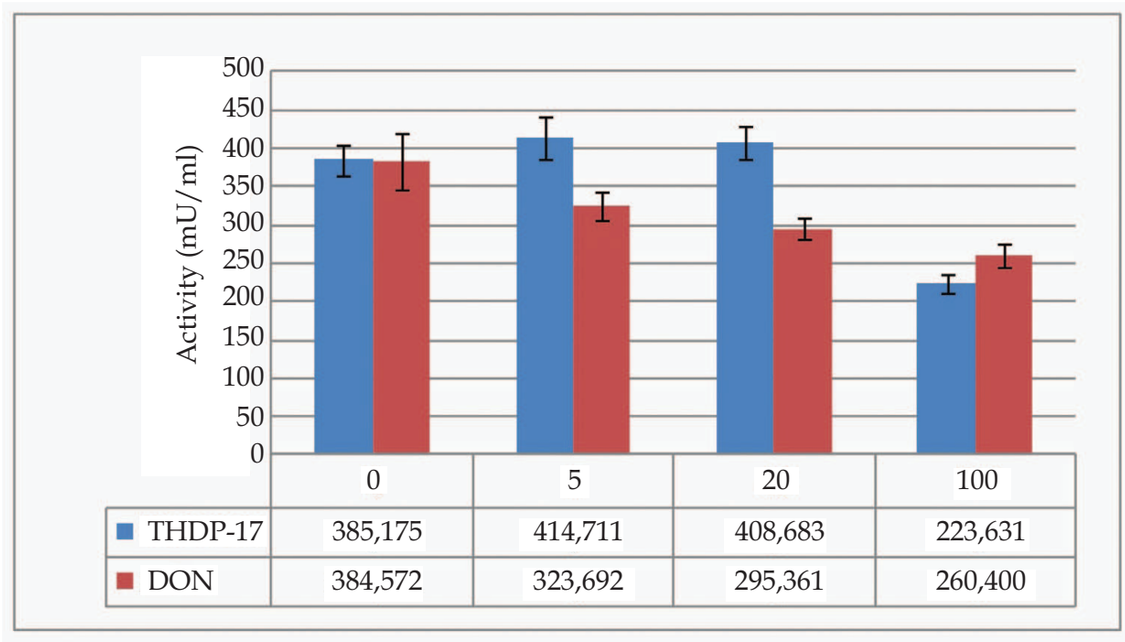

Fig. 3. Inhibition of GA activity following 48 hrs incubation with either THDP 17 or DON in Caco-2 cells

5. Future research

Glutaminase plays a major role in the cause of hepatic encephalopathy. However, a prospective study is required to evaluate the clinical utility of the genetic marker we have identified for predicting overt hepatic encephalopathy before it can be recommended for clinical practice.

In the gut, intestinal glutaminase activity is increased in patients with cirrhosis and correlates with minimal hepatic encephalopathy (Romero-Gomez, Ramos-Guerrero et al. 2004). Glutaminase is also a key factor in the brain. Glutamine synthesis detoxifies ammonia in the brain, but glutaminase transforms glutamine into ammonia, glutamate, and free radicals in the mitochondria, and these byproducts are implicated in mitochondria dysfunction and further neurotransmission impairment (Trojan horse hypothesis) (Albrecht and Norenberg 2006).

Studies to date have indicated that OP is safe and patient studies in minimal HE and HE are needed to establish whether OP or glutaminase inhibitors such TPHD-17 (a promising GA inhibitor) could be used as a treatment for this significant complication of liver disease.

6. Conclusion

We identified a microsatellite in the promoter region of the glutaminase gene that is linked to the development of overt hepatic encephalopathy in patients with cirrhosis. This

promoter is associated with an increase in enzyme activity when the long allele is present. This genetic marker might help identify patients at risk for overt hepatic encephalopathy so that they could be more carefully monitored and could receive intensive treatment and/or define priority in liver transplant waiting list. However, additional studies are needed before this biomarker can be recommended for use in clinical practice.

Our studies support the role of glutaminase in HE and the use of OP, a novel treatment in developing for HE in reducing plasma ammonia. The mechanism by which OP directly reduces ammonia levels in cirrhosis is by increasing muscle GS activity, subsequently trapping and increasing ammonia excretion as phenylacetylglutamine, with the concomitant normalization of gut GA activity. The reduction on ammonia (by OP) leads to a reduction in ICP in ALF and is associated with an improvement in inflammation in the context of chronic liver disease. Moreover, OP modulates iNOS and NFkB mechanisms and prevents LPS-induced brain edema in cirrhotic rats.

And finally, we present a new molecule, THDP-17 as a new therapeutic option for the treatment of hepatic encephalopathy as a GA partial inhibitor that is able to cross the cellular and mitochondrial membranes.

In summary, we believe that further investigation of GA is warranted, because increased knowledge of the pathways involved might lead to the uncovering of new drug targets (such as OP and THDP-17) and other treatments for hepatic encephalopathy. These findings suggest developing approaches to target GA to prevent ammonia release and subsequent HE as a valid therapeutic strategy in the management of patients with liver disease.

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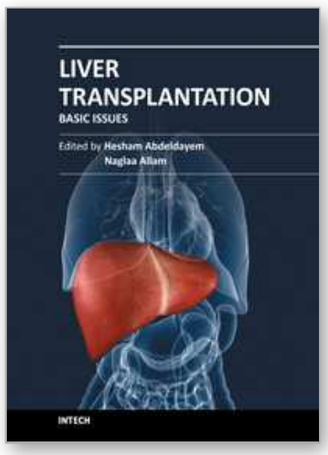

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This book covers a wide spectrum of topics including history of liver transplantation, ischemia-reperfusion injury, immunology of liver transplantation, viral hepatitis and liver transplantation, other indications for liver transplantation, prognostic factors and perioperative period. The authors of the chapters are experts in their respective fields. They are proponents covering different aspects of liver transplantation and come from many centers across the world. The interdisciplinary approach and the authority of the contributors resulted in a valuable reference to anyone interested in developing a global view in liver transplantation including medical students, residents, fellows, nurses, and practicing physicians and surgeons as well as researchers in the field of liver transplantation.

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