Clinical Validation of Blood/Brain Glutamate Grabbing in Acute Ischemic Stroke

Andrés da Silva-Candal,[1] Amparo Pérez,[2] María Santamaría,[1] Clara Correa-Paz,[1] Manuel Rodríguez-Yáñez,[1] Ana Ardá[3], María Pérez-Mato,[1] José Brea,[2] Eddy Sotelo,[4] Tomás Sobrino,[1] Mabel Loza,[2] José Castillo,[1]* Francisco Campos.[1]*

Affiliations:

[1]Clinical Neurosciences Research Laboratory (LINC), Department of Neurology, Clinical University Hospital, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain.

[2]Drug Screening Platform/Biofarma Research Group, Centro Singular de Investigación en Medicina Molecular y Enfermedades Crónicas (CIMUS), Universidade de Santiago de Compostela, Santiago de Compostela, Spain.

[3]Molecular Recognition & Host−Pathogen Interactions Unit, CIC bioGUNE, Bizkaia Technology Park, Building 801A, Derio, Spain.

[4]Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CIQUS). Departamento de Química Orgánica, Facultade de Farmacia, Universidade de Santiago de Compostela, Santiago de Compostela, Spain

Mailing address:

Prof. José Castillo and Dr. Francisco Campos
Clinical Neurosciences Research Laboratory
Hospital Clínico Universitario
Travesa da Choupana s/n
15706 Santiago de Compostela
Spain
E-mail: jose.castillo.sanchez@sergas.es, francisco.campos.perez@sergas.es

One Sentence Summary: glutamate grabbers on blood glutamate reduction in stroke patents.

ABSTRACT:

In the search of pharmacologic treatments for acute ischemic stroke, hundreds of experimental protective strategies with successful effect have been reported, but none of the neuroprotective

agents studied resulted in improved outcome for patients. Repositioning of drugs already known to be safe provides a lower risk alternative. This strategy allows researchers to search drugs already approved becoming a promising alternative to traditional approaches for drug development against stroke. The concept of blood/brain glutamate grabbing or scavenging is well recognized as a novel and attractive protective strategy to reduce the excitotoxic effect of glutamate overload in the brain after an ischemic damage. However, the translation of glutamate grabber drugs to clinical practice has not been tested so far. In this translational study, a high throughput screening (HTS) was performed in 1120 compounds regarding their glutamate grabbing capacity. Riboflavin was identified as the main hit and tested in healthy animals, showing a blood glutamate reduction after administration. Ischemic animal models treated with riboflavin (1 mg/kg) confirmed that blood glutamate reduction was associated with a significant reduction of infarct size. A final proof of concept clinical trial with 25 control (placebo) and 25 riboflavin (20mg) treated stroke patients confirmed the reduction in the blood glutamate levels and a trend to functional recovery the compared with placebo group. This translational study, represents the first human demonstration of the efficacy of glutamate grabbers on blood glutamate reduction in stroke patents, hoping can help to contribute to the clinical development of this novel protective therapy.

INTRODUCTION

Ischemic stroke is a neurologic disease caused by a sharp decline of the blood flow leading to cell death and brain damage. Currently, stroke is the one of the most important causes of

mortality and morbidity in developed countries, a situation that is especially worrying with the increasing incidence of the progressive aging of the population. Nowadays, pharmacological or mechanical reperfusion therapies are the unique effective treatment during the acute phase of ischemic stroke, associated with good outcome in 40–60% of cases.[1] However, these treatments are only applicable to <10% of patients in developed countries due to the narrow therapeutic window and because requires specialized hospital staff not always available in all centers.[1-3]

After ischemic stroke, there is a rapid elevation of glutamate into the extracellular space followed by a marked increase in intracellular calcium, which provokes a neuronal death through an excitotoxicity mechanism.[4] Consequently, calcium and glutamate antagonists have been widely studied as protective agents in experimental models of cerebral ischemia with encouraging results. Unfortunately, they failed or displayed severe adverse effects when they were tested in clinical trials. Nevertheless, because of the central role of glutamate in the ischemic cascade, the mitigation of glutamate excitotoxicity remains one of the most promising strategies for the development of effective treatments to minimize neurological damage following an acute ischemic stroke. [5]

Nowadays, the concept of blood/brain glutamate grabbing or scavenging is well recognized as a novel and attractive protective strategy to reduce the excitotoxic effect of excess extracellular glutamate that accumulates in the brain after an ischemic damage.[6, 7]

The blood/brain glutamate grabbing mechanism is based on the lowering of blood glutamate concentration that leads to a larger natural glutamate gradient between the brain and blood, facilitating the efflux of excess extracellular brain glutamate into the blood. Reduction of glutamate concentration in blood is induced through the activity of the blood-resident enzyme, glutamate oxaloacetate transaminase 1 (GOT1), which catalyzes the reversible transformation of oxaloacetate (Oxal) and glutamate to aspartate and α-ketoglutarate. Thus, the administration of both Oxal and/or recombinant GOT1 (rGOT1) in ischemic animal models leads to a metabolization and reduction of glutamate in blood and lowering of glutamate in the cerebral parenchyma, which is associated with a reduction of the ischemic lesion and sensorimotor recovery.[6, 8-11]

The protective efficacy of this strategy has been widely shown by independent laboratories in different types of ischemic animal models,[8, 11-17] and it has also been tested in other pathologic models associated with increase of glutamate in brain, such as traumatic brain injury,[18, 19] subarachnoid hemorrhage,[20, 21] glioma,[22] or amyotrophic lateral sclerosis[23] with successful results. In a previous retrospective studies with >400 stroke patients, we have also found that those patients with good outcome at 3 months after stroke, showed higher GOT activity (>18

U/l) in serum and lower glutamate concentration respect to those with poor outcome,[24, 25] that represented the first clinical evidence of the protective effect of this therapeutic mechanism.

The main advantage of this novel therapeutic strategy is that occurs in the blood circulation and therefore does not affect the normal brain neurophysiology, as it has been described for other drug treatments used against glutamate excitotoxicity; and more importantly, contrary to thrombolytic therapy (rtPA), they can be given immediately in cases of suspected ischemic stroke without previous neuroimaging needed during the diagnostic of ischemic and hemorrhagic strokes.[6, 7]

Despite of all preclinical studies and the clinical observational evidences that supports the therapeutic efficacy of the reduction of blood glutamate; the translation of glutamate grabber drugs to clinical practice has critical steps before their use in humans. In case of Oxal, this molecule acts as a potent inhibitor of the Succinate dehydrogenase (SDH), (as well-known as Complex II), an enzyme that participates in both the citric acid cycle and the electron transport chain,[26] limiting its therapeutic applicability in humans.

The use of a recombinant enzyme as rGOT for humans involves also a high financial support and risk of investment for the sponsors that limit its translational application. In addition, the repeated failure of protective drugs against glutamate excitotoxicity in clinical trials has reduced the trust of pharmaceutical companies and other sponsors to support new drugs for acute phase of stroke.

Aiming to demonstrate the clinical efficacy of blood glutamate grabbers in stroke patients, in this study we proposed to use a pharmacological strategy known as drug repositioning based on the high-throughput screening (HTS) of chemical library with the aim to find new glutamate grabbing drugs. This strategy allows researchers to search drugs already approved for FDA, EMA or other agencies, with a well-known bioavailability and safety for humans which can be immediately used in patients, reducing significantly the cost and risk of investment.[27-29]

Following this pharmacological strategy, in this study, more than one thousand known drugs were tested based on their capability to interact on GOT reaction then, the protective efficacy of the selected drug to reduce blood glutamate was probed in an ischemic animal model and finally tested in a proof of concept clinical trial with stroke patients.

RESULTS

High-throughput screening of a new blood glutamate grabber.

The 1120 chemically and pharmacologically diverse compounds (ca. 90% being FDA approved drugs) from the Prestwick Chemical Library (PCL)[30] were evaluated in an HTS assay to assess their capability to interact on GOT activity. GOT activity was measured indirectly through the inhibition of glutamate formation by means of a commercial assay kit. In this activity assay, an amino group is transferred of from aspartate to α-ketoglutarate. The products of this reversible transamination reaction are Oxal and glutamate, and the glutamate is detected in a reaction that concomitantly converts a nearly colorless probe to color. Therefore, a reduction on glutamate formation will be detected for those compounds able to interfere on GOT activity. In addition, only those compounds that showed a percentage of inhibition higher than the average plus three standard deviations (>28.2%) were considered as hits. A total of 6 compounds met the requirements for inhibition of the glutamate formation as it is showed in the Fig. 1A. The compounds selected were the following: apomorphine, dobutamine, doxycycline, methyldopa, nifedipine and riboflavin.

To validate these results, same experiments were repeated with these selected hits (data no shown). Finally, to confirm the specificity of the selected hits on GOT activity, the 6 compounds were commercially obtained and a dose-response study on GOT activity was performed. Five of the 6 hits tested presented a sigmoidal curve (Fig. 1B). The IC_{50} calculated for these 5 hits (Fig. 1 C) showed that the compound with lower IC_{50} value (1.96) correspond to riboflavin (as well-known as vitamin B_2). IC_{50} of the other 5 hits are indicted in the Fig. 1C.

Analysis of riboflavin on succinate dehydrogenase activity

One of the most important limitation of Oxal for human use was that this molecule acts an inhibitor of the SDH. Although the 6 selected hits are drugs already used in humans, it was evaluated if any of them could interfere with the activity of this enzyme. For that, a dose-response study was performed on SDH activity with the 6 commercial compounds identified (apomorphine, nifedipine, methyldopa, dobutamine, riboflavin and doxycycline). Oxal was used as positive control of SDH inhibition. Analysis of SDH activity showed that Oxal, at a concentration of 10 μM, induced a significant inhibition (considered as 100%) of SDH, while apomorphine, nifedipine and riboflavin presented an inhibition of 30/40% (respect to Oxaloacetate inhibition). Methyldopa, dobutamine and doxycycline did not affect the SDH activity (see Fig. 1 D). IC_{50} values could be only calculated for those compounds that showed a sigmoidal curve; apomorphine and riboflavin (Fig.1 E and F).

Riboflavin reduces glutamate serum levels in healthy animals

Once selected riboflavin as a potential and novel blood glutamate grabber and once determined its low affinity for SDH, this hit was intravenously injected in healthy rats to determine if the effect observed *in vitro* resulted in a blood glutamate reduction under *in vivo* conditions.

In our previous studies we have found that 35 mg/kg of Oxal was an effective dose to induce a reduction of blood glutamate.[8] Based on this study, we chose three different doses (1, 10 and 50 mg/kg, animal weight) to test the effect of riboflavin on blood glutamate. Saline treatment was used as control group and Oxal (35 mg/kg) as positive control for blood glutamate reduction. The three doses tested showed a significant reduction of blood glutamate levels respect to the control group at 2 and 4 hours after administration. However, 1 and 50 mg/Kg doses produced a faster and higher glutamate reduction than Oxal. Thus, while Oxal induced a reduction on glutamate levels (20% respect to the basal levels) at 4 hours after treatment, the reduction induced by riboflavin 1 and 50 mg/Kg was observed at 30 min after administration, being the maximum effect at 2 h, with reduction of blood glutamate around 50% respect to the basal levels (Fig. 2A). The lack of dose-response effect between 1 and 50 mg/Kg led to choose the minimal dose tested (1mg/Kg) for the next protective study in ischemic animal models.

Analysis of riboflavin levels in animals treated with a dose of 1 mg/kg confirmed a peak increase of this treatment in blood at 30 min after the administration (Fig. 2 B). Rats treated with same dose of a clinical form of riboflavin (used for subsequently clinical trial) showed similar profile (figure X of the supplemental data).

Riboflavin treatment reduces the blood glutamate levels and infarct size in ischemic animal models.

To confirm if the decrease on blood glutamate induced by riboflavin might lead to an ischemic therapeutic effect, experimental animals were subjected to an ischemic model of middle cerebral artery occlusion (MCAo) and then were treated with riboflavin 1 mg/kg. Saline treatment was used as control group and Oxal 35 mg/kg as positive control of blood glutamate grabber.

During ischemic surgery, reduction of cerebral blood blow was checked by laser Doppler (sup data fig X) and Magnetic Resonance Angiography (MRA) to confirm the occlusion of middle cerebral artery (MCA); and basal ischemic lesion (before treatment administration) was also determined with magnetic resonance imaging (MRI) (details of the ischemic animal model are indicted in material and methods). All animals included in the study present similar reduction of cerebral blood (>70% respect to the basal levels) and same ischemic damage before treatment administration (treatment was administrated after artery reperfusion).

Animals treated with Oxal and riboflavin showed a significant ($p<0.05$) infarct reduction at 24 hours compared with control group (Fig 3. A and B). This effect persists until day 7, although these differences did not achieve a significant value. In addition, a significant ($p<0.05$) reduction on blood glutamate levels 3 hours after occlusion compared to control was observed in treated group (Fig. 3 C). None of the treatments used, Oxal or riboflavin, caused an alteration on GOT activity compared with the control group. (Fig. 3D). No differences on sensorimotor analysis were observed in treated group compared with the control (Fig. 3 E and F)

Riboflavin does not increase hematoma size in hemorrhagic animal models.

One most important advantages of the blood glutamate grabbers tested so far (Oxal and GOT) is that they can be given immediately in case of suspected ischemic stroke without previous neuroimaging and without risk in case of hemorrhagic stroke.[10]

In this line, to evaluate the effect of riboflavin on cerebral hemorrhagic damage, this new selected drug was tested in a hemorrhagic animal model produced by injecting collagenase into the basal ganglia (see methods for more details). Hemorrhagic animals were treated 1 hour after brain damage with riboflavin 1 mg/kg and the hemorrhagic lesion was compared with the control group treated with saline. MRI assessment of hemorrhagic lesion during 14 days did not show differences between both groups (Fig.4. A and B) No differences were detected in the sensorimotor recovery (Fig.4. C and D).

The effect of riboflavin on GOT activity is mediated through an indirect mechanism.

In the HTS study, riboflavin was identified as a new blood glutamate grabber drug based on its capability to interact with GOT however, whether riboflavin was acting directly on GOT enzyme or through other indirect mechanism is unknown so far.

In order to characterize the effect of riboflavin on GOT activity, a Saturation Transfer Difference Nuclear Magnetic Resonance (STD-NMR) study was performed. This technique allows to detect weak ligand-based interactions between large proteins and potential ligands in the range of kDa-µM/mM**.** With this purpose, firstly riboflavin and GOT enzyme were analyzed obtaining their respective off resonance spectrum and them both compounds were combined and specifically saturated to obtain the On-resonance spectrum. No overlap of the riboflavin Off-resonance and On-resonance spectra was observed, which means that there is not direct interaction between GOT and riboflavin, at least under the experimental conditions used (Fig. 5A). To evaluate whether the effect on GOT activity observed in the HTS study could be the result of the unspecific interaction between riboflavin with any of the unknown compounds included in the commercial GOT assay kit, different chemical forms of riboflavin were tested on glutamate inhibition under the same conditions than performed in the initial HTS assay. For this analysis, 3

compounds that conserve the basic scafold of riboflavin were purchased or synthesized: flavin adenine dinucleotide (FAD), a phosphorylated form of riboflavin; lumichrome, used as an inactive truncated structure of riboflavin and an acetylated analogue form of riboflavin, tetraacetyl riboflavin (TARF) (Fig. 5B). In addition, Oxal and riboflavin were used again as control. Inhibition of glutamate formation induced for Oxal was considered as 100%. Riboflavin induced an inhibition of 41% (respect to Oxal) similar than observed initially, while the % of inhibition (respect to Oxal) for FAD was 54%, for acetylated analogue 54% and 9% for the inactive form, that suggests riboflavin structure plays specific role on GOT activity (Fig. 5C). Same three different analogs of riboflavin (FADS, lumichrome and TARF) were also tested on blood glutamate reduction in healthy animals at same dose (1 mg/kg) used for riboflavin. Riboflavin and saline were used as control groups. In line with the *in vitro* screening, riboflavin, FAD and TARF induced a reduction of blood glutamate levels during hour after treatment administration compared with the control group, while lumichrome did not show effect compared with the control group (Fig. 5D). These *in vivo* results confirm that, although riboflavin does not have a direct interaction on GOT activity, riboflavin structure is critical to induce a reduction on blood glutamate.

Clinical proof of concept validation of riboflavin as a new blood glutamate grabber in stroke patients.

The final aim of this clinical study was to test the previous experimental studies and to validate in stroke patients the effect of riboflavin as a new blood glutamate grabber,.

The clinical study (protocol code JCS-CBG-2014-01) was initially approved by the Ethics Committee of Clinical Research (CEIC) of Galicia on December 20 of 2014. Patients were included following inclusion criteria indicated in the Figure 6. A total of 50 patients were included in the study and randomized upon arrival in 2 different groups 1) intravenous placebo (saline) or intravenous riboflavin (20 mg) both administrated in a single bolus (following similar administration protocol than used in experimental studies). In both groups, demographic, clinical, laboratory, and treatment variables were similar (Table A, supplementary data).

Analysis of serum glutamate levels, considered as main objective of the study was determined at admission 3, 6, 12 and 24h after. Both groups were administrated at the same times (placebo and treated groups), but the decrease in glutamate concentrations was significantly greater in the group of patients treated with riboflavin at 12 hours after treatment administration (Fig. 7 A and B). Linear regression analysis confirmed that the effect of the treatment on blood glutamate reduction was independently, once adjusted for age, NIHSS at admission and recanalizer treatment (B: 51.9; CI 95%: 2.9 - 100.9; p = 0.038).

Riboflavin serum levels were also analyzed showing a peak in riboflavin treated patients immediately after the administration and a progressive decay after 6 hours until reach levels close to the placebo group 24 hours later, evidencing the quick clearance of the treatment. (Fig. 7C).

Safe analysis, included as one of the secondary objective of the study, revealed that 12 patients presented serious events, 9 of them belonging to the placebo group (none association was detected between the presence of adverse and treatment). The mortality at 3 months was 31.8% in the control group and 16.7% in the active group.

Comparative analysis of the percentage improvement of the NIHSS at discharge was higher in riboflavin treated group compared with placebo at discharge was (33.7 ± 43.7 vs. 48.9 ± 42.4, p = 0.050) and at the third month (60.5 ± 47.9 vs. 76.9 ± 30.3, p = 0.268), although these values did not achieve significant differences (Fig. 7 D). The mRS at 3 months was 4 [2, 6] in the control group and 3 [1, 5] in the riboflavin group (p = 0.300), and the percentage of good outcome was higher in the group that received riboflavin (44.4 % vs. 27.3%, p = 0.212) (Figure 7 E). These results confirmed the main hypothesis that riboflavin achieves a greater reduction of blood glutamate levels, especially within 12 hours after administration. Riboflavin administration is also safe and a tendency to better clinical evolution and better functional status at 3 months is verified (see supplemental data for further clinical variables analysis).

DISCUSSION

In the search of pharmacologic treatments for acute ischemic stroke, hundreds of experimental protective strategies with successful effect have been reported, but only a small percentage of them have been clinically probed, such as glutamate antagonists (selfotel and aptiganel), calcium channel blocker (nimodipine) or free-radical scavengers (trilizad and NXY-059).[5] Unfortunately, none of the neuroprotective agents studied resulted in improved outcome for patients, that has led to a decrease of the interest in the develop of new protective agents against stroke due to the high risk of failure and investment.[5]

Repositioning of drugs already known to be safe provides a lower risk alternative.[27] This strategy is particularly well suited to the public sector, where off-patent agents can undergo HTS for their ability to interact with identified molecular targets both in vitro and in vivo.[31] In addition, the drugs or compounds identified can be immediately tested in patients without the need to conduct a toxicity Phase I study and allow to perform a clinical proof of concept validation of the therapeutic target. Different good examples of drugs repositioning for neurological pathologies can be found in the literature, for example, the discovery of β-lactam antibiotics as neuroprotective agents, through their ability to increase the expression of a glutamate

transporter, has allowed to explore the protective effect of ceftriaxone in patients with amyotrophic lateral sclerosis.[32]

The clear advantages of drug repositioning, mainly in terms of low risk of investment has become this strategy in a promising alternative to traditional approaches for drug development against stroke. [27, 33]

Focused on the necessity to evaluate the efficacy blood glutamate scavenging as a novel protecting mechanism for humans, in this study, in vitro drug repositioning strategy has allowed to identify riboflavin as novel blood glutamate gabber. The beneficial effect of riboflavin observed on ischemic animals, its safety in case of ischemic hemorrhagic, the no-toxic effect in humans and the potential protective effect for stroke patients demonstrates that this vitamin represents a promising new treatment for acute ischemic stroke and evidence the drug repositioning as a low-risk strategy to identity new stroke treatments.

Riboflavin is a water-soluble vitamin of the B group (vitamin B_2) essential for numerous biological functions, it is present as an essential constituent of all living cells, and it is widely distributed in small amounts in foods.[34] Riboflavin promotes normal growth and assists in the synthesis of steroids, red blood cells, and glycogen. However, one of the main roles of riboflavin is its role as co-enzyme in the formation of flavocoenzimes in the liver; flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), two bioactive molecules that takes part in several essential oxide-reduction reactions.[34] In fact, due to its potent antioxidant properties, riboflavin is an important source of exogenous antioxidants and it has been suggested for the treatment of migraine,[35] alcoholism[36] and also stroke.[37] In addition, riboflavin does not present toxic or adverse reactions for humans (only increase the coloration of urine at high doses) because riboflavin it is a water-soluble vitamin and excess amounts are excreted.[34]

To understand the interaction between GOT and riboflavin, based on its chemical structure, initially we discard that riboflavin could be acting as substrate of the reaction of GOT-enzymatic amino-transamination. However, it is well known that pyridoxal 5'-phosphate (PLP) (as well-known as vitamin B_6) acts as co-enzyme for amino-transaminase enzymes like GOT,[38] therefore we speculated that, similar than PLP, riboflavin could be acting as a co-enzyme of GOT. We first aimed to confirm the interaction between GOT and riboflavin observed in the HTS study using the same commercial kits; but although useful to confirm the glutamate grabbing effect we consider this method not the rightful tool to elucidate a mechanism of action due to the unknown reagents present in the kit that may interfere with our results. We opted for a more specific approach using STD-NMR. This analysis revealed that there was not a direct interaction between both molecules what discarded the suspected role as co-factor, and demonstrated that the interaction between GOT and riboflavin was mediated through an indirect mechanism. After

these results, an *in vivo* study of the interaction between GOT and riboflavin were considered however, due to the wide participation of riboflavin on cell metabolism, an individual analysis of all molecular pathways that could be mediating on GOT activity was out of the aim of this study. What we could confirm, both in in vitro and in vivo analysis was the specific effect of riboflavin on GOT reaction and on blood glutamate reduction as the three analogs probed showed similar effect than riboflavin but not the inactive form.

It could be suggested that some important limitations of the treatment was that riboflavin only showed a transient reduction on infarct volume after 24 hours in ischemic animals with a trend at 7 days. Due to the quick clearance nature of the riboflavin, we consider that the therapeutic effect could be improved by successive bolus administrations or by a continuous infusion of the treatment. Moreover, in all our experimental studies with ischemic animal models, we always perform a basal ischemic evaluation during artery occlusion (see methods sections) in order to reduce the infarct size variability (inter-group) and to confirm that all included animals before treatment administration present same infarct lesion.[8, 11, 39, 40] This ischemic pre-treatment evaluation allow us to demonstrate and convince that the differences observed on ischemic animals during the follow up period are due to the treatment used and not to the surgery variability. Thus, in this study, a significant reduction of infarct size was observed at 24 hours after ischemia in treated animals and (although no significant) this reduction can be also observed at 7 days, which demonstrates the protective effect of the treatments. Unfortunately, no significant sensorimotor recovery was observed in these animals, which could be considered as a limitation of the in vivo experimental results. We are aware that additional and more sensitive behavioral tests should be included in this experimental protocol to detect more detailed sensorimotor recovery.

Regarding the proof of concept clinical trial, even showing promising results in terms of glutamate reduction and neurological recovery, the low number on patients included (<25/group), limits our definitive conclusions, but these results support a further clinical trial with a large and adequate numbers of stroke patients.[41] Riboflavin therapeutic effect was demonstrated along this work focused in its glutamate grabbing mechanism, a common effect observed in all the experimental stages, from in vitro, to animals and finally confirmed in ischemic patients.[37] However it is naïve to assume that all the therapeutic effect is due to a glutamate reduction, especially in a molecule like riboflavin which exerts multiple protective effect mediated thought it anti-oxidant activity.[5, 42]

The find of riboflavin as a new glutamate grabber and that was a drug already used in human (Vitamin B2 Streuli®) with similar formulation (for i.v. bolus administration) similar than used in the in vivo studies, allowed us to test immediately the reduction of blood glutamate effect in

stroke patients previously observed in ischemic model animals. The reduction of blood glutamate levels observed in patients after riboflavin administration lead to demonstrate that glutamate grabbing is a valid strategy and it opens the way to use similar treatments and reduces the risk of failure of other glutamate grabbing approaches, as the administration of rGOT1, a recombinant enzyme that overcomes the toxicity impairment of Oxal conserving the therapeutic effect and which works directly on glutamate metabolism.

In conclusion, this translational study, which started with an in vitro drug repositioning study, followed by a in vivo experimental analysis and concluded with a proof of concept clinical study, shows for first time a reliable human demonstration of the efficacy of glutamate grabbers on blood glutamate reduction in stroke patents, hoping can help to contribute to the clinical development of this novel protective therapy.

MATERIALS AND METHODS

High-throughput screening assay of glutamate grabbers.

The 1120 chemically and pharmacologically diverse compounds (ca. 90% being FDA approved drugs) from the Prestwick Chemical Library (PCL)[30] were evaluated regarding their capacity to inhibit glutamate formation in presence of the enzyme GOT (Abcam Cat#99147) by using the GOT assay kit (Abcam Cat#105135). Briefly, in this activity assay kit, an amino group is transferred of from aspartate to α-ketoglutarate. The products of this reversible transamination reaction are oxaloacetate and glutamate, and the glutamate is detected in a reaction that concomitantly converts a nearly colorless probe to color (λ_{max} = 450 nm). Experiments were conducted in 384-well format in a Tecan Ultra Evolution Multifunctional reader (Tecan, Austria). The screening window coefficient (Z'-factor) was used as an indicator for assay development and optimization (i.e. setting of enzyme concentration, exposure time, volumes used, etc.) and as a statistical tool for the assay quality assessment.[43] The quality of the assay was considered sufficient for screening and automation when Z' >0.5. (sup data FIGURE X) Hit compounds (or PCL hits) were identified and statistically validated when their HTS scores are higher than the average of the negative controls +3XSD. The primary scores for the PCL hits are based on the mean from three independent experiments each performed in triplicate.

As positive control of glutamate formation, wells without any compound but GOT enzyme ($3.33X10^{-5}$ mg/ml) were used and oxaloacetate 0.66 mM as control of glutamate inhibition formation. Selected concentrations used for GOT and oxaloacetate are indicated in the **Supplementary data**. Each Hit compounds were screened at a concentration of 10^{-4} M. Once completed the HTS, the selected Hits were tested again using new reagents (obtained from Sigma-Aldrich Co.) on GOT activity to validate the results previously obtained.

Succinate dehydrogenase inhibition Hits analysis.

As we have explained in the introduction, one of the most important limitation of oxaloacetate, from a translational point of view, is that this molecule acts as a potent inhibitor of the Succinate dehydrogenase (SDH), an enzyme that participates in both the citric acid cycle and the electron transport chain.[26] Therefore, and in order to avoid potential interactions on SDH activity as oxaloacetate does, the selected Hits obtained from the previous HTS were tested on SDH activity using the Succinate Dehydrogenase Assay Kit (Sigma-Aldrich Co.) following the manufacturer indications (supp data). Briefly, SDH activity was determined by generating a product with absorbance at λ_{max} = 600 nm proportional to the enzymatic activity present. Oxaloacetate 0.66 mM was used as positive control of SDH inhibition.

Nuclear magnetic resonance analysis of GOT

To characterize the protein biding between the GOT and the selected Hit (riboflavin or Vitamin B_2), Saturation-Transfer Difference (STD) NMR technique was used. 1D 1H Saturation Transfer Difference in magnetic resonance (STD-NMR) was performed with a magnetic field of 600MHz. at a temperature of 298K with 20 uM of GOT1 enzyme in buffer TRIS-d11 25mM in D2O, 150mM NaCl, pH7.4 and 4.5 µl of riboflavin solution 212mM in DMSO-d11.

Experimental animals

The animal experiments were conducted in accordance with the recommendations of the European Convention for the Protection of Vertebrate and the current ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). Experiments were approved by the Galician Network Committee for Ethics Research following the Spanish and European Union (EU)

rules (86/609/CEE, 2003/65/CE, 2010/63/EU, RD 1201/2005 and RD53/2013). Male Sprague-Dawley rats weighing between 300 and 350 g (10 to 12 weeks old) were used for this study. Rats were given water and were fed ad libitum. Anesthesia was induced with sevoflurane (5% to 6% for induction and 3% to 4% for maintenance) evaporated in an oxygen–air mixture (30%:70%). Throughout the experimental period, rectal temperature was maintained at 37 ± 0.5°C by means of an electronic thermostat-controlled warming blanket. Body temperature was maintained until animals completely recovered from anesthesia and displayed normal motor activity.

In vivo experimental design and protocols.

Animal groups were defined as represented in Fig. 8.

- In vivo dose-response study in healthy animals.

To validate the effect of the selected Hit (riboflavin) obtained from the HTS on blood glutamate reduction, a dose-response study was performed healthy animals. In this in vivo study, three different doses of riboflavin (Sigma, Ref#24500), 1, 10 and 50 mg/Kg (animal weight) were tested. All riboflavin doses were initially dissolved in a minimal amount of DMSO (100µL) and them, the concentration was adjusted to inject 1 ml per animal, (after adjusting the pH to 7.4 with HCl 1mM). The final concentration of DMSO in the injected volume was <1%. Animals treated with saline (0.9% of NaCl and 0.5% of DMSO) were used as a control group. Oxaloacetate 35 mg/Kg (animal weigh) was used as positive control group of blood glutamate grabber.[8, 24] Oxalacetate solution (pH adjusted to 7.4) was prepared in saline solution (0.9% of NaCl). Glutamate levels were analyzed in blood samples were collected (under anesthesia conditions) from the tail before treatment administration (basal level), and 30 min, 2, 4 and 6 hours after treatment administration. A total of 6 animals per group were included in this dose-response in vivo study.

- Protective evaluation of riboflavin on ischemic animal models.

Once selected the optimal riboflavin dose able to induce a reduction of blood glutamate levels in the previous dose-response study, the protective effect of riboflavin was tested on an ischemic animal model. Ischemic animals were divided in 3 randomized groups. A control group treated with saline solution (0.9% of NaCl), a group treated with an

effective dose of oxaloacetate of 35 mg/Kg, used as positive control group for blood glutamate grabbing and a last group of animals treated with an effective dose of riboflavin 1 mg/Kg (determined previously in the dose-response study). A total of 8 animals per group were included in this study. To determine the protective effect of the treatments, blood glutamate levels, infarct volume and functional deficit were measured in the experimental animals. Treatments were administered immediately after reperfusion (75 min after occlusion). Blood glutamate concentration and GOT activity was determined under basal conditions (before surgery), immediately after reperfusion, and 3 hours, 24h and 7 days after ischemia. Cerebral infarct volume was determined during arterial occlusion to determine the basal ischemic lesion (before treatment administration), and then 24 h and 7 days after ischemia. Sensorimotor test was performed under basal conditions (1 day before surgery), 24 hours and 7 days after ischemia.

Analysis of riboflavin on hemorrhagic animal models: the effect of riboflavin was also tested in hemorrhagic animal model. Hemorrhagic animals were divided in 2 different groups, a control group treated with saline solution (0.9% of NaCl) and a group treated with a dose of riboflavin 1 mg/Kg. A total of 8 animals per group were included in this study. Hemorrhagic volume profile was determined 1 hour, 3 hours, 24 hours, 7 days and 14 days after hemorrhage lesion. Sensorimotor test was performed under basal conditions (1 day before surgery), 24 hours and 7 days and 14 days after hemorrhage induction.

- Study of different riboflavin forms on blood glutamate levels.

In order to validate the specific effect of riboflavin on blood glutamate reduction, an active form of riboflavin (flavin adenine dinucleotide, known as FAD), an inactive form of riboflavin (lumichrome) and an acetylated riboflavin analogue (synthesized as described in a previous work[32]) were tested in healthy animals at a dose of 1 mg/kg, and the effect on blood glutamate was compared with riboflavin. All treatments were administrated intravenous through the jugular vein in a single bolus adjusted to 1 ml. Blood samples were collected from the tail vein in basal conditions, 30 minutes, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 6 hours and 24 hours after treatment. All samples were kept at 4^{o}C after extraction for 45 minutes in serum tubes for clot formation,

immediately centrifuged at 3000 rpm for 7 minutes, serum was collected and frozen at -80^{o}C until glutamate analysis.

- Evaluation of riboflavin clearance profile

To evaluate the clearance profile of riboflavin after drug administration, healthy animals were treated with 1 mg/kg commercial riboflavin used in all the above experiments, and riboflavin treatment used for patients in the subsequent clinical trial. A total of 3 animals per group were included in this study. All treatments were administrated intravenous through the jugular vein in a single bolus adjusted to 1 ml. Samples were collected at basal conditions, 30 min, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 6 hours, 12 hours and 24 hours after treatment. All samples were kept at 4^{o}C after extraction for 45 minutes in serum tubes for clot formation, immediately centrifuged at 3000 rpm for 7 minutes, serum was collected and frozen at -80^{o}C until glutamate analysis.

Cerebral Ischemic animal model

Transient focal ischemia was induced in rats by using the transient MCAo model following the surgical procedures previously described.[8, 11] In brief, under an operating microscope, the left common, external and internal carotid arteries were dissected from connective tissue through a midline neck incision. The left external carotid artery and pterygopalatine artery of the internal carotid artery were separated and ligated by 6-0 silk sutures. A silicon rubber-coated monofilament (403512PK5Re; Doccol Corporation, USA) was inserted through the external carotid into the left common carotid artery and advanced into the internal carotid artery to 20 mm from the bifurcation to occlude the origin of the MCA. The suture was removed after 75 min of occlusion. A laser-Doppler flow probe (tip diameter 1 mm) attached to a flowmeter (PeriFlux 5000, Perimed AB, Stockholm, Sweden) was located over the thinned skull in the MCA territory (4 mm lateral to the bregma) to obtain a continuous measure of relative cerebral flow during the arterial occlusion. Once artery occlusion had been achieved, as indicated by Doppler signal reduction, each animal was carefully moved from the surgical bench to the MRI system for baseline ischemic lesion assessment using apparent diffusion coefficient (ADC) maps. MR angiography (MRA) was also performed to ensure that the artery remained occluded throughout the MR procedure. After MR analysis, animals were

returned to the surgical bench and the Doppler probe was repositioned. Reperfusion was performed 75 min after occlusion onset.

The following exclusion criteria were used: 1) less than 70% reduction in relative cerebral blood flow; 2) arterial malformations, as determined by MRA; 3) baseline lesion volume of less than 25% or greater than 45% with respect to the ipsilateral hemisphere, as measured using ADC maps; 4) failure to complete treatment. All excluded or deceased animals were replaced until the total number of animals indicated for each group was attained.

Experimental procedures were performed following five criteria derived from the Stroke Therapy Academic Industry Roundtable (STAIR) group guidelines for preclinical evaluation of stroke therapeutics:[44, 45]1) cerebral serum flow was measured to confirm the vascular occlusion as an index of the reliability of the ischemic model; 2) animals were randomly assigned to treatment groups of the study; 3) researchers were blinded to treatment administration; 4) researchers were blinded to treatments during outcome assessment; and 5) temperature was controlled during the ischemic period.

Intracerebral hemorrhage

For the Intracerebral hemorrhage (ICH), the intraparenchymal injection of bacterial collagenase model was used. Collagenase disrupts the basal lamina of blood vessels, which leads to the leaking of blood into the surrounding tissue.[46] To perform the model, rats were placed in a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA) under sevoflurane anesthesia. A 1 cm long midline incision was made in the scalp, beginning midway between the eyes and terminating behind the lambda. A cotton swab was used to clear away the soft tissue covering the skull. A Hamilton syringe (Hamilton, Reno, NV, USA; 10 µL) was filled with 1.5 µL of collagenase type VII (sterile-filtered, high purity; Sigma-Aldrich, St Louis, MO, USA) dissolved in saline (0.2 U/µL). The syringe was mounted onto the injection pump and the needle was positioned directly over the bregma. The x, y, and z axis coordinates were all set to zero. The needle was then positioned at the entry point, +0.6 mm anterior and −2.9 mm lateral of the bregma to the right. A small cranial burr hole was drilled through the skull at the entry point. The needle was slowly inserted into the basal ganglia to a depth of 5.5 mm below the surface of the skull, and a volume of 1 µL collagenase was injected at a rate of 0.1 µL/min over

10 minutes. The needle was left in place for 10 minutes and then removed at a rate of 1 mm/min to prevent the reflux of collagenase and blood. The burr hole was filled with bone wax (Ethicon, Somerville, NJ, USA), and the scalp incision was closed. Sham control rats were injected with an equal volume of saline. The rats were placed in an animal box after surgery for recovering in a warm place with access to food.

Blood extraction

All animal blood samples were collected from the tail vein of at different time points according to the experimental group. Samples were keep at 4^{o}C after extraction for 45 minutes in serum tubes for clot formation, and them immediately centrifuged at 3000 rpm for 7 minutes and finally the serum was collected and frozen at -80^{o}C until analysis. For patients, venous blood were collected at different time points and centrifuged at 3000 rpm for 15 minutes at 4^{o}C and frozen until analysis.

MRI Imaging

All studies were conducted on a 9.4T horizontal bore magnet (Bruker BioSpin, Ettlingen, Germany) with 440 mT m-1 gradients and a combination of a linear birdcage resonator (70 mm in diameter) for signal transmission and a 2×2 surface coil array for signal detection. MRI post-processing was performed using ImageJ software (W. Rasband, NIH, USA).

Basal ischemic lesion during MCA occlusion was determined by counting pixels with apparent diffusion coefficient (ADC) values below a threshold in the ipsilateral brain hemisphere. The values of ADC in the healthy rat brain normally do not fall below 0.55 x10-3 mm2 s-1; therefore, this threshold provides a convenient means of segmenting abnormal tissue (Reith et al., 1995). ADC maps were obtained from diffusion-weighted images (DWI) using a spin echo echo-planar imaging sequence (DTI-EPI) with the following acquisition parameters: echo time (TE) = 26.91 ms, repetition time (TR) = 4 s, spectral bandwidth (SW) = 200 KHz, 7 b-values of 0, 300, 600, 900, 1200, 1600, and 2000 s/mm2, flip angle (FA) = 90º, number of averages (NA) = 4, 14 consecutive slices of 1 mm, field of view (FOV) = 24×16 mm2 (with saturation bands to suppress signal outside this FOV), a matrix size of 96×64 (isotropic in-plane resolution of 250 µm/pixel×250 µm/pixel) and implemented with fat suppression option.

To evaluate the status of MCA occlusion in a noninvasive manner, the time-of-flight magnetic resonance angiography (TOF-MRA) was performed. The TOF-MRA scan was performed with a 3D-Flash sequence with an echo time (TE) = 2.5 ms, repetition time (TR) = 15 ms, flip angle (FA) = 20º, number of averages (NA)= 2, spectral bandwidth (SW)= 98 KHz, 1 slice of 14 mm, field of view (FOV) = 30.72×30.72×14 mm3 (with saturation bands to suppress signal outside this FOV), a matrix size of 256×256×58 (resolution of 120 µm/pixel×120 µm/pixel×241 µm/pixel) and implemented without fat suppression option. DWIs and TOF-MRA were acquired during MCAo, at the same time (30±5 min after occlusion).

Ischemic lesions at 24 hours, 7 days and 14 days after ischemia were determined by mean of T2 maps. These maps were calculated from T2 weighted images using a multi-slice multi-echo sequence (MSME) with echo time (TE)= 9 ms, repetition time (TR)= 3 s, 16 echoes with 9 ms echo spacing, flip angle (FA) = 180º, number of averages (NA)= 2, spectral bandwidth (SW)= 75 KHz, 14 slices of 1 mm, field of view (FOV) = 19.2 × 19.2 mm2 (with saturation bands to suppress signal outside this FOV), a matrix size of 192×192 (isotropic in-plane resolution of 100 µm/pixel x 100 µm/pixel) and implemented without fat suppression option. Infarct size was indicated as % of ischemic damage respect to the ipsilateral hemisphere volume, corrected for brain edema.

Lesion size and hematoma growth in hemorrhagic animals were assessment by means T2-weighted images and T2*-weighted images.

T2*-weighted images were acquired using a MGE sequence with 8 echos, first echo time=3.13 ms, echo epacing=3.38 ms, repetition time=1.4 seconds, number of averages=2, and the same geometry parameters as that of T2-weighted images (field-of-view, image matrix, number of slices and thickness).

The image evaluations were performed by a blind researcher.

GOT activity

Blood samples were collected in a microtainerBD (Microtainer K2E Tubes. Ref: 365975, Franklin Lakes, USA) and GOT activity was determined in 32 µL of serum by means of Reflotron GOT (as well named AST) and following the manufacturer's technical specifications (Roche, Basel, Switzerland).

Glutamate serum levels

Serum glutamate concentration was determined by high performance liquid chromatography (1260 Infinity II, Agilent Technologies, Santa Clara, USA) using the AccQ-Tag™ Precolumn derivatization method for amino acid analysis (Waters, Milford, MA), following the manufacture indications.

Riboflavin serum levels analysis

Detection of riboflavin serum levels was performed with an HPLC 1290 Infinity Agilent with a C18 Column 1.7 micron particle size; dimensions: 2.1 x 50 mm, a mobile phase flow of 0.3 mL/min and a gradient performed with water-methanol. The Injected volume was 7µL microliters and the chromatogram time was 5.6 minutes. A 6430 Agilent Triple Quad was used for mass spectroscopy. Detection was performed with electrospray in positive mode. Riboflavin showed a transition of 377,4 > 243,3 and a linear range between 0 - 250 ng/ml with a lower limit of quantification of 0,5 ng/ml.

Animal sensorimotoric test.

Neurological damage and motor deficit in ischemic animals were assessed by 2 complementary test, Bederson scale, and cylinder test.

Bederson test, is a neurological scale designed to determine the motor deficit in animals after stroke, assessment is focusing in forelimb flexion, circling behavior and lateral push. A grading scale of 0 to 8 is using to determine the cerebral damage and motor deficit, a score of 0 represents an animal with no neurological damage and 8 represents a high neurological damage.

Cylinder test, asses' neurological motor damage by the forelimb use in the exploratory behavior of the animals while inside of a cylinder. Use of the forelimb is expressed as lateral index, measuring the number of times the animal touches the wall of the cylinder with the contralateral forelimb to the lesion divided by the total number of touches with both forelimbs. Ischemic animals usually show an asymmetry use due to ischemic damage, using mostly the not impaired forelimb.

A baseline of each test was performed at least 1-3 days prior to any surgery or invasive manipulation. All tests were performed during the activity cycle of the animals. Observer was blind to treatments.

Proof of concept clinical trial

To evaluate the effect of riboflavin on blood glutamate levels and determine the potential clinical benefit of this treatment, a proof of concept clinical trial was designed.

- Study design

This was an 18-month interventional unicenter study, randomized, with parallel assignment, double blind, phase IIa study conducted in the Spain at the Neurology Department of the University Clinical Hospital of Santiago de Compostela (ClinicalTrials.gov identifier, NCT02446977) and Spain (EudraCT number, 2014-003123-22) between April 2015 and October 2016. Institutional review board and ethics committee approval was obtained before study initiation. All patients or relatives provided written informed consent. This study enrolled 50 patients (older than 18 years) who met the inclusion criteria for the study indicated in the Fig. 2A SUPPL. All patients included in the clinical trial (placebo and treated) were managed according to our Hospital stroke unit protocol.

Patients with previously defined criteria (supplementary data) were excluded. The follow-up period of each patient was three months. Selected patients were randomized to one of two study arms: the control group (4 mL of sterile pyrogen-free sodium chloride 0.9%), and the experimental group (20 mg of riboflavin into a 4 mL vial), in a ratio of 1:1 administrated as a single bolus. The assignment to one or another treatment was carried out through a randomized computerized system. Both vials were adequately masked. The dose of riboflavin (Vitamin B2 Streuli®) chosen for this trial was obtained from one of the most frequently formulas used for extrapolation from animal data to therapeutic effects in humans, which is based on the index (Km).[47] Based on this formula (Human equivalent doses (HED)=Animals dose (1 mg/Kg)X(Animals Km=6/Human Km=37), it was estimated a riboflavin human dose of 0.162mg/Kg. Considering that the average weight of stroke patient is 70 Kg, 11,3 mg of riboflavin was need/patient. In addition, due to each vial of riboflavin (Vitamin B2 Streuli®) is dispensed as ampoule of 10mg/2ml, finally we treated each patient with 4 mL of riboflavin (20 mg), and with 4 mL saline in the placebo group.

- Objectives

The main objective is to check whether riboflavin administration induces a reduction in blood glutamate concentration. For that, the blood glutamate concentrations obtained before treatment administration was compared with levels at 3 ± 1, 6 ± 1, 12 ± 3 and 24 ± 6 hours of the administered between the riboflavin and placebo arms. Secondary endpoints included safety (collection of adverse events), clinical improvement (determined by National Institute of Health Stroke Scale - NIHSS) at discharge [(NIHSS basal - NIHSS at

discharge) / NIHSS basal x 100], and at 3 months [(NIHSS basal - NIHSS at 3 months) / NIHSS basal x 100], and functional status at 3 months (modified Rankin scale at 90 ± 5 days).

- Visits and procedures

Patients with suspected stroke of less than 3 hours of evolution were potential candidates for inclusion in the study. Each potential participant was examined and evaluated to determine eligibility. Regardless of the arm to which they were assigned, all patients performed the detailed visits in Figure A (supplementary data).

For the determination of serum glutamate in all patients, a total of five blood samples were collected: a baseline sample (prior to randomization) and the rest at 3 ± 1, 6 ± 1, 12 ± 3 and 24 ±6 hours after receipt of the investigational medication. Blood samples were obtained by venipuncture in BD Vacutainer tubes of a volume of 10 ml. The samples were immediately stores at 4 ºC for further centrifugation which was performed in an interval of not more than 12 hours, at 3000 rpm, for 10 minutes and at a constant temperature of 4 ºC. The obtained serum was stores at -80 ºC until the moment of the analysis.

- Safety assessment

Was performed according to the standards of good clinical practice and current legislation, identifying events and adverse reactions, which were classified based on their causal relationship with the drug according to the Karch and Lasagna algorithm.[4]

- Sample size and statistical analysis

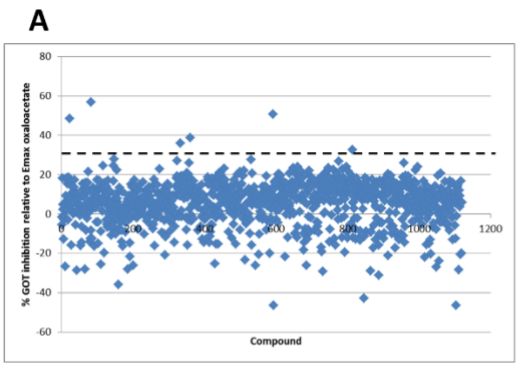
For the determination of sample size, we rely on preclinical studies.[5-8] The protocol was designed to obtain a difference in glutamate concentration of 35% over placebo. Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a bilateral contrast, 25 patients were required in each group. A follow-up loss rate of 10% was estimated.

All data will be computed in a descriptive manner and tabulated independently according to the treatment group. The value $p < 0.05$ will be used as statistically significant. Categorical data will be grouped into contingency tables presenting values and percentages. Continuous data will be summarized using the mean and standard deviation or median and 25th and 75th percentiles as appropriate.

The analysis of adverse events, vital signs and descriptive laboratory results will be performed on the safety population and will be summarized by treatment group. The population to be included for the analysis of the objectives will be the one that has been randomized, will follow the principle of intention to treat (ITT) and of LOCF (Last Observation

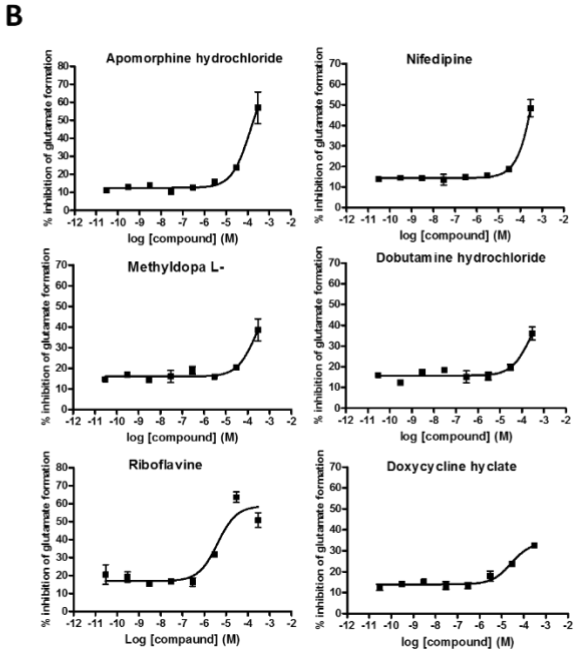
Carrier Forward). The safety population includes all patients who received the medication under study.

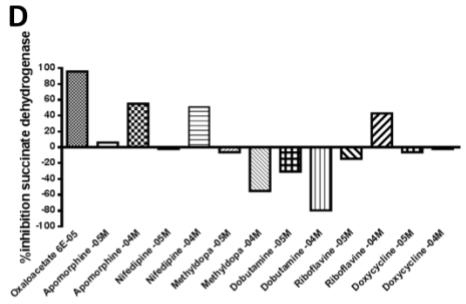
FIGURE LEGENDS



C

Compound	Maximum inhibition of GOT (%)	IC_{50} (µM)
R (-) Apomorphine hydrochloride hemihydrate	80,20	58,1
Nifedipine	42,30	---
L (-) Methyldopa	55,71	117,1
Dobutamine hydrocloride	43,68	81,7
Riboflavin	64,69	1,96
Doxycycline hyclate	52,06	15,1





F

Compound	Maximum inhibition of SDH (%)	IC_{50} (µM)
R (-) Apomorphine hydrochloride hemihydrate	84,31	6,2
Nifedipine	66,35	---
L (-) Methyldopa	36,44	---
Dobutamine hydrocloride	54,90	---
Riboflavin	41	10,8
Doxycycline hyclate	---	---

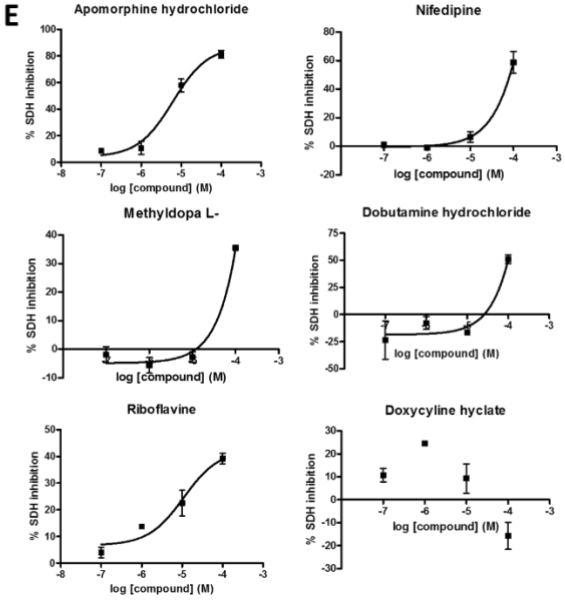


Figure 1. High throughput screening of 1120 compounds, based in the capacity to inhibit the glutamate formation in the reaction catalyzed by GOT enzyme. **(A)** Positive Hits for glutamate formation inhibition obtained in the HTS assay. **(B)** Concentration-dependent results and inhibition curves of the main 6 hits obtained. **(C)** Maximum inhibition of GOT enzyme performed by each Hit and IC_{50} obtained with crescent concentrations. **(D)** Secondary screening based on toxicity of SDH enzyme represented as % of inhibition at two different concentrations normalized to oxaloacetate inhibition **(E)** Inhibition curves

of SDH enzyme obtained with crescent concentrations of each Hit. **(F)** Maximum inhibition of SDH enzyme expressed in % and IC_{50} for each hit.

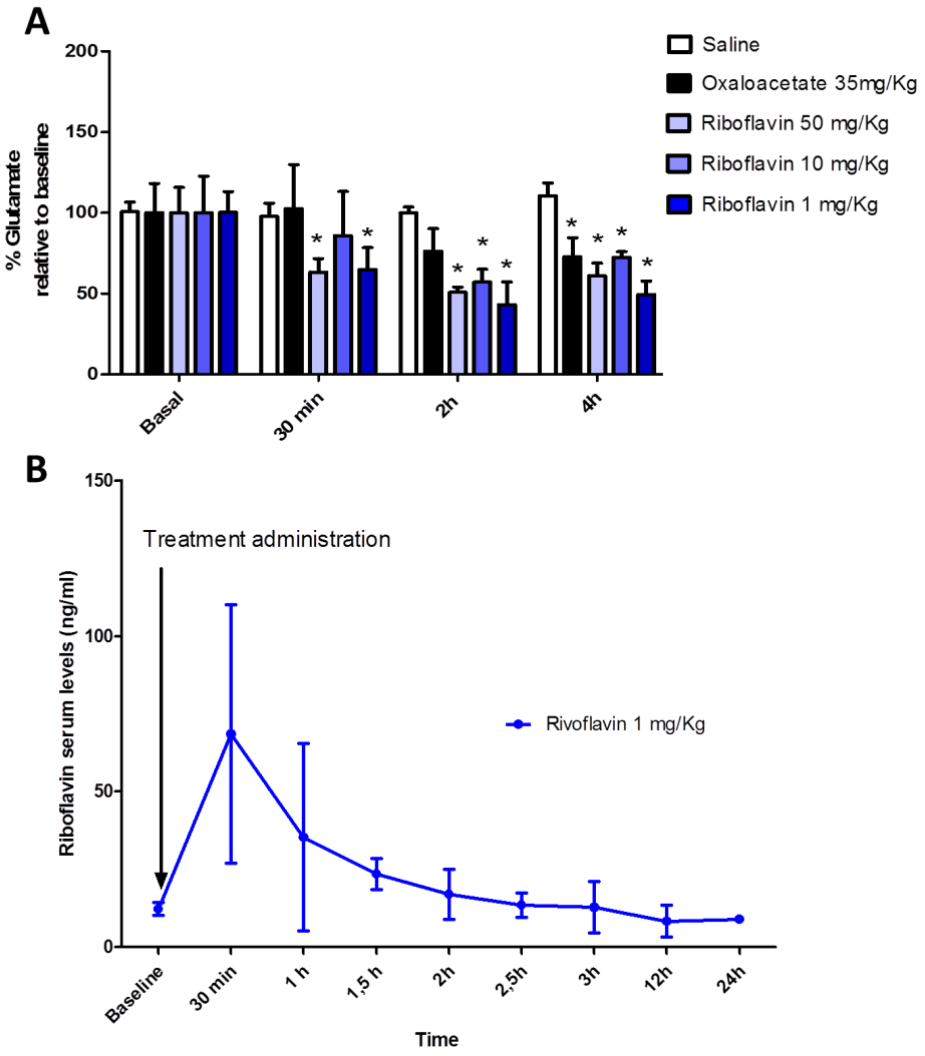


Figure 2. Determination of the glutamate grabbing effect in healthy animals. **(A)** Glutamate serum of animals treated with saline, oxaloacetate 35 mg/Kg, and 3 doses of riboflavin 50, 10 and 1 mg/Kg. **(B)** Riboflavin clearance profile in serum after riboflavin 1mg/Kg intravenous injection.

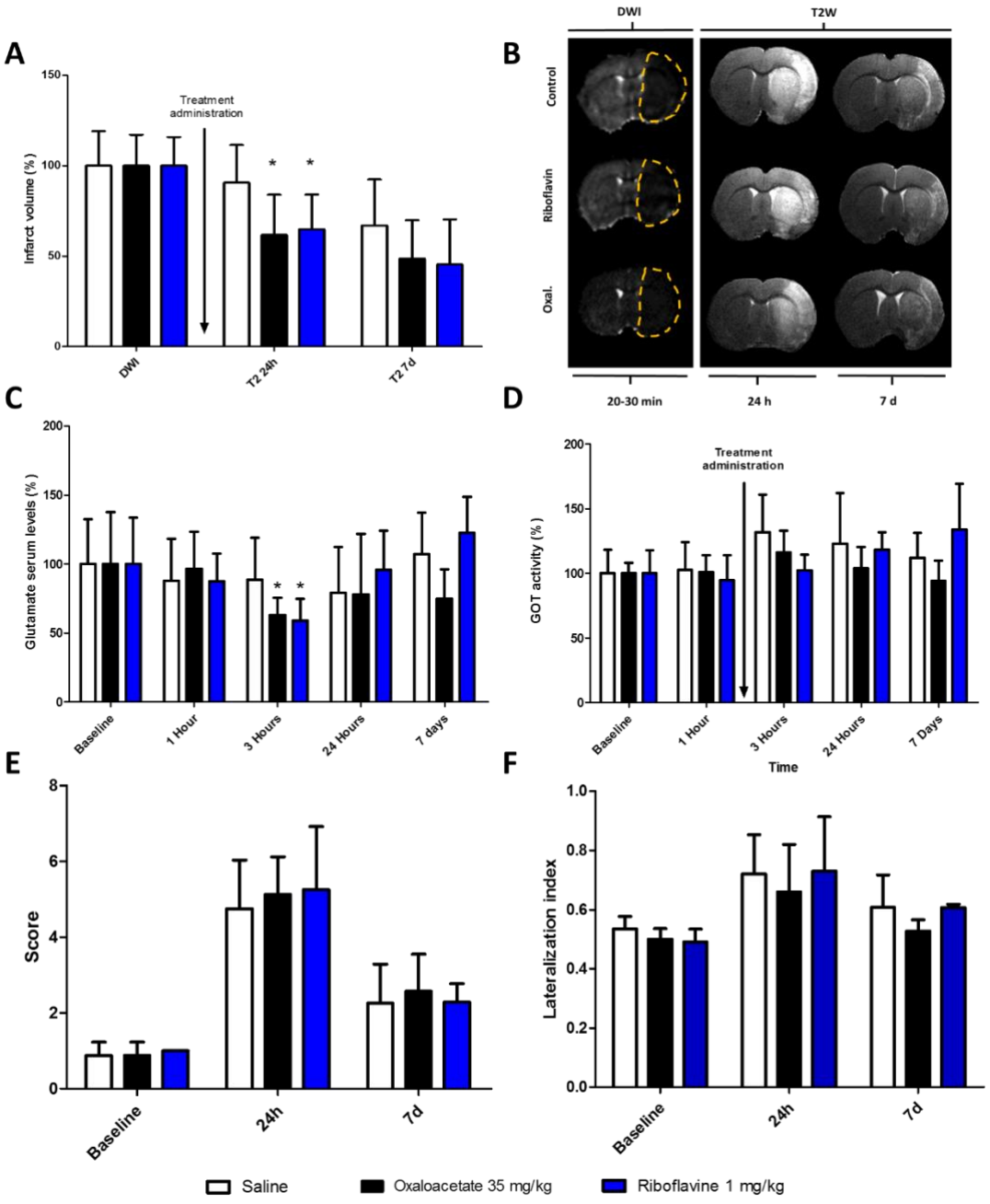
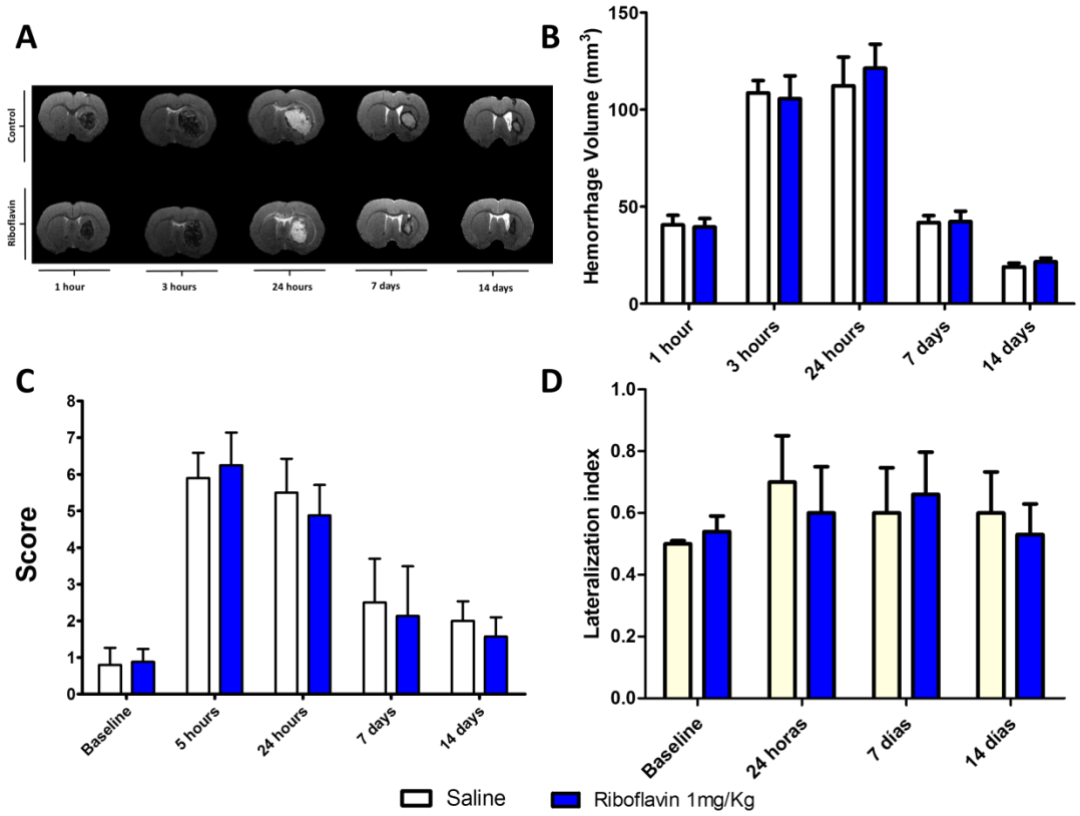


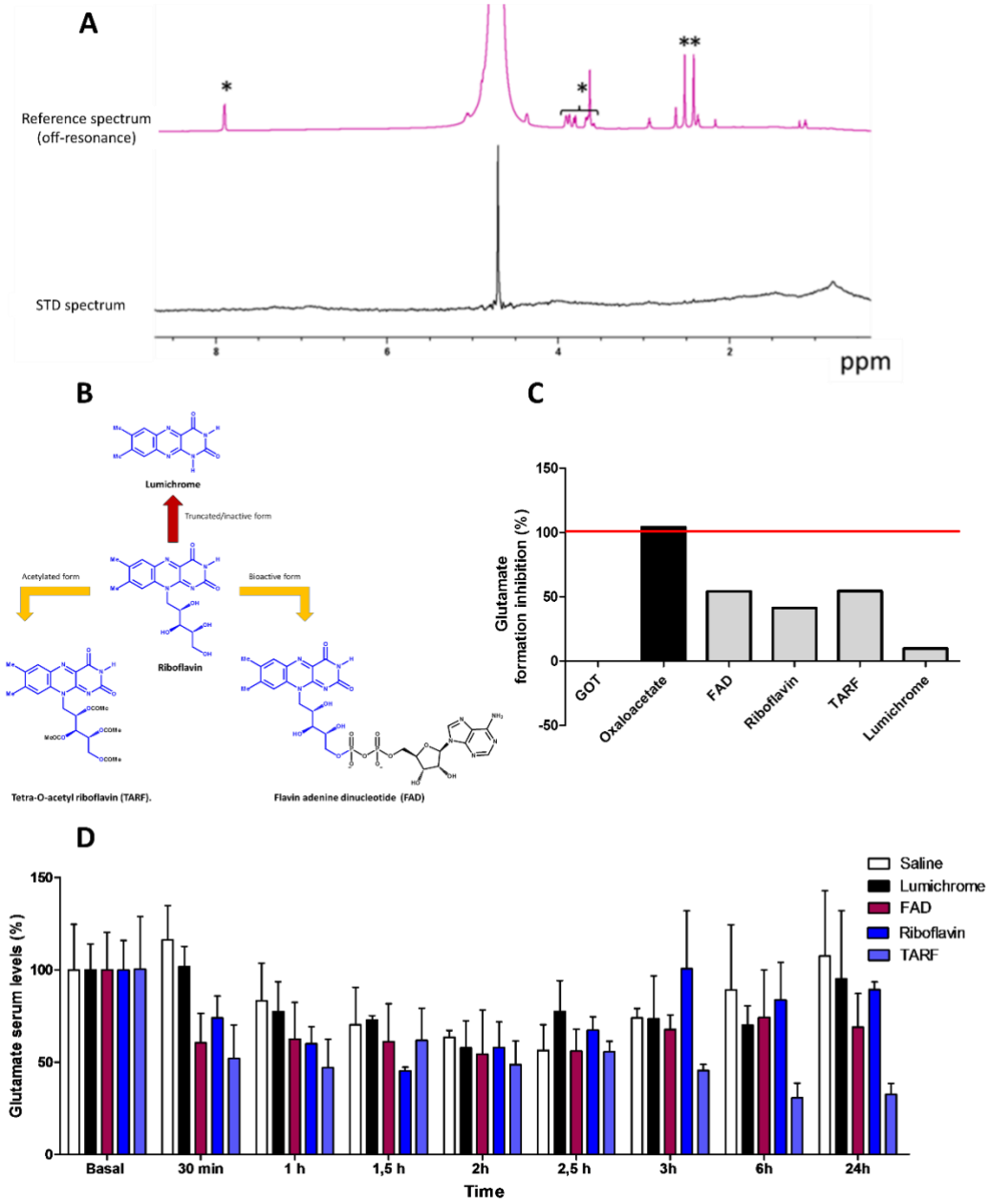
Figure 3. Evaluation of riboflavin as glutamate grabber in ischemic conditions. **(A)** Infarct volumes of ischemic animals represented as %, normalized to the basal DWI lesion. Riboflavin 1mg/kg showed a significant infarct reduction after 24 hours in T2WI compared to control group. **(B)** MRI of baseline ADC/DWI and T2WI 24 hours and 7 days after ischemic induction. **(C)** Glutamate serum levels of ischemic animals represented as % normalized to baseline levels; animals treated with riboflavin showed a significant reduction of glutamate levels 2 hours after administration compared to control group. **(D)** GOT activity of ischemic animals represented as percentage normalized to baseline levels; no increase of activity was detected in any of the groups. **(E)** Bederson scale and

759 **(F)** Cylinder Test, no functional improvement was detected through the sensorimotoric
760 test performed.



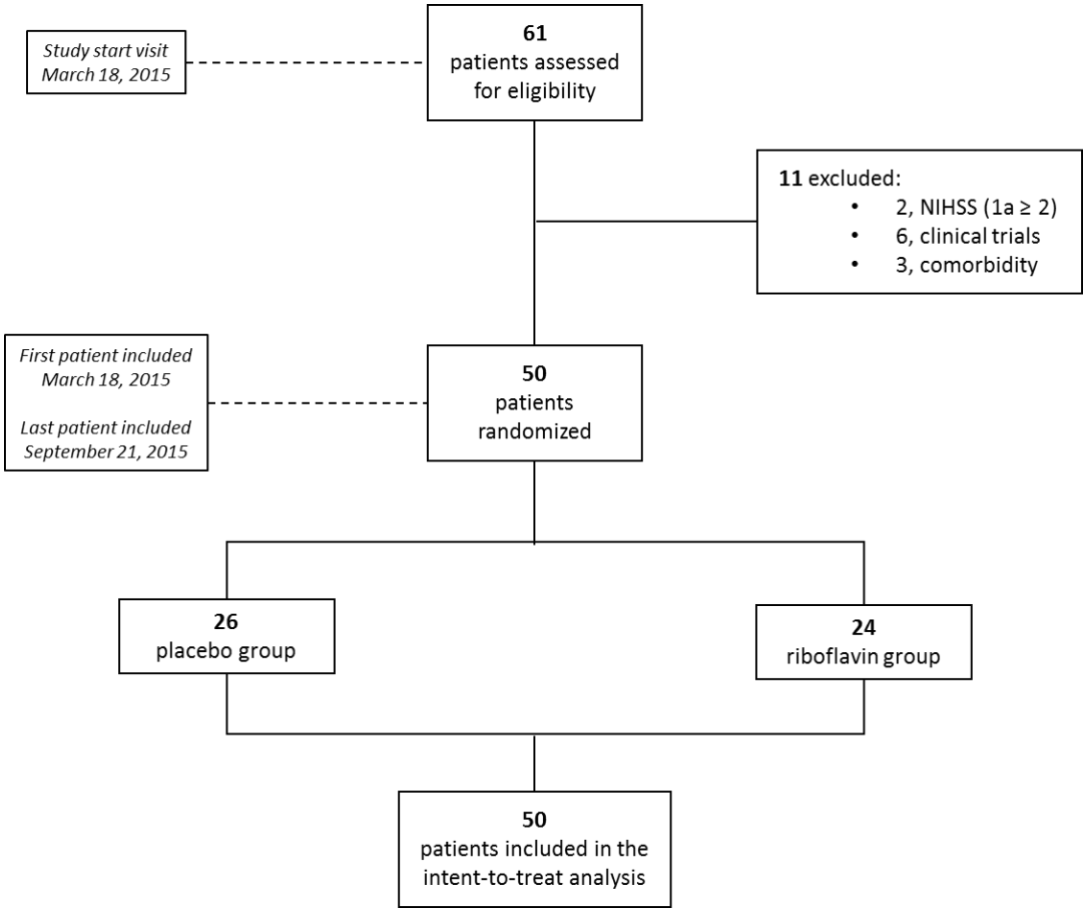
761

762 **Figure 4.** Evaluation of riboflavin effect in hemorrhagic induced animals. **(A)** T2Wi of
763 hemorrhagic animals treated with saline and riboflavin 1 mg/kg. **(B)** Hemorrhage volume
764 profile of treated animals with no differences between treated and control groups. **(C)**
765 Bederson scale and **(D)** Cylinder Test; no functional improvement was observed in
766 riboflavin treated animals compared to control group.



767

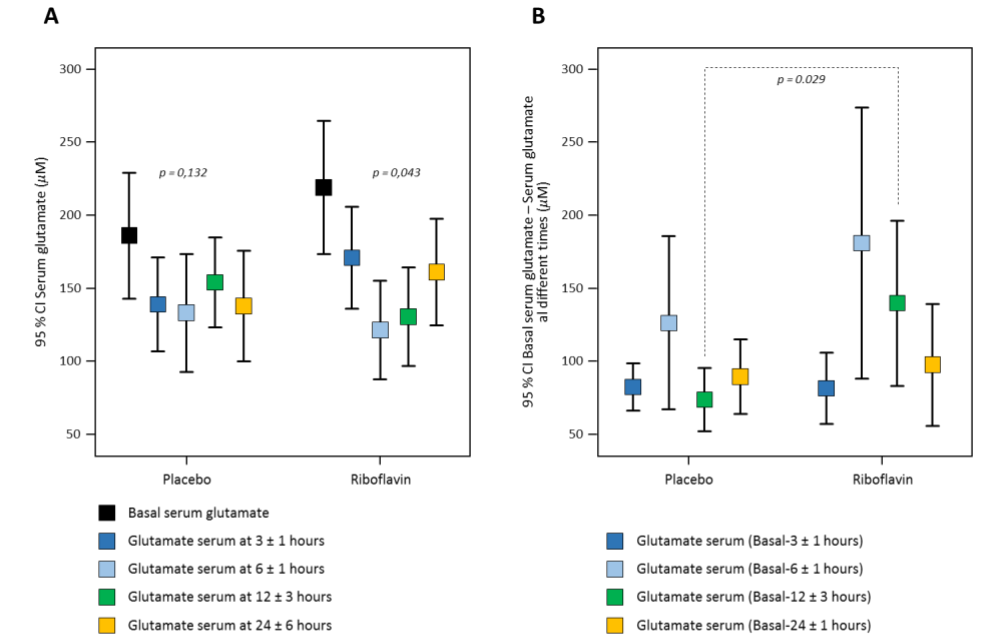
768 **Figure 5.** Determination of the specific effect of riboflavin as glutamate grabber and possible
769 interactions. **(A)** Resonance spectrum of riboflavin and GOT with no weak interaction
770 between them obtained in a STD-NMR experiment. **(B)** Different variations of riboflavin
771 structures used for determine the specific effect of riboflavin as a glutamate grabber. **(C)**
772 GOT activity inhibition by different riboflavin forms, FAD, Riboflavin, TARF, and Lumichrome,
773 oxaloacetate was used as a positive control for inhibition of glutamate formation. Values
774 are represented in % normalized to positive control (only GOT) **(D)** Glutamate serum levels
775 of animals treated with Lumichrome, FAD, Riboflavin, and TARF all at 1 mg/Kg. Saline treated
776 animals were the control group.

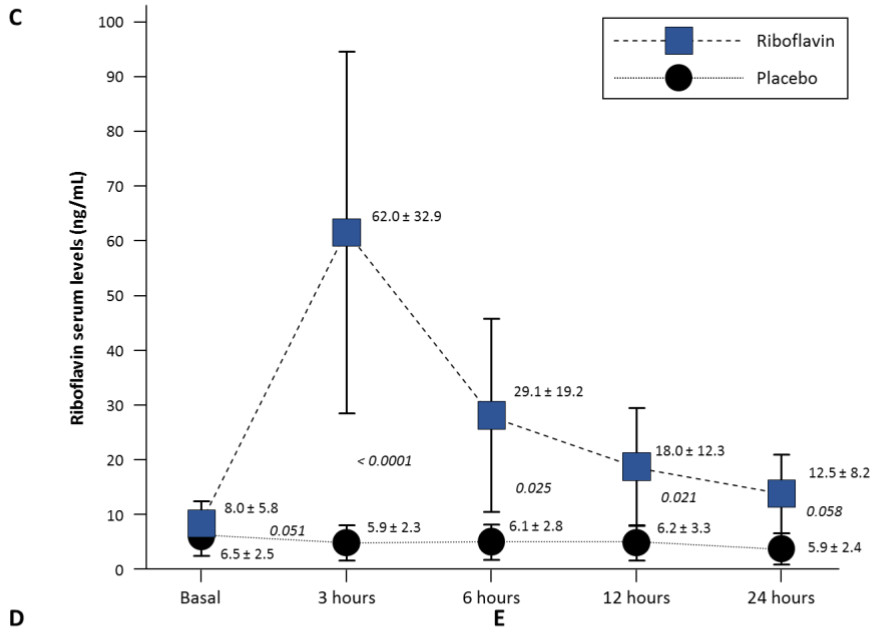


777

778 **Figure 6.** Proof of concept clinical trial flow chart. Patients included in the two arms and the

779 main exclusion causes.


A
95 % CI Serum glutamate (µM)
p = 0,132
p = 0,043
Placebo
Riboflavin
Basal serum glutamate
Glutamate serum at 3 ± 1 hours
Glutamate serum at 6 ± 1 hours
Glutamate serum at 12 ± 3 hours
Glutamate serum at 24 ± 6 hours
B
95 % CI Basal serum glutamate – Serum glutamate al different times (µM)
p = 0.029
Placebo
Riboflavin
Glutamate serum (Basal-3 ± 1 hours)
Glutamate serum (Basal-6 ± 1 hours)
Glutamate serum (Basal-12 ± 3 hours)
Glutamate serum (Basal-24 ± 1 hours)


C
Riboflavin
Placebo
Riboflavin serum levels (ng/mL)
62.0 ± 32.9
29.1 ± 19.2
18.0 ± 12.3
12.5 ± 8.2
8.0 ± 5.8
6.5 ± 2.5
5.9 ± 2.3
6.1 ± 2.8
6.2 ± 3.3
5.9 ± 2.4
< 0.0001
0.025
0.021
0.058
0.051
Basal
3 hours
6 hours
12 hours
24 hours

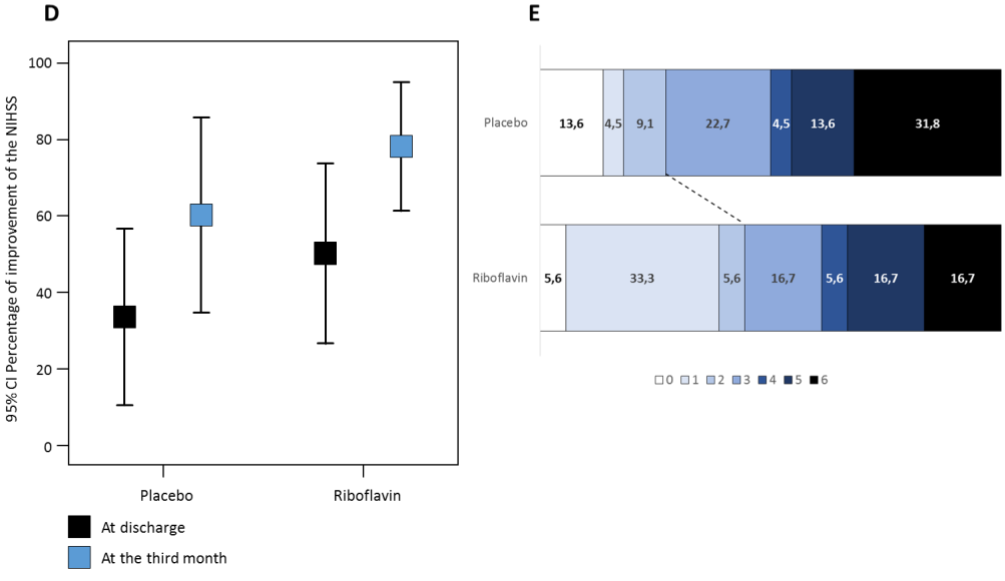

D
95% CI Percentage of improvement of the NIHSS
Placebo
Riboflavin
At discharge
At the third month
E
Placebo
13,6
4,5
9,1
22,7
4,5
13,6
31,8
Riboflavin
5,6
33,3
5,6
16,7
5,6
16,7
16,7
0 1 2 3 4 5 6

Figure 7. Proof of concept clinical trial main results **(A)** Glutamate serum levels of both groups at different time points. Riboflavin treated groups shows a significant reduction of glutamate ($p<0.043$) in a one way ANOVA test. **(B)** Glutamate serum levels difference between baseline levels and each of the different time points. Difference at 12 hours reach significant levels in treated group compared to placebo. **(C)** Riboflavin clearance profile in serum of treated group compared to ischemic

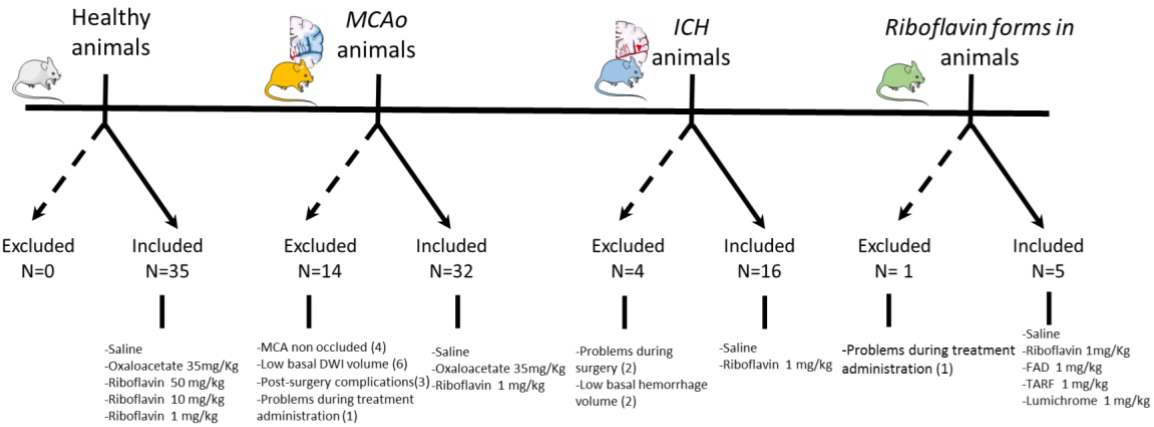


Figure 8. Experimental animal design with included and excluded animals, reason of exclusion and groups of the included animals.

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