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հայցման ատենախոսության

ՍԵՂՄԱԳԻՐ

ԵՐԵՎԱՆ - 2022

NATIONAL ACADEMY OF SCIENCE OF REPUBLIC OF ARMENENIA
INSTITUTE OF MOLECULAR BIOLOGY

DANIELYAN KRISTINE EDGAR

PROPHYLACTIC AND THERAPEUTIC EFFECT OF THE NOVEL COMPOUNDS IN THE EXPERIMENTAL STROKE

SYNOPSIS

of Dissertation Submitted for the Degree
of Doctor of Biological Sciences (D.Sc.) in the Field of
03.00.04. “Biochemistry”

YEREVAN - 2022

Ատենախոսության թեման հաստատվել է ՀՀ ԳԱԱ Հ. Բունիաթյանի անվան կենսաքիմիայի ինստիտուտի գիտական խորհրդում:

Գիտական խորհրդատու՝	կենս. գիտ. դոկտոր Սամվել Գրիգորի Չաիլյան
Պաշտոնական ընդդիմախոսներ՝	կենս. գիտ. դոկտոր, պրոֆ. Մաքսիմ Արշալույսի Սիմոնյան կենս. գիտ. դոկտոր, պրոֆ. Ջոն Սարգսի Սարգսյան բնական գիտ. դոկտոր, պրոֆ. Կարստեն Քուլմզի
Առաջատար կազմակերպություն՝	Հայ-Ռուսական համալսարան

Ատենախոսության պաշտպանությունը տեղի կունենա 2022 թ. դեկտեմբերի 19-ին, ժամը 14:00-ին, ՀՀ ԳԱԱ մոլեկուլային կենսաբանության ինստիտուտում, 042 մասնագիտական խորհրդի նիստում (ՀՀ 0014, ք. Երևան, Հասրաթյան 7):

Ատենախոսությանը կարելի է ծանոթանալ ՀՀ ԳԱԱ մոլեկուլային կենսաբանության ինստիտուտի գրադարանում և http://molbiol.sci.am/ կայքում:

Ատենախոսության սեղմագիրն առաքվել է 2022 թ. նոյեմբերի 7-ին:

042 մասնագիտական խորհրդի գիտական քարտուղար,
կենս. գիտ. թեկնածու Զ.Ա. Խաչատրյան

Dissertation topic approved at the Scientific Council of the Institute of Biochemistry named after Hr. Buniatian, NAS RA.

Scientific Consultatnt:	D.Sc., Prof. Samvel Grigor Chailyan
Official opponents:	D.Sc., Prof. Maxim Arshaluys Simonyan D.Sc., Prof. John Sargis Sarkissian Dr. rer. nat., Prof. Carsten Culmsee
Leading organization:	Russian-Armenian University

The defense of the dissertation will be held on 19 December 2022, at 14:00 at the session of the specialized council 042 acting in the Institute of Molecular Biology NAS RA (Hasratyan 7, 0014, Yerevan, RA).

The dissertation is available at the library of the Institute of Molecular Biology NAS RA and at the website http://molbiol.sci.am/.

Synopsis was sent out on 7 November 2022.

Scientific secretary of the specialized council 042
PhD Z.A. Khachatryan

DESCRIPTION OF THE WORK

Applicability of results. Armenia is a country with the total population of 3,215,800 (April 2006 est.) in the Caucasus, which is the region in Eurasia situated in the heart of Western Asia and Eastern Europe.

Poststroke treatment in Armenia includes influencing on the rennin-angiotensine system (captopril, enalapril, perindopril, valsartan, losartan, lisinopril, ramipril), calcium channels (amilodipine), antibiotics (ceftriaxone), analgetics (analgin, dimedrol), aspirin (cardiomagnyl), compounds improving the metabolic processes (cerebrolisyn), antioxidants (mexidol), for the diabetic patients with the stroke (diabeton), compounds minimizing blood glucose, etc. From 2015 alteplase, the derivate of tissue type plasminogen activator, has been utilized in Armenia, along with the thromboectomy. These two treatment attitudes dramatically decreased the mortality rate of stoke patients in Armenia.

However, stroke remains one of the leading causes of the death in the entire word. Thus, new solutions for the treatment as well as the prevention of pathology are supposed to be discovered.

My study includes investigations of not only the mechanisms of the stroke development, prevention but also creation and improvement of existing and new compounds, interfering and preventing stroke.

The work is consisting from the description of the medicines, targeting almost all stages of pathology development.

The presented work includes evaluation and delineation of the neuroprotectants such as albumin and peptide PRP-1, the compound, which for the first time was isolated from the bovine hypothalamus by A.A. Galoyan and co-workers (Galoyan 2004).

Creation and evaluation of the effective and safe thrombolytics is another path of the results, presented in my work. It was suggested, coupling the thrombolytics with erythrocytes, which can be entrapped in the newly forming clot, can lyse the thrombi from the within. In addition, alteplase, coupled with RBC, as the one of the best biological carrier, was guaranteeing the long and safe circulation. Above mentioned hypothesis was proved also by the other modifications, such as anti CR-1 antibodies coupled with the alteplase, along with anti-PECAM short structures scFv-uPA as the stroke prophylactic and therapeutic agents.

The interchangeable dependence of purines and pyrimidies metabolism was demonstrated earlier by Zollner, et al (Elion, Kelley et al. 1978). Elion and coworkers showed that **purine** isomers can be converted to various nucleotides, which influence **pyrimidine** metabolism and contra verse **(Muir, Harrow et al. 2008).** Thus, targeting an enzyme of purine catabolism consequentially influences the pyrimidine catabolism.

It was shown, allopurinol, which is the isomer of **purine**- hypoxanthine, might be oxidized to the corresponding isomer of xanthine, called oxypurinol. Both substances inhibit Xanthine Oxidoreductase (XOR; EC 1.17.3.2), which decreases the formation of uric acid, ROS, lowering uric acid concentration in the plasma and excretion into the gut and in the urine, and elevating the excretion of hypoxanthine and xanthine in the urine. For the first time, based on the work it was proved, that pyridoxine is directly inhibiting the activity of XOR and that inhibition might increase the number of the alive, as well as newly formed neuronal cells in the hippocampal and cortical cell cultures.

Another important enzyme of nucleotides synthesis – PRPS-1 (Phosphoribosyl Pyrophosphate Synthetase 1; EC 2.7.6.1) was studied as well. It was proved experimentally that in pathological conditions activation of PRPS-1 and inhibition of XO activity, leads to the enrichment of the nucleotide pool, which triggers the key mechanisms for regenerative cells proliferation.

Proline Rich Peptide-1 (PRP-1) was investigated in the conditions of oxidative stress of the neuronal cell culture and in vivo, in the conditions of experimental stroke (in vivo results are not included). It was concluded, PRP-1 is neuroprotective.

The entire work is devoted to the investigation of the mechanisms of regeneration, development and creation of the new pro-medical compounds, targeting different stages of the stroke.

THE AIMS OF THE WORK

1. Investigate and find the key regulatory enzymes in purine and pyrimidine metabolism.
2. Establishment of the pattern of biochemical mechanisms of regulation by the effectors of purine and pyrimidine metabolism
3. Investigation of the regulatory purine/pyrimidine metabolism-linked regenerative some mechanisms of the experimental stroke.
4. Investigation of PRP-1 abilities in the processes of neuroprotection in the settings of experimental stroke.
5. To study the albumin's brain blood barrier preserving abilities.
6. Delineation of the albumin particles's abilities as the medicines` carriers.
7. Targeting the thrombolysis mechanisms in the experimental stroke.
8. Creation of tPA and urokinase safe analogs for the experimental stroke treatment.

NOVELTY OF THE WORK

For the first time it is clarified the role of purine/pyrimidine metabolism in the processes of the cell proliferation and regeneration.

The effect of subcomponents of vitamin B complex, as the inhibitors of XO complex were analyzed on the processes of cells proliferation in vitro and in vivo in the settings of the poststroke recovery.

PRP-1 neuroprotective abilities are delineated in vitro.

As a novel direction, there are created and tested in vivo thrombolytic agents as well as their derivates.

The albumin nanoparticles coated with the allopurinol are created for the treatment of stroke in the experimental setting.

MAIN CLINICAL PERSPECTIVE

Cerebrovascular thrombosis is a major cause of mortality and morbidity, including after surgery when the use of thromboprophylaxis is limited by the formidable risk of perioperative bleeding. Nor can thrombolytics (eg, tissue-type plasminogen activator [tPA]) be used prophylactically in this and other high-risk settings because of their short duration of action and risk of causing hemorrhage. Coupling tPA to red blood cells (RBCs) prolongs and confines activity within the bloodstream and enhances resistance to plasma inhibitors, thereby providing a novel thromboprophylactic agent, RBC/tPA. Our results indicate that injection of RBC/tPA into mice before thromboembolic occlusion of the middle carotid artery facilitates clot lysis, assists in rapid and stable cerebrovascular reperfusion, alleviates ischemic brain damage, and eliminates mortality, whereas pretreatment with tPA is not protective even at a 10-fold higher dose at which mortality is aggravated. At protective doses, RBC/tPA did not consume plasma fibrinogen or cause postsurgical bleeding or hemorrhage and toxicity in the central nervous system in rodent models of brain ischemia and thrombosis. These animal studies indicate that RBC/tPA might provide thromboprophylaxis in patients at risk for cerebrovascular thromboembolism in the postoperative period; after transient ischemic attack, myocardial infarction, and stroke; or in the setting of non–ST-elevation acute myocardial infarction, which is characterized by multiple cycles of intravascular rethrombosis and incomplete thrombolysis. Nascent and growing occlusive clots represent the preferable target for RBC/tPA. Use of injectable antithrombotic prodrugs conjugated with RBC binding peptides may further enhance the clinical application of this new approach toward prophylactic thrombolysis within the cerebral and other vasculatures.

It was delineated the main biochemical mechanism triggering the proliferation of the cells, which might be used in the medicine for the stimulation of the regenerative processes. Vitamin B6 might be used in the stroke clinic as the antioxidative compound.

RESULTS

Neuroprotection

PRP-1 reduces staurosporine-induced apoptosis

In our investigations we evaluated the neuroprotective abilities of PRP-1 in neuronal cell culture. The primary cultures of neurons from postnatal (P1) rat hippocampi contained astrocytes up to max. 40%. Overall, cell death in neurons was less than 20% (Fig. 1 A). The highest concentration of PRP-1 used in our studies, 10–5 g/ ml, did not reduce this background damage and was not toxic either (**Fig. 1 A**). Searching for a neuroprotective effect of PRP-1 cells were pretreated for 6, 8 and 10 h with 10–5 g/ml of this peptide prior to the induction of cell death. The results showed significant neuroprotection by PRP-1 if added 8 h before staurosporine (**Fig. 1 A**). It was examined the effect of various concentrations of PRP-1 added 2 h prior to staurosporine. Increasing concentrations of PRP-1 resulted in a slight yet dose-dependent and significant reduction of the number of apoptotic cells (**Fig. 1 B**). Staining with Hoechst 33258 revealed the classical apoptotic nuclear features after treatment with staurosporine (**Fig. 1 D**). In contrast, cell death was much less pronounced in vehicle-treated neurons (**Fig. 1 C**) and in neurons treated with PRP-1 (**Fig. 1 E**). PRP-1 and vasopressin are closely related peptides (80– 93% identity, depending on species) and produced by the same neurosecretory nuclei of the hypothalamus. We were using [arg8]vasopressin to address the specificity of neuroprotection described above. Similar to PRP-1 vasopressin also did not influence the basal rate of cell death. However, preincubation of the cells for 8 h with [arg8]vasopressin did not reduce staurosporine-induced apoptosis indicating specificity of PRP-1 for the neuroprotective effect.

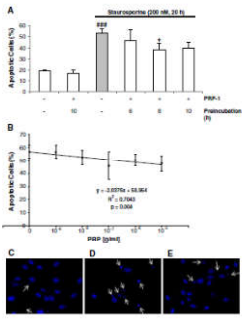

Fig. 1. Effect of PRP-1 on apoptosis of postnatal hippocampal cells. Cell death was induced by staurosporine (200 nM) and monitored after staining with Hoechst 33258. (A) Preincubation. Cells were preincubated with PRP-1 (10– 5 g/ml) for 6, 8 or 10 h prior to the onset of apoptosis by staurosporine. ###P J 0.001, different from untreated cells; +P J 0.05, compared to staurosporine treated cells in the absence of PRP-1 (ANOVA, Scheffe´s test). (B) Concentration dependence. PRP-1 was added up to 10–5 g/ ml. Hoechst 33258 staining of (C) control cells, (D) staurosporine-treated and (E) PRP-protected staurosporinetreated neurons.

Here it is shown, that PRP-1 is a potent regulator of neuronal apoptosis as we provide evidence that PRP-1 significantly can reduce inducible apoptosis of postnatal hippocampal cells. The observed protective effect of PRP-1 on apoptotic cell death was shown to be both time- and dose-dependent. PRP-1 reduced staurosporine-induced apoptosis of hippocampal neuronal apoptosis more pronounced upon long-term pre-treatment (8 h) before the induction of apoptosis.

Albumin Treatment Reduces Neurological Deficit and Protects Blood–Brain Barrier Integrity after Acute Intracortical Hematoma in the Rat. Intracerebral hemorrhage (ICH) constitutes ≈10% to 15% of all strokes and is associated with high morbidity and mortality. Thirty-day case fatality rates of ≈35% have been reported in hospital-based studies and up to 52% in community-

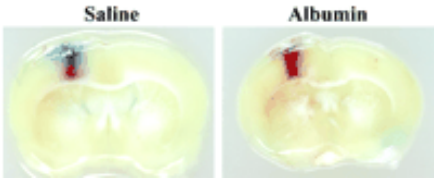

Figure 2. Coronal sections of representative saline- and albumin-treated animals with left intracortical hematoma and 2-day survival, administered Evans blue intravenously 2 hours before they were killed. Evans blue extravasation is grossly visible in the vehicle-treated animal, and substantial inhibition of the dye extravasation is evident in albumin-treated brain.

based studies, (Ginsberg and Bogousslavsky 1998) rates that are 2- to 6-fold higher than in ischemic stroke. Among chronic ICH survivors, nearly half are dependent on outside help in their activities of daily living, and only 20% of patients become independent at 6 months. (Fogelholm, Nuutila et al. 1992) To date, the medical management of ICH has had only a minimal impact on the disease, and the preclinical development of protective strategies has lagged markedly behind ischemic stroke.

Human albumin therapy, in moderate-to-high doses, has proved markedly neuroprotective in experimental models of focal (Belayev, Busto et al. 1997, Belayev, Zhao et al. 1998, Belayev, Liu et al. 2001, Liu, Belayev et al. 2001) and global (Belayev, Saul et al. 1999) cerebral ischemia and in traumatic brain injury.(Belayev, Alonso et al. 1999) In my study, it was extended the therapeutic applications of albumin therapy to the setting of ICH. It is the evidencing, that albumin therapy exerts a substantial protective effect in this setting. The experiments were performed in SD rats with the injection of the whole blood intracranially. The damage of the tissue as well as Blood Brain Barrier (BBB) disruption was evaluated by the injection of Evans Blue, extraction of the last one and spectrophotometrical quantative analysis. It is concluded that preinjection and continued treatment of the animals with albumin reduced brain damage in experimental settings (**Fig. 2**).

Albumin microparticles as the carriers for the allopurinol. In comparison with the synthetic polymers, proteins have several advantages: they might be degraded by the naturally occurred enzymes into the peptides. In contrast to protaines, chemically synthesized nanomolecules may accumulate in the body and result in toxic degradation products (Elzoghby, Samy et al. 2012). Electrostatic interactions, hydrophobic attractions and covalent bindings are the mechanisms allowing the attachment of the drug to the carrier. Also, these nanoparticles are able to be modified and presented the attached molecules to the surface of the targeted cells (Elzoghby, Samy et al. 2012).

In the current my work it is introduced the method for the formation of the albumin particles, which are coated with the allopurinol solution.

The assumption is that the albumin nanoparticles carrying allopurinol will not possess with side-negative effects including triggering the liver necrosis. Also, the frequency of the treatment after utility of the coated by allopurinol particles in comparison with the free floating albumin, will be less which will raise the efficacy of this medicine.

It is evaluated the number of the formed particles after mild changes of the experimental conditions. After 24 hours of mixture incubation, the particles were centrifuged for 15 minutes, RPM=8000. The precipitate was resuspended in water and the samples were evaluated with the utility of the light as well as contrast phase microscope. The maximal number of the particles was observed in the samples with the addition of 20 mg of the protein 699.00 ±35.87. The results indicated that in the samples with the addition of 200 mg of the albumin the number was equal to 468.00 ± 51.87, whereas addition of 500 mg resulted with the consequential formation of 288.76 ± 17.83 particles (**Fig.3**). It is quite interesting to mention that shape of the particles was spheroid. These particles as

have showed investigations might attach up to 97-100 % of allopurinol and stayed stable up to 24 hours.

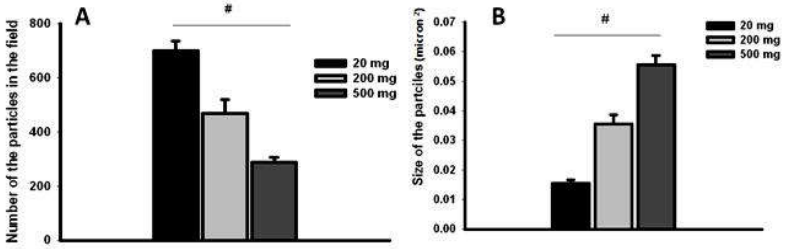

Figure 3. Determination of the particle size after addition of different quantities of albumin. A. The number of the particles was calculated based on the micropictures and after utility of Pixcavator 6.0 program. The significances between groups for 20, 200, 500 mg of added albumin, reflected by p were lower than 0.05 (ONE-WAY-ANOVA). B. The size of the particles, surface area, is presented as the microns 2. The significances between groups for 20, 200, 500 mg of added albumin, reflected by p were lower than 0.05 (ONE-WAY-ANOVA).

Thus, it is adopted the method for the generation 1-5 micron spheroid particles. The method to

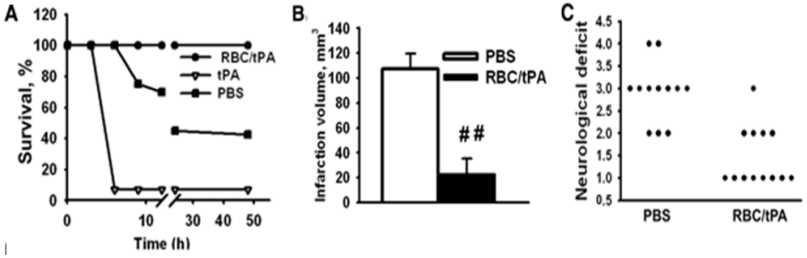

Figure 4. Cerebral thromboprophylaxis by RBC/tPA protects against CNS damage. A, RBC/tPA eliminates postembolic mortality. Survival of mice injected with PBS (▪; n=32), tPA (▽; n=13), or RBC/tPA (•; n=13) 30 minutes before emboli. The difference between RBC/tPA and PBS- and tPA-treated groups is significant (P<0.001). B, RBC/tPA reduces volume of postembolic infarction in the brain. Total infarct volume determined by triphenyltetrazolium chloride staining of brain tissue sections 48 hours after emboli in the RBC/tPA-treated (closed bar; n=7) and in survivors of PBS-treated groups (open bar; n=10) (##P<0.01). C, RBC/tPA alleviates postembolic neurological deficit. Neurological deficit in RBC/tPA-treated (right; n=13) and PBS-treated (left; n=12) survivors 48 hours after emboli, as described in Methods (minimal score, 1; maximal score, 4). The difference between the 2 groups is significant (P<0.001).

assess the allopurinol quantity in the coated particles was developed, which might be useful in the future not only for the treatment of stroke but also ischemic stroke.

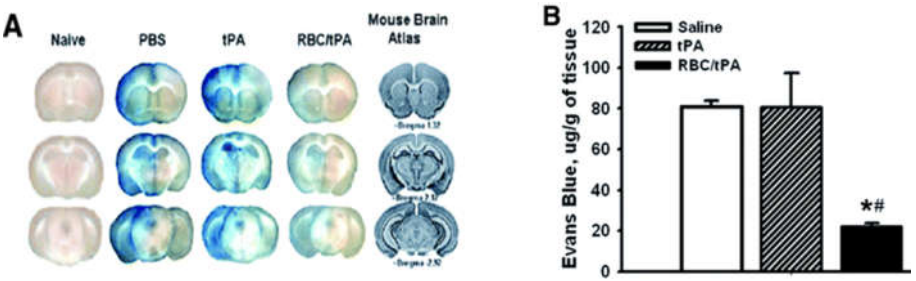

Figure 5. RBC/tPA thromboprophylaxis reduces postembolic cerebral edema. Mice were injected with PBS, 2 mg/kg tPA, or 0.2 mg/kg RBC/tPA through the femoral vein. Thirty minutes later, emboli were injected via the right MCA. Evans blue was injected intravenously 3 hours after emboli, and extravasation of dye into the brain was measured 2 hours later. A, Representative brain sections of naïve mice injected with Evans blue (first set), mice injected before emboli with PBS (second set), tPA before emboli (third set), or RBC/tPA before emboli (fourth set). B, Level of Evans blue extracted from brain of mice injected 30 minutes before emboli with PBS (open bar), soluble tPA (hatched bar), or RBC/tPA (closed bar). Between-group statistical differences are indicated (**P*<0.01 vs PBS; #*P*<0.01 vs tPA). n=4 mice per group.

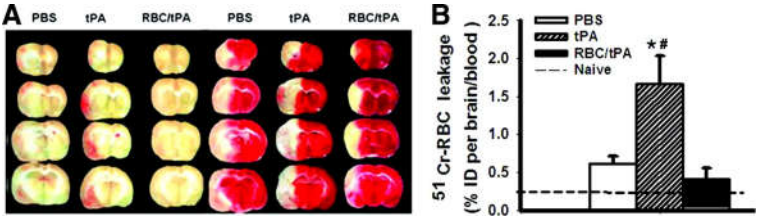

Figure 6. RBC/tPA does not cause hemorrhagic transformation after cerebral thrombosis in SHRs. SHRs were injected via the MCA with a 3-cm fibrin clot, followed 6 hours later by an intravenous injection of PBS, 10 mg/kg tPA, or 0.05 mg/kg RBC/tPA, each mixed with a suspension of ^{51}Cr-labeled rat RBCs (≈2×10^5 cpm/rat). Rats were killed 18 hours later and perfused with citrate/PBS to remove residual blood. A, Typical images of hemorrhages and infarctions in rat brain tissue sections from the indicated groups of SHRs. The area of hemorrhage was measured in fresh brain sections (reddish color in the 3 sets on the left), and the infarction zone was determined with triphenyltetrazolium chloride staining (white in the 3 sets on the right). B, RBC/tPA does not provoke RBC extravasation in the postthrombotic SHR brain. ^{51}Cr-RBC uptake in the brain was measured in postthrombotic SHRs injected with PBS (open bar; n=6), tPA (hatched bar; n=5), or RBC/tPA (closed bar; n=4). The dashed line shows the background level of ^{51}Cr-RBC retention in the brain. Data are shown as percent of ^{51}Cr brain uptake normalized to the level in blood to account for differences in brain enlargement (percentage of injected ^{51}Cr detected in the brain divided by percentage of injected ^{51}Cr per 1 g blood and multiplied by 100). tPA treatment significantly elevated ^{51}Cr-RBC extravasation vs groups treated with PBS (**P*<0.05) and RBC/tPA (#*P*<0.05).

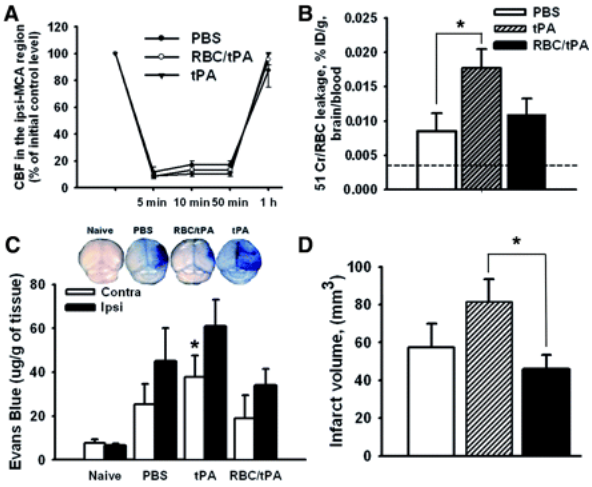

Figure 7. RBC/tPA does not adversely affect ischemic brain. The MCA was occluded for 1 hour with a silicon-coated nylon filament. After filament removal, mice were immediately injected intravenously with PBS, 0.2 mg/kg RBC/tPA, or 10 mg/kg tPA. Mice were injected 3 hours later with ^{51}Cr-RBC and Evans blue, killed 2 hours later, and perfused with PBS-heparin at death. Extravasation of ^{51}Cr-labeled RBC (B) and dye (C) into the brain and infarct volume (D) were measured and calculated as described in Figures 5A, 2B, and 3B, respectively. A, Similar extent of ischemia in experimental groups. Laser Doppler analysis of vascular occlusion and reperfusion. B, RBC/tPA does not exacerbate RBC extravasation in the postischemic mouse brain. Mice were injected with PBS (open bar; n=6), tPA (hatched bar; n=3), or RBC/tPA (closed bar; n=3). The dashed line shows the background level of ^{51}Cr-RBC retention in the brain of intact mice. In contrast to RBC/tPA, tPA significantly increased the ^{51}Cr-RBC leakage in brain (**P*<0.05 vs PBS). C, RBC/tPA does not enhance Evans blue uptake in the postischemic brain in mice. The Evans blue content in the ipsilateral (closed bars) and contralateral (open bars) hemispheres in naïve mice (n=4) and in postischemic mice injected with PBS (n=6), tPA (n=4), or RBC/tPA (n=3). The difference between postischemic and naïve mice in the contralateral hemisphere is significant in tPA-injected mice (**P*<0.05). The difference between postischemic and naïve mice is significant in the ipsilateral hemisphere (*P*<0.05) for PBS- and RBC/tPA-injected mice and in tPA-injected mice (*P*<0.005). d, RBC/tPA does not augment postischemic infarct volume. Infarct size was measured 48 hours after ischemia in a separate cohort of mice injected with PBS (open bar; n=4), tPA (hatched bar; n=3), or RBC/tPA (closed bar; n=4). Compared with tPA injection, RBC/tPA injection resulted in significantly lower infarct volume (**P*<0.05).

Thrombolytic agents
RBC –coupled tPA

Coupling to red blood cells (RBCs) converts tPA into a prophylactic agent, RBC/tPA, that effectively lyses nascent thrombi that otherwise may cause sustained vascular occlusion.(Murciano, Medinilla et al. 2003, Zaitsev, Danielyan et al. 2006) The large size of RBC/tPA precludes it from entering and lysing preexisting clots and prevents extravasation, thereby limiting CNS toxicity. Studies in animals have shown that tPA carriage by RBCs prolongs its circulation by orders of magnitude, permitting prophylactic administration; permits tPA access to the interior of nascent intravascular clots, which are then rapidly lysed from within; and blocks tPA penetration into hemostatic clots.(Murciano, Medinilla et al. 2003, Ganguly, Krasik et al. 2005, Ganguly, Goel et al. 2006, Zaitsev, Danielyan et al. 2006, Ganguly, Murciano et al. 2007) We sought to characterize the effectiveness and toxicity of prophylactic administration of RBC/tPA in animal models of cerebral thromboembolism and ischemia (**Figures 4, 5, 6, 7**).

Human complement receptor type 1–directed loading of tissue plasminogen activator on circulating erythrocytes for prophylactic fibrinolysis

Ex vivo tPA conjugation to isolated RBCs followed by reinjection of the RBC/tPA complexes may be applicable to settings in which the risk of thrombosis can be anticipated and transfusion is part of routine clinical care (eg, patients with sickle cell disease). However, phlebotomy, ex vivo loading, and reinfusion of the modified RBCs are impractical in most settings where the use of such therapy could be envisioned. We hypothesized that targeting PAs directly to circulating RBCs would markedly improve the speed, safety, dosing, efficacy, and utility of this drug-delivery strategy.

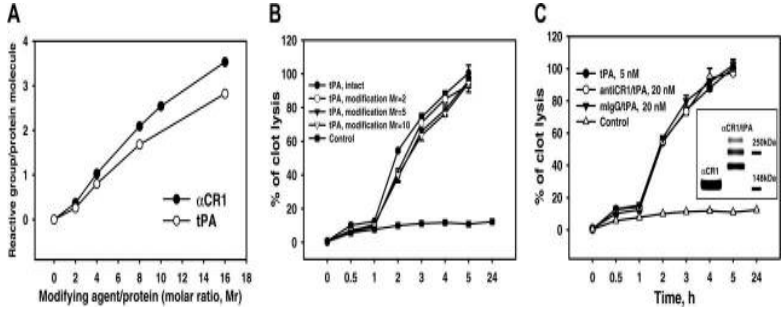

Figure 8. Synthesis and fibrinolytic activity of anti-CR1/tPA and IgG/tPA conjugates. (A) Modification curves of anti-CR1 IgG and tPA at indicated molar ratios (Mr) of SATA and SMCC, respectively, determined with Ellman reagent. (B) In vitro lysis of ^{125}I-fibrinogen clots by 5 nM tPA or SMCC-modified tPA. (C) In vitro fibrinolysis of ^{125}I-fibrinogen clots by indicated amounts of tPA or tPA conjugates. The data in this and the following figures are presented as the mean plus or minus SEM. Unless specified otherwise, in vitro fibrinolysis data show results of triplicates for each condition. The insert shows anti-CR1/tPA analysis using a 4% to 12% gradient SDS-PAGE run in Tris-Glycine buffer under nonreducing conditions. The results show maternal anti-CR1 IgG (left band) and the anti-Cr1/tPA conjugate (right band) containing species with 1, 2, or 3 molecules of tPA per molecule of IgG.

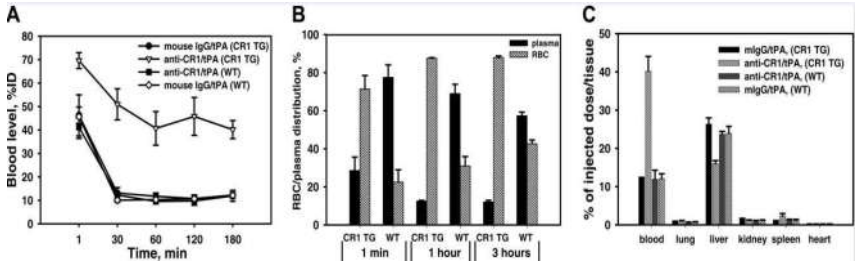

Figure 9. Pharmacokinetics of anti-CR1/^{125}I-tPA and IgG/^{125}I-tPA in TgN-hCR1 mice versus WT mice. (A) Blood clearance of conjugates. Wild-type (WT) and TgN-hCR1 mice (CR1 TG) were injected with ^{125}I-tPA–containing conjugates, and percent of the injected dose (%ID) in the blood was measured. (B) Percent of ^{125}Iodine recovered in plasma (■) versus RBC pellet (hatched bars) in blood obtained 1 minute, 1 hour, or 3 hours after injection of ^{125}I-labeled conjugates in TgN-hCR1 or WT mice. (C) Organ distribution of anti-CR1/^{125}I-tPA versus IgG/^{125}I-tPA 3 hours after injection in TgN-hCR1 and WT mice. (The number of animals in all experiments is 5 per group.)

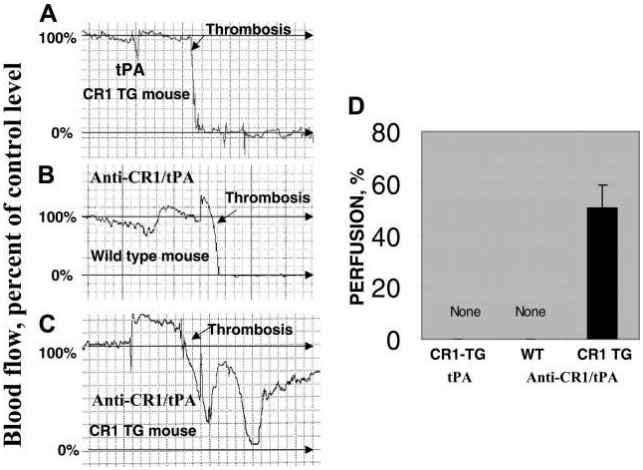

Figure 10. Coupling of tPA to circulating RBCs reduces rebleeding. (A) Soluble tPA caused greater lysis of hemostatic clots than anti-CR1/tPA. Segments from the tails of TgN-hCR1 mice were amputated, and 5 minutes after hemostasis was attained, the tails were immersed into warm saline. PBS, tPA, or anti-CR1/tPA (2 mg/kg tPA each) was injected through the jugular vein. The amount of hemoglobin released from the tail over the ensuing hour was measured. (B, C) Schematic comparison of vascular delivery of tPA and anti-CR1/tPA. (B) Rapid clearance by liver, among other reasons, prohibits prophylactic use and dictates injection of large doses of tPA, which diffuses into hemostatic clots and tissues, causing bleeding and side effects. (C) Injected anti-CR1/tPA binds predominantly to RBCs, circulates for a prolonged time without access to preexisting hemostatic clots and extravascular tissues, while incorporating into and dissolving nascent intravascular clots.

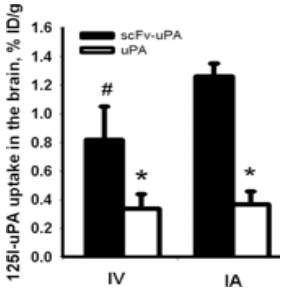

Figure. 11. Accumulation of anti-PECAM scFv-uPA in the cerebral vasculature in mice. Brain uptake 1 h after injection of ^{125}I-labeled scFv-uPA (closed bars) or equimolar amounts of ^{125}I-uPA (open bars) in anesthetized mice via the carotid artery (i.a.) or jugular vein (i.v.). Unless specified otherwise, the data are shown as mean ± S.E.M. n = 3 mice per group; *, $p < 0.05$ scFv-uPA versus uPA; #, $p < 0.05$ i.a. versus i.v. for scFv-uPA.

To achieve this goal, we conjugated tPA to a monoclonal antibody (mAb 7G9) directed against complement receptor type 1 (CR1),(Fearon 1980, Lindorfer, Hahn et al. 2001) generating an anti-CR1/tPA conjugate.

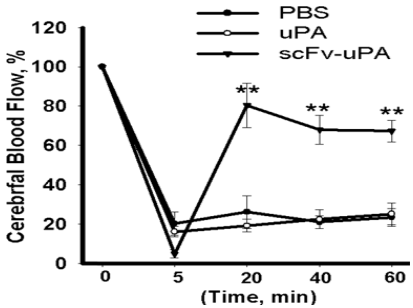

Figure. 12. Prophylactic cerebrovascular immunotargeting of anti-PECAM scFv-uPA facilitates post-thrombotic reperfusion. Mice were given an i.a. injection of PBS (closed circles), 4 mg/kg uPA (open circles), or 4 mg/kg scFv-uPA (closed triangles) followed 10 min later by an i.a. injection of fibrin thrombi. Cerebral blood flow in the MCA distribution was measured by Doppler ultrasound prior (time 0) and at the indicated times after injection of thrombi. **, $p < 0.01$ for scFv-uPA versus PBS and uPA (n = 4, 10, and 4 per group, respectively).

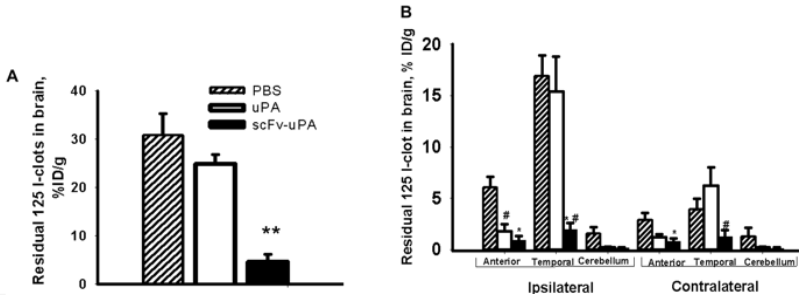

Figure. 13. Fibrinolytic effect of anti-PECAM scFv-uPA in the cerebral vasculature. A, residual ^{125}I-labeled fibrin emboli in the brain 1 h after injection of PBS (hatched bar), 4 mg/kg uPA (open bar), or 4 mg/kg scFv-uPA (closed bar) into the right MCA 10 min before embolization. Asterisks indicate $p < 0.01$ for scFv-uPA versus PBS and uPA (n = 10, 5, and 4 mice per group, respectively). B, distribution of residual ^{125}I-labeled fibrin emboli in the indicated areas of ipsilateral (right) and contralateral (left) hemispheres. *, $p < 0.05$ scFv-uPA versus PBS and uPA; **, $p < 0.01$ scFv-uPA versus PBS; and #, $p < 0.05$ uPA versus PBS.

site for tPA in blood (Fearon 1980, Nickells, Hauhart et al. 1998, Krych-Goldberg and Atkinson 2001, Lindorfer, Hahn et al. 2001); the remaining CR1 is expressed on neutrophils and lymphocytes at levels of 5×10^3 and 2×10^4 copies per cell, but these cell types are found in blood at 1/1000 of the number of RBCs. Previous in vivo studies in nonhuman primates demonstrate that diverse ligands can be coupled to circulating RBCs using anti-CR1 conjugates without damage or shortening their survival.(Taylor, Sutherland et al. 1991, Reist, Liang et al. 1994, Ferguson, Martin et al. 1995)

In the present study, I tested the hypothesis that injection of anti-CR1/tPA conjugates will generate fibrinolytically active RBCs in vivo without compromising RBC survival. Using for this goal transgenic mice expressing human CR1 on their RBCs (Repik, Pincus et al. 2005) (TgN-hCR1), we found that a single prophylactic injection of anti-CR1/tPA (but not nontargeted tPA) does provide specific-loading of RBCs in CR1-positive mice (**Figures 8,9**). This maneuver affords rapid dissolution of subsequently forming pulmonary emboli and prevents occlusive arterial thrombi from developing, while causing far less bleeding than nontargeted tPA. These data in animals indicate that tPA immunotargeting to circulating RBCs is, in principle, feasible, effective, and safe, and might provide a practical new approach to prophylactic fibrinolysis (**Figures 10**).

Delivery of Anti-Platelet-Endothelial Cell Adhesion Molecule Single-Chain Variable Fragment-Urokinase Fusion Protein to the Cerebral Vasculature Lyses Arterial Clots and Attenuates Postischemic Brain Edem

Efficacy and safety of current means to prevent cerebrovascular thrombosis in patients at high risk of stroke are suboptimal. In theory, anchoring fibrinolytic plasminogen activators to the luminal surface of the cerebral endothelium might arrest formation of occlusive clots in this setting. It is tested this approach using the recombinant construct antiplatelet-endothelial cell adhesion molecule (PECAM) single-chain variable fragment (scFv)-urokinase-type plasminogen activator (uPA), fusing low-molecular-weight single-chain urokinase-type plasminogen activator with a scFv of an antibody directed to the stably expressed endothelial surface determinant PECAM-1, implicated in inflammation and thrombosis. Studies in mice showed that scFv-uPA, but not unconjugated uPA 1) accumulates in the brain after intravascular injection, 2) lyses clots lodged in the cerebral arterial vasculature without hemorrhagic complications, 3) provides rapid and stable cerebral reperfusion,

and 4) alleviates post-thrombotic brain edema. Effective and safe thromboprophylaxis in the cerebral arterial circulation by anti-PECAM scFv-uPA represents a prototype of a new paradigm to prevent recurrent cerebrovascular thrombosis. Prevention of cerebrovascular thrombosis remains a major unmet need. Situations in which the risk is high, e.g., after transient ischemic attack, myocardial infarction, and post-cardiac bypass surgery, among others, have been identified (Johnston 2002, Kang, Chalela et al. 2005). However, the brain is extremely vulnerable to hemorrhage, neurotoxicity, and disruption of the blood-brain barrier (BBB), leading to cerebral edema, which narrows the therapeutic margin of existing modalities and restricts their use to a fraction of patients in need of medical intervention (Lo, Dalkara et al. 2003, Wang, Lee et al. 2003). To date, the possibility of using plasminogen activators (PA) for prophylaxis of cerebrovascular thrombosis has not been feasible due to their short half-life and untoward incidence of hemorrhagic and neurotoxic complications.

The use of gene delivery to the endothelium to generate PA expression facilitates arterial thrombolysis in animal models (Waugh, Kattash et al. 1999). This observation supports the hypothesis that if feasible, stable localization of a PA along the luminal surface of the cerebral endothelium would enhance its natural antithrombotic mechanisms (Selhub, Jaques et al. 1999), helping prevent ischemic stroke. Targeted delivery of PA to endothelial luminal surface may be especially helpful in settings where the propensity for recurrent thrombosis is high and the acuity of the risk makes gene therapy unsuitable.

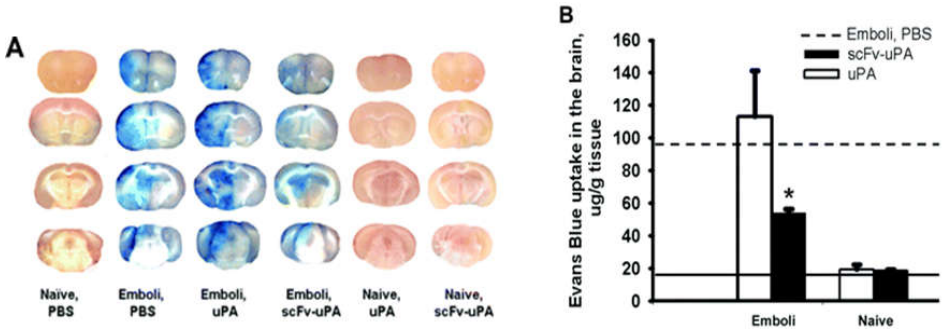

Figure. 14. Effect of targeted anti-PECAM scFv-uPA versus nontargeted uPA on post-thrombotic brain edema. Mice were given an i.a. injection of PBS, 4 mg/kg uPA, or 4 mg/kg scFv-uPA followed 10 min later by an i.a. injection of fibrin emboli. In control groups, emboli injection after drugs was omitted (naive mice). Evans blue dye was extracted 5 h after injection of drugs. A, characteristic sections of the brain of animals from each experimental group: naive animals (injected with PBS), control (emboli and PBS), animals injected with uPA followed by emboli (uPA; emboli), scFv-uPA injected (mentioned as scFv-uPA, emboli), naive animals (uPA, naive and scFv-uPA, naive). B, amount of Evans blue dye extracted from the brain tissue homogenates. n = 4, 6, 4, 4, 4, and 4 mice per group, respectively; *, $p < 0.05$ scFv-uPA versus saline and uPA-treated groups. Solid line shows basal level of the dye uptake in the brain of naive mice, and dashed line shows the level of dye accumulation in the brain of emboli-injected animals without pretreatment by either uPA or scFv-uPA.

Vascular immunotargeting of PA fused with antibody to platelet-endothelial cell adhesion molecule-1 single-chain variable fragment (anti-PECAM scFv-PA) has been shown to provide thromboprophylaxis in the pulmonary circulation (Danielyan, Ganguly et al. 2008), but analogous approaches to deliver PA to the cerebral endothelium have not been reported. PECAM is stably

expressed on endothelial surface in all blood vessels, including cerebral arteries (Giri, Shen Y et al. 2000). The endothelium neither internalizes PECAM nor anti-PECAM scFv-PA (*Muzykantov*, *Christofidou-Solomidou* et al. *1999,* Ding, Gottstein et al. 2005), thereby maintaining intravascular activity of PECAM-anchored drugs.

To determine whether we could use this approach to protect the cerebral vasculature from thrombotic occlusion, I tested an anti-PECAM scFv fused with low-molecular-weight single-chain urokinase-type PA (scFv-uPA), a prodrug that expresses essentially no activity until cleaved by plasmin (Pannell and Gurewich 1987, Danielyan, Ganguly et al. 2008). We tested whether PECAM-directed targeting 1) delivers uPA to the cerebral vasculature; 2) facilitates lysis of cerebral arterial clots without causing intracerebral hemorrhage; and 3) accelerates reperfusion, thereby alleviating postischemic cerebral edema. Experimental results are evidencing, anti-PECAM scFv-PA is able to afford fibrinolysis in the brain after effective accumulation, restores the CBF as well as protects the BBB from the damage (**Figures 11, 12, 13, 14**).

Vitamins

Vitamin B-Complex Initiates Growth and Development of Human Embryonic Brain Cells Growth of the cells and development on the 4th and 12th days.

It was studied the combined effect of subcomponents of vitamin B complex on the growth, development, and death of human embryonic brain-derived cells (E90) cultured using a modified method of Matson. Cell death was detected by trypan blue staining. According to results, vitamin B-complex in low-doses promote the development, maturation, and enlargement of human embryonic brain cells, on the one hand, and increases the percent of cell death, which attests to accelerated maturation and metabolism, on the other.

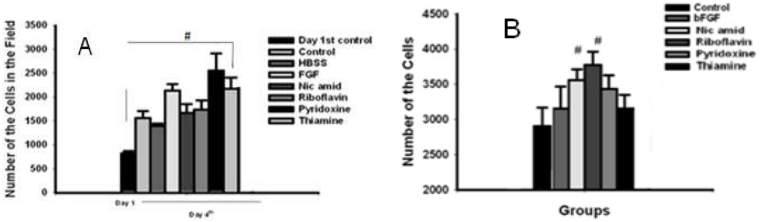

Figure. 15. A. The influence of the single components of vitamin B complex on the growth and development of the human embryonic brain cells. A. Presented are the pictures of the brain cells. Pictures are taken on day 1st and day 4th. Polarizing microscope Bipolar PI (PZO,Warsaw,Poland; magnification 60x1.25x40) was used. The cells were grown on the glass in the Petry dishes, covered with the Poly-L-Lysine. The Neurobasal/B27 containing bFGF , Nicotinamide , Riboflavin , Pyridoxin , Thiamine or low concentration of vitamin B complex was used as cell media. The number of the cells in the field was counted using the Pixcavator program, allowing to calculate authomatically the size as well as the number of the cells. t-student test t was used to calculate the statistical significance of the results . P2, P4, P5 were higher than 0.05, whereas P1 between Control from day 1st and Control from day 4th were <0.05. P3, P6, P7, P8 were significantly higher in comparison with the Control and lower than 0.04. **B. The presence of the single components of vitamin B complex on the growth and development of the human embryonic brain cells on day 12th.** $P < 0.05$ was considered significant.

Biologically active vitamin B-complex containing a certain dose ratio of vitamins B is used in

medical practice as a dietary supplement since 1930 (Elvehjem 1935); complex treatment with these vitamins is much more effective than their individual application. Many laboratories studied the effect of individual components of vitamin B complex on the growth and development of the nervous tissue or cell cultures. It was demonstrated that thiamine deficiency can serve as the basis for modeling of chronic oxidative metabolism disorders leading to neurodegeneration (Belayev, Zhao et al. 1998). Moreover, thiamine deficiency can cause death of neurons, rather than astrocytes, microglia, or endothelial cells lining the inner wall of blood vessels in the brain (Belayev, Zhao et al. 1998). In rats, thiamine deficiency during pregnancy induces death of brain cells. Oxythiamine treatment after thiamine deficiency promoted survival of hippocampal neurons (Geng, Saito et al. 1995). Neurotrophic effect of pyridoxal phosphate was suppressed with picrotoxin and ifenprodil. Aminohydroxyacetic acid, an inhibitor of pyridoxalphosphate-dependent enzymes, initiated death of nerve cells by itself. It was assumed that vitamin B6 contributes to neuronal survival by coenzyme stimulation of enzymes responsible for the neurotransmitter synthesis (Geng, Saito et al. 1995). It was shown that vitamin B complex initiates the cells' growth and maturation. Results presented in this part of the work indicate that the all components of vitamin B complex are responsible for these functions. During the early period of the growth, important components responsible for these functions were thiamine and pyridoxine, riboflavin as well as nicotinamide. However, the most vivid difference might be detected in the groups treated with the pyridoxine and thiamine (**Figure 15**).

The size of the embryonic bodies, as well as smaller cells, was sensitive to the all components of vitamin B subcomponents. At day 12^{th} the size of the small cells' fraction treated with the subcomponets of vitamin B complex was smaller in comparison with the control groups. Instead, the average size of the embryonic bodies in comparison with day 4^{th} increased by 20% in the groups treated with the subcomponents, indicating the fact that small cells were congregating together and forming larger embryonic bodies.

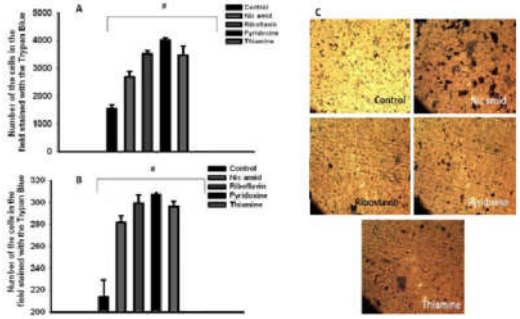

Figure 16. Trypan Blue staining of the human embryonic brain cells and cell bodies at day 12th. It has been used Polarizing microscope Bipolar PI (PZO,Warsaw,Poland; magnification 60x1.25x40). The cells were grown on the glass in the Petry dishes covered with the Poly-L-Lysine. The medium for cells served the Neurobasal/B27 or HBSS, containing Nicotinamide, Riboflavin, Pyridoxin, Thiamine. The number of the cells in the field were counted by the utility of the Pixcavator programm, allowing authomatically to calculatate the size as well as the number of the cells. Results were calculated utilizing SigmaStat 3.5. We have used one –way-ANOVA for the comparison of the groups vs control or the groups between each other. The entire cells' fraction was removed from the wells, mixed for every group and 1ul of the fruction was pictured in the Hemocytometer. **A. Trypan Blue staining of the fruction of the cells at day 4th.** P1 is the comparison of the results between the control and the all other groups. (**p<0.001**). **P <0.05 was** between Nicotinamide and Ribovlavin, **p (<0.05)–** Nicotinamide and Pyridoxine, p (>0.05)- Nicotinamid and Thiamine, p (>0.05)- Riboflavin and Pyridoxine, p7 (>0.05)- Riboflavin and Thiamine, p (>0.05) - Pyridoxine and Thiamine. **B. Trypan Blue staining of the cells bodies on day 12th.** P1 is the comparison of the results between the Control and the all other groups. **p<0.001**. p (>0.05)- between Nicotinamide and Ribovlavin, p (>0.05)– Nicotin amid and Pyridoxine, p (>0.05)- Nicotinamide and Thiamine, p (>0.05)- Riboflavin and Pyridoxine, p(>0.05)- Riboflavin and Thiamine, p (>0.05) - Pyridoxine Thiamine,**C. The micropictures of the mixed fraction of the cells and cells' bodies.**

In comparison with the all other subcomponents of vitamin B complex, only in pyridoxine containing samples XO activity was specifically inhibited by allopurinol in cell culture. Moreover, pyridoxine by itself was inhibiting formation of uric acid. In all the other samples XO activity wasn't inhibited with no any concentrations of allpurinol, probably due to the initiation of the alternative ways of uric acid formation (**Figure 17**).

Similarly to allopurinol pyridoxine treatment during the early stages of the treatment was initiating the increase in the number of the cells, whereas in the late stages that process was suppressed. During the late stages the most effective components were riboflavin and nicotinamid . Also, it worth to be mentioned, allopurinol is the classical drug for the treatment of chronic gout (Moriwaki, Yamamoto et al. 1999). The results indicate that the effectiveness of this compound might be diminished in case of simultaneous treatment of the patients along with the Allopurinol, as well as also with riboflavin, nicotinamidee and thiamine, because of possible stimulation of the other biochemical pathways responsible for the formation of uric acid.

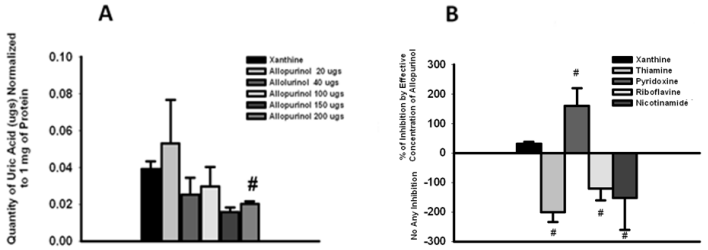

Figure 17. Assessment of XO activity by the estimation of uric acid formation in the presence of allopurinol and vitamin B complex subcomponents. Inhibition of uric acid formation in the human brain derived (E90) cells by the utility of allopurinol. The first black column represents the ugs of uric acid, formed in the presence of the substrate of XO – xanthine. Further application of allopurinol in the concentration equal to 0,0024uM, 0,0048uM; 0,0097 uM, 0,018 uM, 0,036 uM was in the concentration dependent manner decreasing the total quantity of uric acid, normalized to 1 mg of the protein. Statistically significant difference was observed during the comparison of the control group vs Allopurinol, applied in the concentration of 0,036 uM ($p<0,05$). **Persentile of inhibition** of uric **acid formation in the pressence of vitamin B subcomponents.** The first black bar shows the persentile of uric acid formation during the 40 minutes of incubation in the presence of xanthine and allopurinol (31, 5±6,9). The second gray bar situated above the X-axis indicates the persentile of pyridoxine dependent , initiated by allopurinol inhibition of XO, which is compared and calculated based on the quantity of uric acid formated in the presence of pyridoxine. The persentiles with the sign "minus" show no inhibition of uric acid formation, because of the presence in the samples riboflavin, thiamine, nicotinamide. The all comparisons between control and experimental groups are statistically significant and $p<0,05$.

Influence of the allopurinol and pyridoxine on the activity of XO and on the entire purine catabolism pathway

There are numerous publications indicating the evidence about the primer regulating role of the hypoxanthine/xanthine in the purine metabolic pathway (Edwards, Recker et al. 1979, Edwards, Recker et al. 1981, Harrison and Simmonds 1985, Bleisch, Sillero et al. 1994).

For instance, Edwards NL et. al have performed the small clinical trial with the infusion of the radiolabeld [8-(14)C] adenine to four patients with gout as well as to the patients suffering from Lesch-Nyhan syndrome. Five days after infusion it became clear that the mean cumulative excretion of radioactivity after adenine administration to patients not receiving and receiving (off and on) allopurinol therapy was 6.1% and 3.6% of infused radioactivity for gouty subjects and 15.9% and 20.8% for the Lesch-Nyhan patients.

Edwards NL and co-workers (Edwards, Recker et al. 1981) have suggested that purine salvage is a major contributor to increased purine excretion and that the purine catabolic pathway responds differently to an increased substrate load in hypoxanthine-guanine phosphoribosyltransferase deficiency.

Another group has shown that allopurinol treatment might promote the excretion of xanthine /hypoxanthine as well as the 8-hydroxy-7-methyl guanine but not any other minor purines into the blood circulations (Harrison and Simmonds 1985).

Bleisch et all have shown that allopurinol, besides inhibiting uric acid synthesis, reduced the rate of degradation of AMP (Bleisch, Sillero et al. 1994).

Extremely interesting is the work related to the reduction of allopurinol-induced oroticaciduria by dietary RNA (Zollnerd 1982, Salerno and Crifò 2002, Kilic, Kilic et al. 2005).

A. **Evaluation of the inhibiting abilities of the allopurinol of the entire purine catabolism.**

By my study with the utility of the different initial, not primer substrates for the XO I noticed increase in the formation of the XO product – uric acid in vitro conditions (**Figure 18**) (Gyongyan, Manucharyan et al. 2013).

Taking into the consideration, that the utility of allopurinol allows to reduce activity of XOR significantly, I proved that XOR is the key regulative enzyme in the purine catabolism pathway.

Also, to prove whether pure catabolism related enzyme is able to react with the non-primer substrates, I checked the activity of purified XOR in the presence of histidine, one of the substrates, which was stimulating the activity of the enzyme very strongly.

It was noticed 10 times elevation of XOR activity in statistically significant way (control - 1.63±0.20, xanthine - 2.16±0.11, allopurinol - 1.81±0.05, $p<0.05$). In the presence of histidine we haven't noticed any activity (histidine - 1.77±0.30).

Activity of XO in the rat brain in the presence of different substrates was the following in comparison with the control (blank control-0.964±0.051, xanthine- 2.119±0.081, allopurinol in low concentration -1.695±0.117, allopurinol in high concentration- 1.520±0.058, $p<0.05$); for histidine - 3.302±0.192, in the presence of allopurinol in low and high concentrations - 2.279±0.134, 1.783±0.077, respectfully; for riboflavin 1.257±0.058 (with allopurinol in low concentration - 0.716±0.125, in high concentration 0.994±0.192), for adenosine - 2.937±0.076 (with allopurinol in low and high concentrations - 1.899±0.227 and 2.119±0.102), for desoxyadenosine - 2.411±0.373, (with allopurinol - 2.031±0.102, 2.0310±0.064 (**Fig. 18**). In comparison with the control all substrates elevated the XOR activity in statistically significant way, where as allopurinol was able to suppress formation of uric acid.

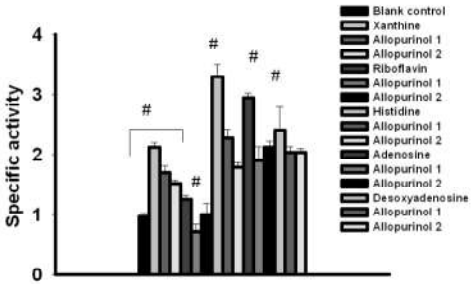

Figure 18. Evaluation of XO specific activity in the presence of none-primer subtsrates. The experiments were performed with the utility of the spectrophotometer Perkin-Elmer Coleman Model6|20 and 6|35 Junior II Spectrophotometer (λ=660 nm) for all type of the spectrophotometric investigations. The first black column represents the 11 specific activity of XOR, in the presence of xanthine (0.06 ug/ml). The all other substrates were applied in the same quantity. Allopurinol was used for the inhibition of XO activity in the concentration equal to the concentration of the substrates (Allopurinol 1) or higher twice (Allopurinol 2). ONE-WAY-ANOVA, t-test was applied 0for delineation of statistical significance of the experimental results for all type of the investigations. Results were considered statistically significant when $p<0,05$.

Taking into the consideration, that the utility of allopurinol allows significantly reducing activity of

XOR, I proved that XOR is the key, rate-limiting, regulative enzyme in the purine catabolism pathway.

B. Evaluation of the inhibiting abilities of the pyridoxine of the entire purine catabolism

In the second set of the experiments it was assessed the activity of the same enzyme in the presence of the possible inhibitor - pyridoxine, as well as the substrates, which are catabolizing via the purine pathway.

Clearly, the addition pyridoxine into the mixture did provoke the inhibition of the XO. In comparison with the blank control (5.0299e-3±1.4215e-3) addition of the riboflavin along with the pyridoxine was inhibiting the enzyme activity (2.2715e-3±1.1444e-3). Similarly, the inhibition of the enzyme was noticed in the presence of the adenosine (2.6610e-3±6.1656e-4), desoxy-adenosine (7.6259e-4±4.4994e-4) as well as aspartate (8.1127e-4±3.5362e-4), (**Figure 19**).

C. Determination of Ki for purified XO.

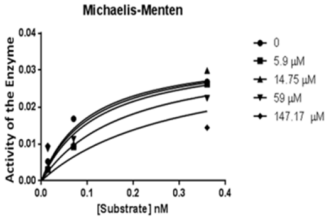

Figure 19. Determination of Ki for purified XO. The experiments were performed with the utility of the spectrophotometer Perkin-Elmer Coleman Model6|20 and 6|35 Junior II Spectrophotometer (λ=660 nm) for all type of the spectrophotometric investigations. **Determination of Ki for purified XO.** of XO substrate- xanthine and cofactor Mo^{2+} there was calculated the activity of the enzyme and normalized to 1 mg of the protein. A. Results reflecting the Km, Vmax determination. Data are also reflecting the Ki determination.

The activity of purified from the liver XO was evaluated in the presence of 4 different concentrations of pyridoxine: 5.9 uM, 14.75 uM, 59 uM, 147,17 uM. Km for the enzyme and xanthine as a substrate was equal to 0.051 nM, Vmax= 0.0279, Ki (0.0076 mM). Taking into the consideration that the utility of the pyridoxine might vary from (Board. 1998) 0.1-2 mg/day depending on the age, sex as well as pregnancy and after intramascular injection approximetely 1/10 of the dosage passes into the blood circulation and only 0.1-3% might reach the brain parenchyma and we were working with purified enzyme fruition we have used indicated in the figures (**Figure 19**) doses of pyridoxine.

Influence of purine and pyrimidine catabolism on the cells proliferation and experimental stroke outcome

Dependence of cell survival on correlative activities of xanthine oxidase and dihydopyrimidine dehydrogenase in human brain-derived cell culture

XO as well as XDH are two enzymes responsible for the last steps of purine metabolism, and hydroxylation of a wide variety of pyrimidines, pterins, and aldehyde substrates. There are different forms of the same enzyme Xanthine Oxidoreductase (XOR; EC. 1.17.1.4), which was isolated from a wide range of organisms, from bacteria to man. Moriwaki Y et al. demonstrated the presence of XO in the brain as well as in other organs (Hille and Nishino 1995). The mammalian enzymes, which catalyze the hydroxylation of hypoxanthine and xanthine, the last two steps in the formation of urate, are synthesized as the dehydrogenase form and exist mostly as such in the cell, but can be

readily converted to the oxidase form (XO) by oxidation of sulfhydryl residues or by proteolysis. XDH shows a preference for NADH reduction at the FAD reaction site, whereas XO fails to react with NADH and exclusively uses dioxygen as its substrate, leading to the formation of superoxide anion and hydrogen peroxide (Yong, Power et al. 2001).

Numerous publications provide evidence about the feedback mechanism-based regulation of the purine catabolism owing to the regulation of XO activity, for instance by its classical inhibitor - allopurinol (Simmonds and Bowyer 1974, Zollnerd 1982, Harrison and Simmonds 1985, Reiter, Löffler et al. 1986, Löffler and Gröbner 1988, Yong, Power et al. 2001, Kilic, Kilic et al. 2005), (Leung and Schramm 1978, Sabina, Hanks et al. 1979, Khalil and Khodr 2001).

The mechanism of reactive oxygen species (ROS) production is mediated partially by the activity of XO. Also, ROS, except for their very well-known deleterious role in organisms, are involved in the delayed recovery of injured nerves in old rats as well as in tissue repair (Moriwaki, Yamamoto et al. 1999).

Furthermore, an involvement of XO in cellular proliferation and differentiation has been suggested (Danielyan and Kevorkian 2011) for this enzyme.

We also investigated the impact of the activity of XOR on the processes of cell survival and proliferation in vitro.

As a comparative study, we have chosen the other enzyme, dihydropyrimidine dehydrogenase (DPD; EC. 1.3.1.2, which is NADP+ dependent) which is, also, the one of the final enzymes responsible for pyrimidine catabolism and might be inhibited by dipyridamol (Traut and Loechel 1984). There are at least 3 pathways where DPD plays an important role: 1. Pyrimidine catabolism (Traut and Loechel 1984), 2. Synthesis of beta alanine (Katzan, Hammer et al. 2004) and 3. Metabolism of 5-FU (Katzan, Hammer et al. 2004).

Fluorouracil is a non-native substrate of DPD, which diminishes the proliferative activities of cancer cells (Van Kuilenburg, Vreken et al. 1999).

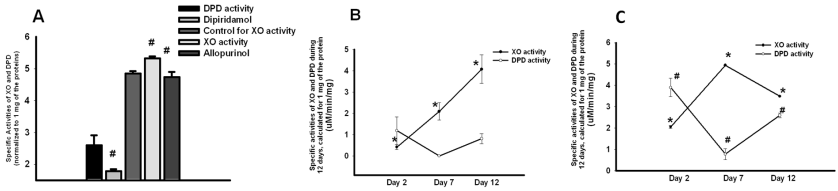

Fig.20. Dynamic correlation of XO and DPD activities in human brain-derived cell culture. Cells were grown for 2, 7, 12 days w/o or with inhibitors in concentration 10 ug/ml. The experiments were performed with a spectrophotometer Perkin-Elmer Coleman Model 6|20 and 6|35 Junior II Spectrophotometer. Human embryonic (E12) brain-derived cells were grown in Neurobasal/B27 medium. **A. Activities of XO and DPD in cell culture.** The sample contained NADPH (4 mM) as well as thymine or/and dipyridamole, in a concentration equal to 2.5 mM for determination of DPD activity. Experiments were carried out in the presence of Tris buffer (0.1 M; pH=7.4). The content of xanthine and/or allopurinol in the final samples was equal to 0.6 ug for XO activity estimation. **B. Determination of DPD and XO activities in cells culture treated with allopurinol and dipyridamole over 12 days.** Delineation of p values inside the groups pointed to the statistical significance between the XO activity between day 2 vs day 7 (p=0.05), day 2 vs day 12 (p=0.035), and day 7 vs day 12 (p=0.05). **C. Determination of DPD and XO activities in cell culture over 12 days.** P values for XO were equal to 0.0005, 0.0019, and 0.0001 for days 2 vs 7, day 7 vs 12, and day 2 vs 12, respectively. For DPD activity statistical significance was estimated for days 2 vs 7, day 7 vs 12, and day 2 vs 12 (p=0.001, p=0.0038,p= 0.01).

The gene DPYD, which encodes DPD, is the initial and rate-limiting enzyme in the catabolism of the pyrimidine bases: uracil and thymine (Shiotani and Weber 1981). DPD was isolated from the liver of rats (Podschun, Wahler et al. 1989), pigs (Cipolla, Porter et al. 1997), cattle (Cipolla, Porter et al. 1997), as well as human (Lu and Tsirka 2002). Mostly DPD is localized in the cytoplasm of the cells (Podschun, Cook et al. 1990). It was determined that the pig and human enzyme contain 1025 amino acids and have molecular mass equal to 111 kDa. This enzyme exists as a homodimer, containing the subunit with a molecular mass equal to 107.00. DPD contains two distinct binding sites for NADPH/NADP and FAD in the N-terminus, as well as a central peptide domain corresponding to the uracil-binding site (Spector, Harrington et al. 1993).

Along with the other organ systems, DPD is localized in the brain (Yamada, Fukushima et al. 2003).

Yamada H et al. studied DPD gene expression during liver regeneration and proved a vivid correlation between the regenerative proliferative processes and expression, and synthesis as well as activity of DPD (Tennant 1964).

Thus, we have investigated the dynamics of both XO as well as DPD activities in the human brain-derived cell culture and highlighted their correlation with the cell death occurrence and proliferation.

Evaluation of cell survival and death in human brain-derived cell culture

Inhibition of XO activities with both low and high concentrations of allopurinol during the days 1-12 and 7-12 diminished cell death with statistical significance (control- 3500±400; treatment during the period 1-12 days: low concentration of allopurinol - 2400±400, high- 1000±200 6-12: low concentration of allopurinol - 390±67, high- 900 ±200; $p<0.05$ between all groups vs. control in accordance with one-way ANOVA, **Table 1**).

Treatment of the cells with low concentrations of dipyridamole decreased the number of the dead cells for the 1-12 and 7-12 day periods of treatments (control- 120±8, dipyridamole treatment through days 7-12 (48±6), dipyridamole treatment through days 1-12 (73±7), (p=3.07221E-08, control vs dipyridamoledays 6-12; p=0.000126909, for control vs dipyridamole, days 1-12).

Treatment of cells with the high concentration of dipyridamole and stained with Trypan Blue also indicated a decrease in cells number in the culture (control- 97±8, dipyridamole treatment through days 7-12 - 26±10, dipyridamole treatment through days 1-12 - 12±2, p=4.50558E-05, control vs dipyridamole days 7-12; p=1.35309E-08, for control vs dipyridamole, days 1-12), in contrast to dipyridamole treatment with low concentration during days 1-7 (control 36±5, dipyridamole 94±8, p=3.65022E-06).

Allopurinol in low concentration during the days 1-12 treatment period initiated the growth of the cells (2350±40 vs control 2120±23, $p<0.05$). The high concentration of allopurinol produced no significant effects (1900± 200).

Evaluation of XO and DPD activities in human brain-derived cell culture

Based on the results reflecting the most effective treatment time periods for the cells protection and proliferation, we have monitored the activities of XO and DPD in the cell culture homogenate.

First, we delineated whether allopurinol and dipyridamol are effectively able to diminish activities of XO as well as DPD.

After calorimetric reaction we noticed, that in comparison with the control, in the presence of the natural substrate xanthine, the activity of XO increased after 45 minutes of incubation with enzymatic mixture, which was in a statistically significant way diminished with its classical inhibitor allopurinol (4.85±0.08, 5.32±0.07,4.7±0.2, $p<0.05$, **Fig.20.A**).

s Inhibitors	Concentrations of the inhibitors	Periods of the Cell Culture Treatment			
		Days 1-12			
		Number of the cells in the field (% from control), p values		Number of the dead cells in the field (% from control), p values	
Allopurinol treatment	high	80±9	no any stat. significance	29±7	0.035
	low	111±2	0.045	67±16	0.025
Dipyridamol treatment	high	74±4	2.38769E-07	18±3	1.35309E-08
	low	92±3	0.034871093	61±6	0.000126909
		Days 7-12			
Allopurinol treatment	high	94±2	no any stat. significance	25±6	0.017
	low	70±5	no any stat. significance	11±2	0.025
Dipyridamol treatment	high	88±4	0.007522114	26.±10	4.50558E-05
	low	84±3	0.000153147	40±5	3.07221E-08
		Days 1-7			
Allopurinol treatment	high	70±3	no any stat. significance	33±4	no any stat. significance
	low	80±3	1.84977E-05	132±14	no any stat. significance
Dipyridamol treatment	high	67±3	no any stat. significance	275±9	no any stat. significance
	low	79±3	1.03251E-05	258±21	3.65022E-06

Table 1. Evaluation of cell death and proliferation in human brain-derived cell culture in the presence and absence of DPD as well as XO inhibitors at different time periods over 12 days. We used Boeco Trinocular Microscope with a phase contrast adapter (BM-180/T/I/SP). The phase contrast pictures (100X objective, total magnification 2500x) were taken on day 12. Results were calculated from observation of the five different fields from the plates for every group. Cells were stained with 2% Trypan Blue dye for visualization of the viable/nonviable cells. The cells were grown on glass in Petri dishes covered with Poly-L-Lysine. We have used cell Neurobasal/B27 (Gibco, Invitrogen) medium.

The activity of DPD measured by NADPH absorbance decrease was inhibited after dipyridamole addition to the mixture (2.6±0.3, 1.79±0.06, $p<0.05$, **Fig. 20. A**).

Interestingly, the activity of XO in the normal cell culture, not treated with the inhibitors, was increasing from days 2nd to 7th and from days 2nd to 12th, with the bending point on day 7th (2.05±0.06, 4.95±0.02, 3.49±0.00, p=0.0005, p=0.0019,p= 0.0001 for days 2 vs 7, 7 vs 12, 2 vs 12), whereas DPD activity was decreasing from days 2 to 7 and day 2 to 12, with the bending point on day 7^{h} (3.9±0.4, 0.878±03, 2.60±0.11, p values for day 2 vs 7, day 7vs 12, and day 2 vs 12 was equal to 0.001, 0.0038,0.01, **Fig. 20 C**).

The trend wasn't changed for DPD activity in the dipyridamoe-treated cell culture ($p<0.05$, one-way ANOVA), although the values of activity were lower than the ones in the treated with the inhibitor cell culture (1.2±0.6, 0.00±0.00, 0.8±0.2, **Fig. 20 B**). The activity of XO in contrast to DPD, was increasing during the entire period of the cell culturing (0.4±0.1, 2.0±0.4, 4±1, for day 2 vs 7, p=0.05, for day 2 vs 12 p=0.035, and for day 7 vs 12 p=0.05, respectfully). However, the initial values of XO activity was lower than in those not treated with allopurinol in cell culture ($p<0.05$).

In addition, it is noticeable that XO activity was increasing over 12 days until it reached the value comparable with the activity of XO in the normal, non- treated cell culture.

The analysis by Pearson correlative function has demonstrated that in the non-treated cell culture the correlation between the DPD and XO activities exists but it is not strong and is called “small, negative” (-0.265), in contrast to the “strong, negative" correlation of enzymes’ activities in cell cultures treated with inhibitors (-0.9997).

During our investigations with human brain-derived cell culture, it was noticed a reversed correlation between activities of XO and DPD over 12 days under normal conditions.

It is not stated that the negative correlation has a compensatory nature; however, it is obvious from the figure that as a final regulator enzyme, XO activity remains high for a very long period in comparison with DPD activity and starts dropping only after day 7, remaining high even on day 12 in comparison with the activity registered on day 2. This stable activity might be explained by the domination of ATP as well as GTP as the main source of the energy in the organism in contrast to TTP or UTP.

A similar increase of XO activity in rats during aging was observed by Vida et all (Moriwaki, Yamamoto et al. 1999).

In cell culture treated with inhibitors, we registered an increase in XO activity over the 12 day time period in contrast to DPD activity, which reached its peak level only on day 12 and was lower on day 2 in comparison with its activity in non-treated cell culture.

This discrepancy of XO and DPD activities was reflected very well in the cell culture survival results. Only 12 days’ treatment with allopurinol increased the cell number in comparison with the control andin contrast to the dipyridamole-treated cell culture groups. Under the same conditions, there was a decrease in dead cell number vs control.

Generally, during periods of cells treatment through days 1-12 and 7-12 we observed the prevention of cell death with both inhibitors, whereas during treatment period through days 7-12, an elevation of the percentage of dead cells was observed.

It is well known that XO might initiate formation of synapses and connections between neuronal cells (Jin, Wang et al. 2010) and introduce regenerative or developmental activities.

Thus, in the newly seeded cell culture, where interactions between the cells are not formed, the activity of the above-mentioned enzyme is vitally important. Inhibition of the last one may not just fail to preserve the cell survival but can even stimulate their death. In addition, the 6^{th} and 7^{th} days are vitally important for the survival and development of the cells, and changes in the feeding medium might initiate cell death.

Allopurinol and pyridoxine effect on the proliferative activity of the cells evaluated by flow cytometry

Further, it was checked allopurinol and pyridoxine pro-proliferatice effects by the utility of flow cytometry and Ki-67 proliferative marker the stimulating abilities of pyridoxine and allopurinol for neuronal/glial human embryo brain derived cells. As a consequence, based on the long lasing investigation cited above and the result, presented below, we have concluded that inhibitors might initiate cells proliferation. Interestingly, the best results are obtained after treatment of the cell culture in time period from days 1-6 and detection of the results on day 12^{th}. Feeding of human embryo brain derived cells with the inhibitors from days 1-12 was beneficial too, however, to the the less extent (**figure 21**).

Thus, it is proposed that the time period of the treatment is extremely important.

Activation of XO by epinephrine triggers cells proliferation as are evidencing obtained results. In some studies alfa 2-agonistis able to stimulate breast tumor cells proliferation (Szpunar, Burke et al. 2013). It is suggested, that in pathological state and, even during different aggravating for cells conditions, XO might be activated differently. Also, it is necessary to take into the

considerationthat XOR acts as a dual enzyme: it is performing its action as a dehydrogenase and as an oxidase, mostly in pathological conditions (Suzuki, Kidwell et al. 2005). Coefficient of inhibition for this enzyme by different inhibitors are widely ranging as well as the Vmax of the reactions (http://www.brenda-enzymes.org/enzyme.php?ecno=1.17.3.2), assuming different mechanism of its regulation. However, during biochemical experiments (figure 3) it is proved once again that XO is the rate-limiting enzyme and activation of it might have an impact on the entire purine catabolism.

Pyridoxine had no any statistically significant effect on cells growing experiments. Previously, it was noticed proproliferative effect of pyridoxine; however, the implied concentrations (100x higher) didn't have any impact on the significance of the obtained current results. Previously, it was demonstrated that inhibition of XOR activity with allopurinol might stimulate in vitro processes of cells proliferation (Zaitsev, Danielyan et al. 2006), whereas the inhibition of dihydropyrimidine dehydrogenase might prevent brain derived cells from death (Zaitsev, Danielyan et al. 2006).

In the comparable concentration with epinephrine, pyridoxine had no any effect. Pyridoxine behaves as a dual effect possessing compound for XO: in the low concentration it inhibits the enzyme, where as in the high concentration it doesn't possess any effect or even activate it. It was delineated pyridoxine-XO link by the utility of the theophylline, assuming that it will deplete the pyridoxine and we will observe activation of XO. However, in vivo results reflected right opposite picture, which considered even more complicated interaction between these compounds. We can just speculate that theophylline by triggering ATP synthesis might also initiate activation of purine catabolism and pyridoxine quantity depletion is the secondary for this vitally important process.

Here, it is proved and presented the fundamental mechanism of purine catabolism regulation via XO in the presence of epinephrine and pyridoxine, which might be utilized for regenerative processes, prevention of cells proliferation in the settings of cancer development and PD treatment.

Protective abilities of pyridoxine in experimental oxidative stress settings in vivo and in vitro

1. Calculation of the cell number in vitro after treatment with hydrogen peroxide. The human brain derived neuronal cells containing culture was maintained for 7 days. At day 7th the culture was treated with the 3 % of hydrogen peroxide for 24 hours. The number of the cells without staining was calculated based on the microscopy pictures of the visual field. The ocular tube for all groups was placed on same height from the microscopy preparative table and the light supply was maintained on the same level.

In comparison with the control (2680.00±45.34) in the peroxide treated group (1631.89±111.77), the number of the cells was dramatically decreased (**figure 22 A**). Allopurinol treatment 15 minutes before (1852.38±94.79) and 15 minutes after (1950.38±33.67) in not statistically significant way increased the cells number. Addition of pyridoxine into the cells media increased the number of the cells in statistically significant way, moreover, treatment with pyridoxine before peroxide addition was less efficinet (2106.88±79.64) than after (2392.38±104.01), ($p<0.05$). 2. **Calculation of the death cells number in culture.** One hour treatment of the cell culture with hydrogen peroxide (740.33±86.16) in comparison with the control group (245.90±31.72) strongly increased the number of the death cell in the culture in statistically significant way ($p<0.05$), (**figure 22B**). Treatment with the allopurinol before (556.78±16.17), as well as after (536.70±49.79) addition of hydrogen peroxide as well as treatment with pyridoxine after modeling of oxidative stress (630.22±71.84) were less effective in comparison with the treatment with the B6 before oxidative stress initiation (231.50±53.77), ($p<0.05$, ONE WAY ANOVA for the comparison of the peroxide control group with the treatment all 4 groups), ($p<0.05$, t-test for the comparison of control vs peroxide negative control group).

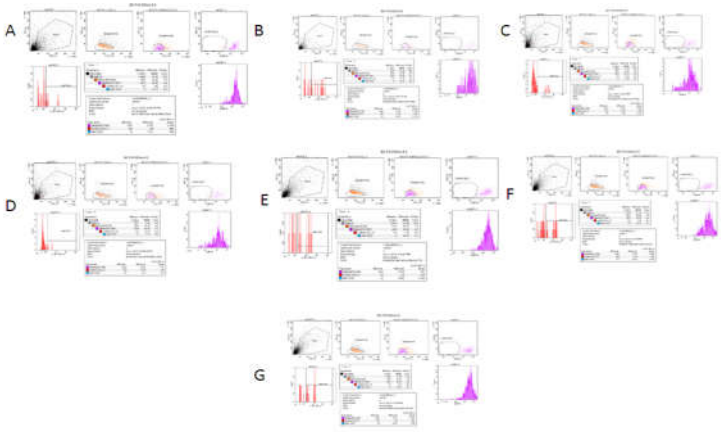

Figure 21. Side Scattering (SSC) and Forward Scattering (FSC) pictures of the human brain derived cell culture cells stained with Hoechst and Ki-67. A. The control group of cell culture. **B.** Culture treated with allopurinol from days 1-6. Detection was performed on day 12th. **C.** Treatment of the cell culture with pyridoxine from days 1-12. **D.** Treatment of the cells with allopurinol from days from days 6 – 12. **E.** Treatment of the cells with allopurinol from days 1-12. **F.** Treatment with the pyridoxine from days 6-12. Treatment of the cells with pyridoxine from days 1-12.

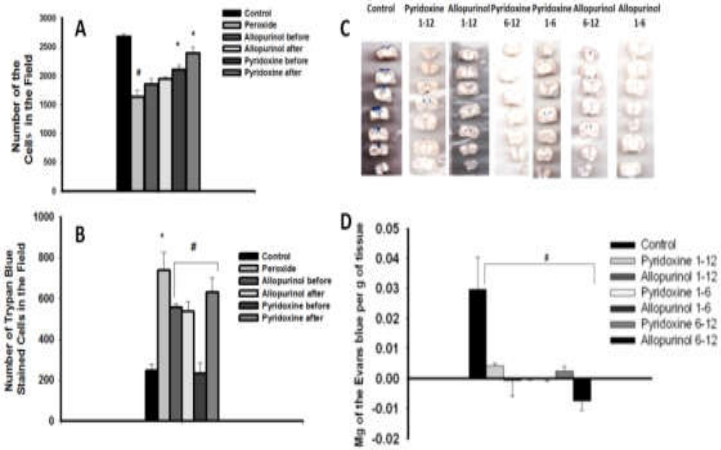

Figure 22. A. Calculation of the cells number in vitro after treatment with hydrogen peroxide. The pictures were analyzed by the Pixcavator 4 program with the maintenance of the same values for contrast, light intensity as well as dots size inclusion parameters. **B. Calculation of the death cells number in the culture.** The cells were stained with Trypan Blue (0.04%) solution and their numbers were calculated. **C. In vivo evaluation of new and classical inhibitors XO. Representative pictures of the whole brains of the animals. D. Evans Blue extraction values from the animal's brains represented as ug per gram of the tissue.**

2. In vivo evaluation of XO new and classical inhibitory effects. After Evans Blue extraction, the values of the ipsiateral hemisphere were extracted from the numbers of the contralateral hemisphere to exclude the brain perfusion experimental errors (**figure 22 C, D**). Animals were treated in 3 different time periods: days 1-12, 1-6, and 6-12. In comparison with the control, all treatment periods were effective for allopurinol as well as pyridixine (0.029756 ± 0.010616 - control, 0.004131 ± 0.000803 - pyridoxine 1-12, 0.00062 ± 0.042976 - allpopurinol 1-12, -8.5E-05 ± 0.000353 – pyridixine 1-6 , -8.9E-05 ± 0.000816 – allopurinol 1-6, 0.002451 ± 0.001573 – pyridixine 6-12, -0.00742 ± 0.003123 allopurinol 6-12), ($p<0.05$).

Based on the presented results, it is proved, that pyridoxine as well as allopurinol, were protective against oxidative stress probably due to the XO inhibition.

However, the utility of allopurinol as well as pyridoxine during different time periods after brain damage didn't have any significant effect for the protection of BBB from peroxide determined damage.

The antioxidative abilities of pyridixine are proposed by the work which might be applicable for the treatment of stroke in the future.

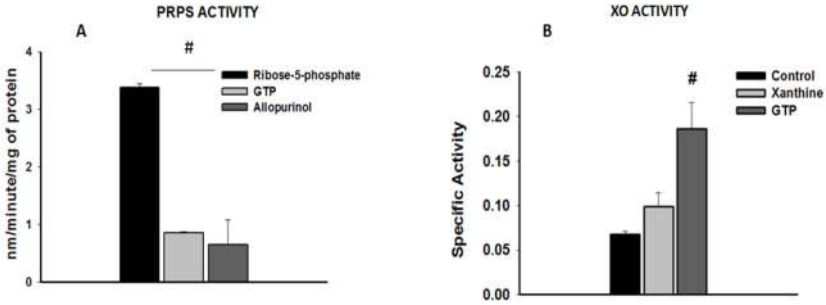

Figure 23. Activities of XO and PRPS in the intact animals brains. A. PRPS activity. B. XO activity. The experiments were performed with the utility of spectrophotometer Cary 60 UV/VIS, Agilent (USA). The absorption of the light was evaluated with the utility of 660 nm of the wavelength for XO activity determination and 340 nm for PRPS. ONE-WAY-ANOVA, t-test for delineation of statistical significance was applied for experimental results for all type of the investigations. The number of animals (N) in the groups was varied from 4-6.

Correlational activity of XO and PRPS-1 in the normal and pathological conditions

XO activity was determined by the formation of uric acid per minute per mg of the protein. 0.0156 xanthine, 0.1863±0.0296-xanthine+GTP). Interestingly, allopurinol and GTP were diminishing the activity of PRPS 1 in the brain tissue of intact animals in statistically significant way (3.3854±0.0617, 0.8542±0.0265, 0.6536± 0.4311, $p<0.05$), (**Figure 23**).

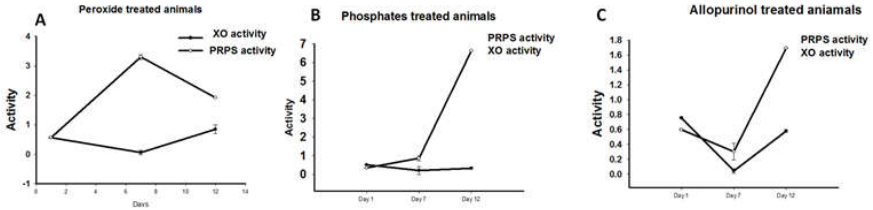

Figure 24. Activities of XO and PRPS in the peroxide injected animals' brains. A. Correlative activities of XO and PRPS on 1st day. B. Correlative activities of XO and PRPS on day 7th. C. Correlative activities of XO and PRPS on day 12th.

The experiments were performed with the utility of spectrophotometer Cary 60 UV/VIS, Agilent (USA). The absorption of the light was evaluated with the utility of 660 nm of the wavelength for XO activity determination and 340 nm for PRPS. ONE-WAY-ANOVA, t-test for delineation of statistical significance of the experimental results for all type of the investigations were applied. The number of animals (N) in the groups was varied from 4-6.

It's necessary to take into consideration that patients with hypoxanthine–guanine phosphoribosyl transferase (HGPRT) deficiency or Lesch-Nyhan disease (MIM 300322) having uric acid overproduction similar to PRPS-I superactivity can also have mental retardation and hypotonia, as described in patients with Arts syndrome (de Brouwer, van Bokhoven et al. 2010). Thus, the phosphate related elevation of PRPS activity and simultaneous decrease of XO activity will promote synthesis of purines and prevent above mentioned negative side effects related with overproduction of uric acid. During the second time period of our study, taking into the consideration that phosphates inhibit XO activity after peroxide brain impairment and activate PRPS ,we have decided instead of allopurinol and activator of PRPS – phosphates to use only phosphates. These salts inhibit by the feed-back mechanism catabolism of purines meanwhile activating the synthesis, promote the formation of the nucleotides rich environment within the cells.

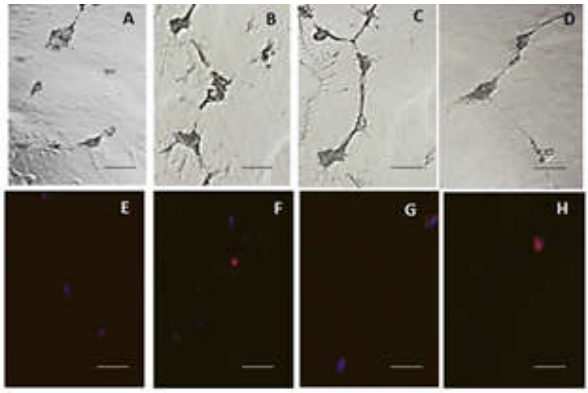

Figure 25. In vivo experiments, representing the Ki-67 staining in 4 groups: A, E. Peroxide intracranial injection. B, F. Treatment by phosphates from days 1-7. C. G. Pyridoxine injections from days 7-12. D,H. Pyridoxine injections from days 1-12.The mark is equal to 10 micron.

In the normal conditions in control group theactivity of XO was elevated by GTP in comparison with the blank control group and enzyme+xanthine containing conditions ($0.0673\pm3.5607^{e-3}$ - control, 0.0988±
As it was proposed, the Ki-67 was injected into the 4th ventricle and the integration of the marker was visualized after 12 days of the time period. We have dissected the tissue from
the dentate gyrus of the hippocampus and from the subventricular zone of the lateral ventricle, made cells suspension and investigated by fluorescent microscope with Texas Red matching filter system (excitation 633-647 and emission = 660 nm). **Figure 25** depicts the contrast phase as well we fluorescence microscopy pictures of 4 groups: control animals with 3% hydrogen peroxide injection (A; E), impaired animals treated with phosphates from days 1-7 (B; F), 7-12 (C; G) and 1-12 (D;H). The survival of the animals in the group of the animals treated with phosphates from day 1-12 was lower (results are not presented). From 5000 cells the higher amount of red colored cells were visualized in the group of the animals treated from days 1-7.
It is interesting that there are experimental evidences that PRPS 1 is regulator of the the glioma stem cells proliferative and apoptotic processes. The mice implanted with PRPS1 knockdown T98G or U87MG stem cells exhibited prolonged survival time and reduced tumor volume (Li, Yan et al. 2016).
Thus the permeability and protection of the BBB with Evans Blue utility was tested for this particular group. We have noticed positive effect of BBB protection after application of phosphates, activators of PRPS-1.

Conclusions

1. PRP-1 reduces staurosporine-induced apoptosis.
2. The protective effect of PRP-1 against apoptotic cell death was shown to be both time- and dose-dependent. Neuroprotection was more pronounced after prolonged pretreatment of the cells with PRP-1 before the induction of apoptosis with staurosporine. ***PRP-1 in pharmacological model of***
3. Quantitative analyses of TH neurons' numbers and statistical comparisons confirmed the predominant ipsilateral loss of DA neurons in the substantia nigra pars compacta during the short-term study in PDC model. It was observed that intracerebral injection of PRP-1 at low concentration offered efficient neuroprotection in PD.
4. Albumin treatment reduces neurological deficit and protects blood–brain barrier integrity after acute intracortical hematoma in the rats
5. It was shown, that prompt therapy with moderate-dose human albumin reduces neurological deficits and improves BBB integrity in the setting of acute intracortical hematoma in the rat.
6. Creation of albumin microparticles as the carriers for the allopurinol
7. It was adopted the method for the generation 1-5 micron spheroid particles. It was developed method to assess the allopurinol quantity in the coated particles, which might be useful in the future not only for the treatment of stroke.
8. RBC/tPA provides effective and safe cerebrovascular thromboprophylaxis, whereas tPA is not ineffective even at a 10-fold higher dose.
9. RBC carriage also prevents uptake of tPA by the brain.

10. Regardless of the mechanism, tPA causes profound bleeding from surgical wounds, is deleterious if given as soon as 1 hour after induction of cerebral ischemia by mechanical occlusion of the MCA, and is ineffective and deleterious if given >5 minutes before thrombotic occlusion.
11. RBC/tPA prophylaxis shows no detectable toxicity and protected against thromboembolism. RBC/tPA, but not tPA, retained its fibrinolytic activity for hours after injection in mice and rats because of prolonged pharmacokinetics and protection against plasma inhibitors by the RBC glycocalyx.
12. Anti-CR1/tPA conjugate, but not control conjugate (mIgG/tPA), bound to human RBCs (1.2 x 10(3) tPA molecules/cell at saturation), endowing them with fibrinolytic activity (90% clot lysis versus 20% by naive RBCs).
13. In vivo, more than 40% of anti-CR1/(125)I-tPA remains within the circulation (approximately 90% bound to RBCs) 3 hours after injection in transgenic mice expressing human CR1 (TgN-hCR1) versus less than 10% in wild-type (WT) mice, without RBC damage.
14. Approximately 90% of mIgG/(125)I-tPA is cleared from the circulation within 30 minutes in both WT and TgN-hCR1 mice.
15. Anti-CR1/tPA accelerats lysis of pulmonary emboli and prevented stable occlusive carotid arterial thrombi from forming after injection in TgN-hCR1 mice, but not in WT mice, whereas soluble tPA and mIgG/tPA are ineffective.
16. Anti-CR1/tPA causes 20-fold less rebleeding in TgN-hCR1 mice than the same dose of tPA.
17. CR1-directed immunotargeting of PAs to circulating RBCs provides a safe and practical means to deliver fibrinolytics for thromboprophylaxis in settings characterized by a high imminent risk of thrombosis.
18. Studies in mice showed that scFv-uPA, but not unconjugated uPA accumulates in the brain after intravascular injection,
19. Lyses clots lodged in the cerebral arterial vasculature without hemorrhagic complications, provides rapid and stable cerebral reperfusion, alleviates post-thrombotic brain edema.
20. Effective and safe thromboprophylaxis in the cerebral arterial circulation by anti-PECAM scFv-UPA represents a prototype of a new paradigm to prevent recurrent cerebrovascular thrombosis.
21. XOR inhibition stimulates growth and development of human brain derived cells. Treatment with the low concentration of allopurinol will guarantee the survival of the cells, decrease the number of the death cells and promotes the proliferative processes.
22. Low doses of pyridoxine as well as allopurinol prevented mortality of the animals (n=4-6 pergroup).
23. Low doses of pyridoxine and allopurinol prevented BBB disruption and extrvasation of Evans Blue bound albumin into the brain parenchyma.
24. All components of vitamin B complex (thiamine, riboflavin, pyridoxine, nicotinamide) are able to initiate cells development and growth, however, they are supposed to be selectively utilized in time dependent manner to guarantee the highest effectiveness of the cell proliferation, differentiation and maturation.
25. PRPS-1 and XO activity regulation impact on the cells proliferation and BBB integrity.

26. GTP as well as allopurinol in the normal conditions suppresses PRPS 1 activity.
27. GTP in the normal conditions activates XO activity.
28. In peroxide treated as well as phosphate groups it is notable negative correlation of XO and PRPS1activties (-0.63459; -0.1907 – Pearson coefficient).
29. In allopurinol treated groups during time it is notable positive correlation with the slight XO increase and dramatic PRPS 1 activities elevations (0.466748).
30. PRPS-1 activation and XO inhibition stimulates the recovery processes after experimental stroke.

PEER-REVIEWED PUBLICATIONS
The entire thesis is the reflection of mentioned below publications!

Chapters

Translational Research in Stroke. Editors: Lapchak, P.A, Yang, G.Y. Chapter 26. Danielyan, K.E. Do We Have a Chance to Translate Bench-top Results to the Clinic Adequately? An Opinion. Springer. 2017. Pp. 555-578.

Molecular Nutritions: Vitamins. Editor: Patel, P. Chapter 16. Danielyan K.E., Chailyan S.G. New Properties of Vitamin B6 or Pyridoxinein Experimental Oxidative Stress in the Brain. Elsevier; 2020 (paper version). Pp. 301-322.

Articles

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2. Danielyan, K.E., Chailyan, S.G. Delineation of Effectors Impact on the Human Brain Derived Phosphoribosylpyrophosphate Synthetase Activity.// Biomedical Journal of Scientific and Technical Research. 2019; 24(1), 17918-17926.
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Դանիելյան Քրիստինե Էդգարի
Նոր միացությունների կանխարգելիչ և բուժիչ ազդեցությունը փորձարարական ինսուլտի պայմաններում
Ամփոփում

Հանգուցային բառեր. ***ինսուլտ, փորձարարական ինսուլտ, թրոմբոլիտիկներ, հակաօքսիդանտային միացություններ, նյարդապաշտպանիչ միացություն, դեղերի կրիչներ, ռեգեներատիվ միացություններ, պուրինների նյութափոխանակություն, պիրիմիդինների նյութափոխանակություն***

Հետինսուլտի բուժումը Հայաստանում ներառում է ռենին-անգիոտենզին համակարգի (կապտոպրիլ, էնալապրիլ, պերինդոպրիլ, վալսարտան, լոզարտան, լիզինոպրիլ, ռամիպրիլ), կալցիումի ուղիների վրա ներազդող դեղեր (ամիլոդիպին), հակաբիոտիկներ (ցեֆտրիաքսոն), ցավազրկողներ (անալգին, դիմեդրինիլ), նյութափոխանակության գործընթացները բարելավող միացություններ (ցերեբրոլիզին), հակաօքսիդանտներ (մեքսիդոլ), դիաբետի սիմպտոմները նվազեցնող դեղեր (դիաբետոն) և այլն: Հայաստանում 2015 թվականից օգտագործվում է ալտեպլազը՝ հյուսվածքային տիպի պլազմինոգեն ակտիվատորի ածանցյալը թրոմբոէկտոմիայի հետ համատեղ: Բուժման այս երկու մոտեցումները կտրուկ նվազեցրին Հայաստանում ինսուլտով հիվանդների մահացության մակարդակը:

Այնուամենայնիվ, ինսուլտը շարունակում է մնալ մահվան հիմնական պատճառներից մեկը ողջ աշխարհում: Այսպիսով, պարզ է դառնում, որ բուժման, ինչպես նաև պաթոլոգիայի կանխարգելման համար նոր լուծումներ են անհրաժեշտ:

Աշխատանքը ընդգրկել է ինսուլտի գրեթե բոլոր փուլերը թիրախավորող նոր դեղամիջոցների ստեղծման և փորձարկման նկարագրումը:

Ատենախոսության հիմնական նպատակն է հանդիսացել՝ պուրինների և պիրիմիդինների նյութափոխանակության հիմնական կարգավորող ֆերմենտների հայտնաբերումը և ուսումնասիրությունը, այս ֆերմենտների վրա ներազդող էֆեկտորների հայտնագործումը և ուսումնասիրությունը, պուրինների և պիրիմիդիններ նյութափոխանակության կարգավորման կենսաքիմիական մեխանիզմների օրինաչափության հաստատումը, պուրինների/պիրիմիդինների նյութափոխանակության հետ կապված որոշ ռեգեներատիվ մեխանիզմների ուսումնասիրությունը փորձարարական ինսուլտի պայմաններում, ՊՀՊ-1-ի կարողությունների ուսումնասիրությունը նեյրոպաշտպանության գործընթացներում փորձարարական ինսուլտի ժամանակ, ալբումինի հեմատոէնցեֆալիկ պատնեշը պաշտպանող հատկությունների ուսումնասիրությունը, ալբումինային մասնիկների, որպես դեղերի կրիչներ բնութագրումը, փորձարարական ինսուլտի բուժման համար հյուսվածքային պլազմինոգեն ակտիվատորի անալոգների ստեղծումը թրոմբոլիզի մեխանիզմների թիրախավորումը փորձարարական ինսուլտի ժամանակ ապահովելու նպատակով:

Աշխատանքը ընդգրկել է ինսուլտի գրեթե բոլոր փուլերը թիրախավորող նոր դեղամիջոցների ստողծման և փորձարկման նկարագրումը :

Ներկայացված աշխատանքը ներառում է նեյրոպաշտպանիչների ուսումնասիրությունը, ինչպիսիք են ալբումինը և ՊՀՊ-1, որն առաջին անգամ մեկուսացրել և նույնականացրել է խոշոր եղջերավոր կենդանիների հիպոթալամուսից Ա.Ա. Գալոյանը և գործընկերները (Գալոյան 2004 թ.): Բացի այդ, ՊՀՊ-1 (PRP-1) հետազոտվել է իմ

կողմից նեյրոնային բջիջների կուլտուրայի օքսիդատիվ սթրեսի, ինչպես նաև in vivo, փորձարարական ինսուլտի պայմաններում: Եզրակացվել է, որ ՊՀՊ-1-ը հատուկ են նյարդապաշտպան հատկություններ:

Արդյունավետ և անվտանգ թրոմբոլիտիկների ստեղծումն ու գնահատումն նոր ուղղություն է, որը ներկայացված է իմ աշխատանքում: Աշխատանքում ներկայացված են տվյալներ, որոնք վկայում են արդյունավետ թրոմբոլիտիկների զուգակցման կամ կապման մասին էրիթրոցիտների հետ: Այսպիսի կոմպլեքսները կարող են հեշտությամբ ընդգրկվել նոր գոյացող մակարդուկի կառուցվածքային կազմի մեջ և ապահովել քայքայումը ներսից:

Բացի այդ, ալտեպլազը, զուգորդված էրիտրոցիտների հետ, որոնք հանդիսանում են լավագույն կենսաբանական կրիչները մեր օրգանիզմում, երաշխավորում են երկար և անվտանգ դեղի շրջանառությունը:

Վերոհիշյալ վարկածն ապացուցվել է նաև այլ կոմպլեքսների ստեղծմամբ և կիրառմամբ: Հակա CR-1 հակամարմինները զուգակցվել են ալտեպլազի հետ: Մեկ այլ պլասմինոգեն ակտիվատորը՝ ուրոկինազի թեթև շղթան, զուգակցվել է թրոմբոցիտ ենդոթելիալ բջիջներ ադհեզող մոլեկուլային կլանիչների հակամարմինների մի հատվածի հետ՝ ապահովվելով ինսուլտի պրոֆիլակտիկան և բուժումը:

Պուրինների և պիրիմիդինների նյութափոխանակության փոխանակելի կախվածությունը նկարագրել է Զոլները և այլոք (Elion, Kelley et al. 1978): Էլիոնը և գործընկերները ցույց են տվել, որ երկու պուրինային իզոմերներ կարող են փոխակերպվել տարբեր նուկլեոտիդների, որոնք ազդում են պիրիմիդինների նյութափոխանակության վրա (Muir, Harrow et al. 2008): Այսպիսով, պուրինային կատաբոլիզմի ֆերմենտի թիրախավորումը հետևաբար ազդում է պիրիմիդինների կատաբոլիզմի վրա:

Ցույց է տրվել, որ ալոպուրինոլը, որը պուրին-հիպոքսանտինի ածանցյալն է, կարող է օքսիդացվել քսանտինի համապատասխան իզոմերի՝ օքսիպուրինոլի: Երկու նյութերն էլ արգելակում են քսանտինօքսիդորեդուկտազը (ՔՕՌ; XOR; EC 1.17.3.2), որը նվազեցնում է միզաթթվի, ռեակտիվ ազատ ռադիկալների ձևավորումը՝ իջեցնելով միզաթթվի կոնցենտրացիան պլազմայում և արտազատումը աղիքների և մեզի միջոցով: Ներկայացվելիք աշխատանքի հիման վրա ապացուցվեց, որ պիրիդոքսինն ուղղակիորեն արգելակում է ՔՕՌ-ի ակտիվությունը, և որ արգելակումը կարող է մեծացնել կենդանի, ինչպես նաև նոր ձևավորված նեյրոնային բջիջների թիվը հիպոկամպային և կեղևային բջիջների կուլտուրաներում:

Ուսումնասիրվել է նաև նուկլեոտիդների սինթեզի մեկ այլ կարևոր ֆերմենտ՝ Ֆոսֆոռիբոզիլպիրոֆոսֆատ սինթետազ-1 (ՖՌՊՍ-1; PRPS-1 EC: 2.7.6.1): Փորձնականորեն ապացուցվեց, որ ախտաբանական պայմաններում ՖՌՊՍ-1-ի ակտիվացումը և ՔՕՌ-ի ակտիվության արգելակումը հանգեցնում են նուկլեոտիդային պաշարի հարստացմանը, ինչը խթանում է բջիջների վերականգնողական բազմացման մեխանիզմները:

Այսիսով՝ ամբողջ աշխատանքը նվիրված է ինսուլտի տարբեր փուլերի զարգացման կանխարգելմանը կամ բուժմանը նոր մեխանիզմների թիրախավորմամբ կամ նոր միացությունների կիրառմամբ:

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Воздействие новых профилактических и терапевтических соединений при экспериментальном инсульте
Резюме

Ключевые слова: **инсульт, экспериментальный инсульт, тромболитики, антиоксидантные соединения, нейропротекторные соединения, носители лекарств, регенеративные соединения, пуриновый обмен, пиримидиновый обмен**

Целью работы послужило исследование и выявление ключевых регуляторных ферментов метаболизма пуринов и пиримидинов, установление закономерностей биохимических механизмов регуляции пуринового и пиримидинового обмена посредством эффекторов, исследование регуляторных ферментов пуринового/пиримидинового обменов, влияюших на регенеративные механизмы экспериментального инсульта, изучение возможной нейропротекции ПОП-1 в процессах нейропротекции, изучение способности альбумина предотвращать повреждение гематоэнцефалического барьера, разработка альбуминовых частиц, как носителей лекарственных средств, воздействие на механизмы тромболизиса, создание безопасных аналогов тканегого плазминогенного активатора и урокиназы для лечения экспериментального инсульта.

Лечение постинсультных больных в Армении включает воздействие на ренин-ангиотензиновую систему (каптоприл, эналаприл, периндоприл, валсартан, лозартан, лизиноприл, рамиприл), кальциевые каналы (амилодипин), использование антибиотиков (цефтриаксон), анальгетиков (анальгин, димедрол), аспирин (кардиомагнил), препараты, улучшающие обменные процессы (церебролизин), использование антиоксидантов (мексидол), в случае больных сахарным диабетом-применение препаратов, снижающих уровень глюкозы в крови (диабетон). С 2015 г в больницах начали использование тромболитического препарата альтеплазы, производного тканегого плазмногенного активатора вместе с тромбоэктомией. Последние два подхода лечения резко снизили смертность больных с инсультом в Армении.

Тем не менее, инсульт остается одной из ведущих причин смерти во всем мире. Таким образом, предполагается разработка новых подходов создания лечащих препаратов, профилактики патологии, исследвания метаболитических путей развитя инсульта.

Мое исследование включает изучение не только механизмов развития и профилактики инсульта, но и создание и усовершенствование существующих и/или новых соединений, предотвращающих инсульт. Работа состоит из описания лекарственных средств, действующих практически на все стадии развития патологии.

Представленная работа включает оценку и изучение таких нейропротекторов, как альбумин и пептид ПОП-1 (PRP-1), соединение, впервые выделенное из гипоталамуса крупного рогатого скота А.А. Галояном и сотрудниками (Галоян 2004). Кроме того, свойства ПОП-1 были исследовали на культуре нейрональных клеток в условиях окислительного стресса in vitro и in vivo, в условиях экспериментального инсульта (результаты in vivo не предтавлены в работе). На основании результатов мы пришли к заключению, что ПОП-1 обладает нейропротекторным действием.

Создание и оценка эффективных и безопасных тромболитиков – еще одно направление результатов, представленных в моей работе. Было высказано предположение, что сочетание

тромболитиков с эритроцитами, которые в процессе формирования тромба могут быть включены в состав сгустка, способны лизировать тромб извнутри. Кроме того, альтеплаза в сочетании с эритроцитами, которые являются лучшими биологическими носителями лекарств, гарантируют длительную и безопасную циркуляцию в потоке крови в организме. Вышеупомянутая гипотеза была подтверждена также использованием других модификаций плазминогенного активатора и его коньюгатов, таких как комплексное соединение антител против CR-1 и альтеплазы. Следущий коньюгат, предствленный в работе, является сочетанием легкой цепи урокиназы и отрезком антител, созданных против тромбоцитарно-эндотелиальных адгезирующих молекул или рецепторов, который может служить в качестве профилактического и/или терапевтического средства лечения инсульта.

Взаимозависимая связь метаболизма пуринов и пиримидов была продемонстрирована ранее Золльнером и др. (Elion, Kelley et al., 1978). Элион и его коллеги показали, что производные пуринов могут быть преобразованы в различные нуклеотиды, влияющие на метаболизм пиримидинов и наоборот (Muir, Harrow et al. 2008). Таким образом, влияние на ферменты катаболизма пуринов влияет на катаболизм пиримидинов.

Было показано, что аллопуринол, являющийся производным пурин-гипоксантина, может окисляться до соответствующего изомера ксантина, называемого оксипуринолом. Оба вещества ингибируют ксантиноксидоредуктазу (КОР; XOR; EC 1.17.3.2), что снижает образование мочевой кислоты, реактивных свободных радикалов, снижает концентрацию мочевой кислоты в плазме и экскрецию в кишечник и в мочу, а также повышает экскрецию гипоксантина и ксантина в мочу. Впервые на основании работы было доказано, что пиридоксин напрямую ингибирует активность КОР и, что ингибирование может увеличивать количество как живых, так и новообразованных нейрональных клеток в культурах клеток гиппокампа и коры.

Также был изучен другой важный фермент синтеза нуклеотидов – ФРПС-1 (PRPS-1 фосфорибозилпирофосфатсинтетаза-1; EC 2.7.6.1). Экспериментально мною было доказано, что при патологических состояниях активация ФРПС-1 и угнетение активности КОР приводит к обогащению запаса нуклеотидов, что запускает ключевые механизмы регенеративной пролиферации клеток. Вся работа посвящена изучению механизмов регенерации, разработке и созданию новых промедицинских препаратов, направленных на лечение и профилактику разных стадий инсульта.

NOTES