ORIGINALNI RAD

UDK BROJ: 615.213.065
DOI: https://www.doi.org/10.5937/zdravzast53-52916

UTICAJ 2,4-DIAMINOBUTERNE KISELINE NA PROTEINE KOJI UČESTVUJU U RAZVIĆU MORFOMETRIJSKI MERLJIVIH PARAMETARA OKA KOD ZEBRICE: *IN SILICO* ANALIZA

Milica Milošević[1], Nikola Mitović[2], Maša Ristić[3], Ljubica Dimitrijević[4], Sanjin Kovačević[2], Jelena Nešović Ostojić[2], Marija Stanojević[2], Svetolik Spasić[2]

[1] Institut za kardiovaskularne bolesti „Dedinje", Beograd, Republika Srbija
[2] Institut za patološku fiziologiju, Medicinski fakultet Univerzitet u Beogradu, Beograd, Republika Srbija
[3] Klinika za endokrinologiju, dijabetes i bolesti metabolizma, Univerzitetski klinički centar Srbije, Beograd, Republika Srbija
[4] Specijalna Bolnica „Sveti Sava", Beograd, Republika Srbija

[*] Korespondencija: Milica Milošević, Institut za kardiovaskularne bolesti "Dedinje", 11000 Beograd, Republika Srbija; e-mail: milosevic.a.milica13@gmail.com

SAŽETAK

Uvod: 2,4- diaminobuterna kiselina (2,4-DABA), ekscitatorna amino-kiselina sa dokazanim neurotoksičnim efektom se nalazi u vodenim ekosistemima, sa potencijalom za akumulaciju u biljnim i u životinjskim organizmima. S obzirom da je dokazan njen neurotoksični, hepatotoksični i potencijalno kancerogeni efekat postavlja se pitanje moguće embriotoksičnosti. Zahvaljujući velikoj homologiji sa ljudskim genomom, dinamika i morfologija razvića se može proučavati na zebricama (lat. *Danio rerio*), koje predstavljaju dobar model sistem za ispitivanje razvića i razvojnih abnormalnosti.
Cilj: Ispitivanje uticaja 2,4-DABA-e na proteine ključne u razviću oka zebrica pomoću molekularnog dokinga.
Metode: Inicijalno je urađen skrining celokupnog genoma korišćenjem *FINDSITE*^{comb} softvera, preciznom analizom je selektovano 1119 proteina iz baze kojima smo utvrđivali stepen homologije, tkivno specifičnu ekspresiju i vreme ekspresije. Šest proteina koji su ispunili tražene kriterijume, analizirani su u *AutoDock Vina* programu molekularnim dokingom.
Rezultati: Interakcija fzd8a proteina i 2,4-DABA-e ispoljila je najnižu vrednost Gibsove slobodne energije od - 4,6 kCal/mol, dok je najviša od - 3,4 kCal/mol zabeležena u interakciji sa proteinom *pbx4*. Takođe, uočena je sličnost aminokiselinske sekvence u proteinima za koje se vezivala 2,4-DABA, koja se ogledala u aminokiselinama koje u svom sastavu imaju –SH grupu.
Zaključak: Sprovedenim istraživanjem pokazano je da 2,4-DABA može ostvarivati svoje efekte na razvoj oka, tokom celog perioda. Rezultate *in silico* analiza ne treba posmatrati izolovano, već kao početni korak i smernice za istraživanja u in vivo uslovima. Stoga naša studija treba biti dopunjena rezultatima ispitivanja na živim embrionima.

Ključne reči: bioinformatika, razviće oka, 2,4-DABA, zebrice

Uvod

2,4- diaminobuterna kiselina (2,4-DABA) je ekscitatorna amino-kiselina sa dokazanim neurotoksičnim efektom (1). Predstavlja metabolički produkt cijanobakterija (2), a nalazi se i u brojnim ekosistemima kako slatkovodnim, tako i morskim, ali i u uzorcima aerosola i tla u njihovoj blizini (3).

Pored toga primećeno je da ima potencijal akumulacije u živim organizmima, kao što su alge, ribe, krabe, ali i u nekim biljnim organizmima, kao i u organizmima sisara (2). Nalazi se i u otpadnim vodama, a potencijalno i u vodama za piće gde ostvaruje interakciju sa sporednim produktima dezinfekcije vode (3).

Svoj dokazani neurotoksični efekat 2,4-DABA ostvaruje delujući kao nelinearni i nekompetitivni inhibitor transaminaze gama aminobuterne kiseline (GABA), te tako povećava koncentraciju GABA-e. Pored toga dovodi do osmotske lize ćelije, ostvaruje hepatotoksični efekat, a istražuje se potencijalni kancerogeni efekat u nastanku glioma, fibrosarkoma, karcinoma jetre itd. (4).

UDK NUMBER: 615.213.065
DOI: https://www.doi.org/10.5937/zdravzast53-52916

ORIGINAL ARTICLE

THE IMPACT OF 2,4-DIAMINOBUTYRIC ACID ON PROTEINS INVOLVED IN THE DEVELOPMENT OF MORPHOMETRICALLY MEASURABLE EYE PARAMETERS IN ZEBRAFISH: AN IN SILICO ANALYSIS

Milica Milošević[1], Nikola Mitović[2], Maša Ristić[3], Ljubica Dimitrijević[4], Sanjin Kovačević[2], Jelena Nešović Ostojić[2], Marija Stanojević[2], Svetolik Spasić[2]

[1] Institute for Cardiovascular Diseases "Dedinje", Belgrade, Republic of Serbia
[2] Institute for Pathological Physiology, Faculty of Medicine, University of Belgrade, Belgrade, Republic of Serbia
[3] Clinic for Endocrinology, Diabetes, and Metabolic Diseases, University Clinical Center of Serbia, Belgrade, Republic of Serbia
[4] Special Hospital "Saint Sava", Belgrade, Republic of Serbia

[*] Correspondence: Milica Milošević, Institute of Cardiovascular Diseases "Dedinje", 11000 Belgrade, Republic of Serbia; e-mail: milosevic.a.milica13@gmail.com

SUMMARY

Introduction: 2,4-Diaminobutyric acid (2,4-DABA) is an excitatory amino acid with neurotoxic, hepatotoxic, and potentially carcinogenic effects, found in aquatic ecosystems with a tendency to accumulate in plants and animals. Due to its potential impact on development, its embryotoxicity is being studied. Zebrafish (Danio rerio), with high homology to the human genome, serve as an excellent model for studying development and developmental abnormalities.
Objective: To investigate the effect of 2,4-DABA on proteins crucial for zebrafish eye development using molecular docking.
Methods: Proteome screening was conducted using the FINDSITEcomb software, selecting 1119 proteins based on homology, tissue specificity, and expression timing. Six proteins that met the criteria were analyzed using molecular docking in the AutoDock Vina program.
Results: The interaction of the fzd8a protein with 2,4-DABA showed the lowest Gibbs free energy value of -4.6 kCal/mol, while the interaction with the pbx4 protein had the highest value of -3.4 kCal/mol. A similarity was observed in the amino acid sequence of proteins that bind to 2,4-DABA, particularly in those containing an –SH group.
Conclusion: 2,4-DABA may affect eye structure development in zebrafish by interacting with proteins throughout the entire development period. The results of *in silico* analyses provide a basis for further in vivo research, which should be conducted on live embryos to confirm these findings.

Key words: bioinformatics, eye development, 2,4-DABA, zebrafish

Introduction

2,4-Diaminobutyric acid (2,4-DABA) is an excitatory amino acid with a proven neurotoxic effect (1). It is a metabolic product of cyanobacteria (2), and it is found in numerous ecosystems, both freshwater and marine, as well as in the samples of aerosols and soil in their vicinity (3).

In addition, it has been noticed that it has the potential of accumulation in live organisms, such as algae, fish, crabs, as well as in some plants and mammalian organisms (2). It has also been found in waste water, and potentially in drinking water, where it interacts with by-products of water disinfection (3).

2,4-DABA achieves its proven neurotoxic effect by acting as a non-linear and non-competitive inhibitor of GABA (gamma-aminobutyric acid) transaminase activity, thus increasing the concentration of GABA. In addition, it leads to osmotic lysis, achieves the hepatotoxic effect, while its carcinogenic effect is being investigated in the development of glioma, fibrosarcoma, liver cancer, etc. (4).

2,4-DABA ima i pokazani antitumorski efekat, jer dovodi do osmotske lize ćelije nakon ulaska aktivnim transportom pomoću transportera za aminokiselinu alanin (5).

Zebrice (lat. *Danio rerio*) su slatkovodne ribe, dužine 2–4 cm, koje predstavljaju dobar model sistem za ispitivanje razvića i razvojnih abnormalnosti (6). Neke od najznačajnijih karakteristika zebrica kao model sistem za ispitivanje razvića su: veličina, jednostavnost genetičkog manipulisanja, visoka feritlnost, lakoća i ekonomičnost za održavanje, brzo i transparentno razviće (7).

Zahvaljujući velikoj homologiji sa ljudskim genomom, dinamika i morfologija razvića koji se proučavaju na ovim ribama mogu se preneti i na razviće ljudskog embriona (8). Oko embriona nalazi se na prominentnoj poziciji, te je pogodno pratiti njegovo razviće kroz nekoliko morfometrijskih parametara. Parametri koji se mogu pratiti su dijametri oka, dijametri sočiva, njegova površina, oblik i drugo. S obzirom da strukture oka potiču kombinovano od mezoderma i nervne kreste (9) postavlja se pitanje uticaja 2,4-DABA-e ne samo na razvoj struktura oka već i na ostale nervne strukture.

In silico analiza potencijalnih interakcija različitih molekula predstavlja brz način provere mehanizma dejstva određene toksične supstance i spoznavanje patofizioloških procesa koji su posledica istog (10,11).

S obzirom na široku rasprostranjenost 2,4-DABA-e, njenu neurotoksičnost i kancerogenost, ali i neistraženost mehanizma njegovog delovanja, cilj našeg rada je bio ispitivanje interakcije ovog toksina sa proteinima bitnim u razviću oka, mehanizama kojim ova amino-kiselina deluje, kao i mogućih posledica koje će iz njenog dejstva proisteći.

Metode

Preliminarni skrining proteoma

U cilju utvrđivanja mogućih interakcija 2,4-DABA-e i proteina domaćina, odrađen je skrining celog proteoma čoveka, koristeći *FINDISTE*^{comb} softver. Ovaj softver funkcioniše po principu analize unesene hemijske strukture molekula uključujući informacije iz različitih baza podataka (*PDB*, *chEMBL*, *DrugBank*) i upoređujući potencijalne sličnosti u strukturnim domenima između unetog molekula i svih dostupnih proteina. Rezultati se prikazuju tabelarno sa sledećim podacima: mTC vrednost (Tanimoto koeficijent – skor koji ukazuje na meru sličnosti između 2 elementa), preciznost odrađene analize, naziv proteina u *RefSeq* bazi i opis proteina (12).

Za dalje analize su korišćeni rezultati čija je preciznost analize bila preko 70%, što je činilo ukupno 1119 proteina.

Navedene analize su, kao što je gore navedeno, važile za proteine ljudskog organizma. Stoga, koristeći *ZFIN* bazu podataka, utvrdili smo stepen homologije između intereagujućih proteina sa ljudskim i ispitali stopu i tkivnu specifičnost njihove ekspresije kroz period embriogeneze u zebrica (13). Finalno smo utvrdili da sve kriterijume (homologi proteini koji se eksprimiraju tokom embriogeneze u oku zebrica) ispunjava 6 proteina iz naše baze koje smo dalje analizirali. Nazivi, funkcija i lokalizacija ispitivanih proteina su preuzeti sa ZFIN-a i prikazani su u tabeli 1.

Priprema za molekularni doking

Strukture proteina (receptora) dobijene kristalografijom su preuzete sa *PDB* ili AlphaFold sajta. 3D struktura prokain-hidrohlorida (liganda) je preuzeta sa *PubChem*-a (14).

Molekularni doking

Molekularni doking je tehnika koja predviđa orijentaciju, afinitet i interakciju liganda na mestu vezivanja u receptoru (protein). U našem radu smo koristili *AutoDock Vina* program koji se primenjuje za predviđanje navedenih interakcija, što je našlo široku primenu u otkrivanju novih lekova.

Nakon otvaranja programa, strukture proteina (u *PDB* formatu) i liganda (u *SDF* formatu) se ubacuju i modifikuju prema potrebi. Za naše analize smo kod ispitivanih molekula uklonili molekule vode. U većini slučajeva, molekuli vode nemaju značajnu ulogu u vezivanju pa se zbog toga brišu kako bi se olakšala izračunavanja. Pored ovoga, polarni atomi vodonika i naelektrisanja su dodati molekulima receptora pre inicijalizacije procesa u cilju „otvaranja“ što više veznih mesta. Molekularni doking je odrađen pod okolnostima da su sve veze liganda rotirajuće, dok su veze receptora podešene da nemaju ovu mogućnost (15).

Pokretanjem dokinga program započinje simulacije interakcija između različitih funkcionalnih grupa receptora i liganda. Na kraju se prikazuje 10 najreprezentativnijih interakcija sa vrednostima Gibsove slobodne energije i srednjeg kvadratnog

2,4-DABA has been shown to have an antitumor effect, because it leads to osmotic lysis after entering by active transport with the help of aminoacid transporter alanine (5).

Zebrafish (Danio rerio) are freshwater fish, 2-4 cm long, which represent a good model system for the examination of development and developmental abnormalities (6). Some of the most important characteristics of zebrafish that serve as a model system for the examination of development are: size, simplicity of genetic manipulation, high fertility, simple and inexpensive maintenance, fast and transparent development (7).

Thanks to the high homology with the human genome, the dynamics and morphology of development, which are studied in these fish, can also be transferred to the development of the human embryo (8). The eye of the embryo is in a prominent position, and it is convenient to monitor its development through several morphometric parameters. The parameters that can be monitored are the following: the diameters of the eye, the diameters of the lenses, its surface, shape, etc. Considering that eye structures are derived from the mesoderm and neural crest (9), the question of the impact of 2,4-DABA is raised, including both the impact on the development of eye structures and on other neural structures.

In silico analysis of potential interactions between different molecules represents a quick way to check the mechanism of action of certain toxic substances and to realize consequential pathophysiological processes (10,11).

Considering the widespread distribution of 2,4-DABA, its neurotoxicity and carcinogenicity, as well as the unexplored mechanism of its action, the aim of our study was to examine the interaction between this toxin and proteins that are important for eye development, the mechanism by which this amino acid acts, as well as the possible consequences of its action.

Methods

Preliminary screening of the proteome

In order to determine possible interactions between 2,4-DABA and host proteins, screening of the entire human proteome was performed using the FINDSITE^{comb} software. This software works on the principle of analysis of the input chemical molecule structure, including information from different databases (PDB, chEMBL, DrugBank), and comparison of potential similarities in structural domains between the input molecule and all available proteins. The results are presented in tables with the following data: mTC value (the Tanimoto coefficient – score that indicates the measure of similarity between 2 elements), the accuracy of analysis, the name of the protein in the RefSeq database and description of the protein (12).

The results, whose accuracy was over 70%, were used for further analysis, and it included the total of 1119 proteins.

The mentioned analyses were, as stated above, valid for the proteins of the human organism. Therefore, using the ZFIN database, we determined the degree of homology between the proteins interacting with the human ones, and examined the rate and tissue specificity of their expression throughout the period of embryogenesis in zebrafish (13). Finally, we determined that 6 proteins from our database met all the criteria (homologous proteins that are expressed during embryogenesis in the eye of zebrafish), and we further analyzed them. The names, function and localization of the examined proteins were obtained from the ZFIN database and they are shown in Table 1.

Preparation for molecular docking

Protein (receptor) structures, which were obtained by crystallography, were downloaded from the PDB or AlphaFold websites. The 3D structure of procaine-hydrochloride (ligand) was downloaded from PubChem (14).

Molecular docking

Molecular docking is a technique that predicts the orientation, affinity and interaction of ligands at the binding site in a receptor (protein). In our study, we used the AutoDock Vina program, which is used for predicting the mentioned interactions, which has found wide application in discovering new drugs. After opening the program, the structures of proteins (in PDB format) and ligands (in SDF format) are entered and modified as needed. We removed water molecules from the examined molecules in our analyses. In most cases, water molecules do not have a significant role in binding, and therefore, they are removed in order to facilitate calculations. In addition, polar hydrogen atoms and electric charges are

Tabela 1. Podaci o ispitivanim proteinima preuzeti iz ZFIN baze podataka

Oznaka proteina	Pun naziv proteina	Lokalizacija	Funkcija
Lgsn	Lengsin	Transmembranski protein Citoplazma	Učestvuje u morfogenezi sočiva i eksprimira se u njemu
rnf216	Prstenasti protein 216	Citoplazma	Učestvuje u razvoju oka i malog mozga
fzd8a	Uvijeni receptor 8a	Transmembranski protein	Učestvuje u građi oka, u neurogenezi, ekspimira se u nervnoj kresti, CNS-u i digestivnom traktu
cyp1b1	Citohrom P450, familija 1, subfamilija B, polipeptid 1	Mitohondrije, endoplazmatski retikulum	Učestvuje u patogenezi glaukoma.
Lepr	Leptinski receptor	Transmembranski protein	Učestvuje u razvoju čulnih organa Eksprimira se u CNS-u i notohordi
pbx4	Pre-B-ćelijska leukemija transkripcioni faktor 4	Jedro	Učestvuje u razvoju neurona

U tabeli su prikazane oznake, pun naziv, lokalizacija i funkcija proteina koje su ispunile kriterijume (homologi proteini koji se eksprimiraju tokom embriogeneze u oku zebrica); podaci preuzeti iz ZFIN baze podataka.

odstupanja atomskih pozicija (SKOAP). Negativnije vrednosti Gibsove slobodne energije ukazuju na veću šansu spontane reakcije odnosno jače vezivanje između receptora i liganda, dok vrednosti SKOAP-a nam služe za evaluaciju preciznosti odrađenog dokinga (15).

Rezultati

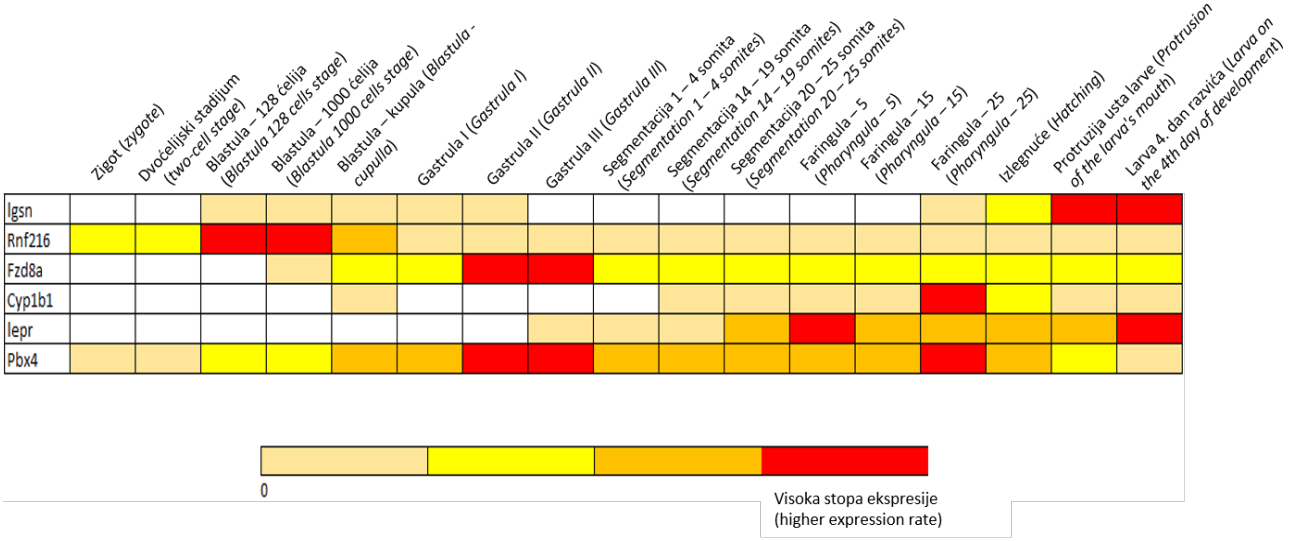
Stepen ekspresije ispitivanih proteina

Na osnovu podataka preuzetih sa *ZFIN*-a, napravljene su toplotne karte koje pokazuju vreme i stepen ekspresije ispitivanih proteina. X-osa karte je podeljena na 17 delova od kojih svaki predstavlja deo razvića u kome se dešavaju neke karakteristične promene. Na Y-osi se nalaze ispitivani proteini (Grafikon 1).

Prema dobijenim rezultatima utvđeno je da postoji difuzna raspoređenost u stopi eksprimiranja ispitivanih proteina. S tim u vezi možemo zaključiti da bi usled kontinuirane ekspozicije tokom perioda embriogeneze 2,4-DABA svoje efekte ostvarivala celom dužinom ovog perioda.

Molekularni doking

Molekularnim dokingom su za poređenja uzete interakcije gde su vrednosti SKOAP-a bile niže od 3 (za veće vrednosti se smatra da je preciznost analize mala).



Grafikon 1. Termalna mapa sa prikazom nivoa ekspresije ispitivanih proteina kroz različite periode embriogeneze (Skala pokazuje stepen ekspresije ispitivanih proteina kroz različite periode embriogeneze.)

Table 1. Data on the examined proteins obtained from the ZFIN database

Protein label	Full protein name	Localization	Function
Lgsn	Lengsin	Transmembrane protein Cytoplasm	Involved in lens morphogenesis and is expressed in it
rnf216	Ring finger protein 216	Cytoplasm	Involved in develepmont of eye and cerebellum
fzd8a	Frizzled receptor 8a	Transmembrane protein	Involved in eye structure, neurogenesis, and is expressed in the neural crest, CNS, and digestive tract
cyp1b1	Cytochrome P450, family 1, subfamily B, polypeptide 1	Mitochondria, endoplasmic reticulum	Involved in eye development, expressed in immature eye structures Involved in the pathogenesis of glaucoma
Lepr	Leptin receptor	Transmembrane protein	Involved in the development of sensory organs Expressed in the CNS and notochord
pbx4	Pre-B-cell leukemia transcription factor 4	Nucleus	Involved in neuron development

The table presents the labels, full names, localization, and functions of the proteins that met the criteria (homologous proteins expressed during zebrafish eye embryogenesis); data obtained from the ZFIN database.

assigned to the molecules of receptors before the initialization of the process aimed at "opening" as many binding sites as possible. Molecular docking was performed under the condition that all bonds of ligands are rotatable, while receptor bonds are set not to have this possibility (15).

By starting docking, the program starts simulations of interactions between different functional groups of receptors and ligands. Finally, the ten most representative interactions are shown with the values of Gibbs free energy and the root mean square deviation (RMSD) of atomic positions. The negative values of Gibbs free energy indicate a greater chance of a spontaneous reaction, that is, stronger binding between the receptor and the ligand, while the RSMD values are used to evaluate the accuracy of conducted docking (15).

Results

The degree of expression of examined proteins

Based on the data obtained from the ZFIN database, heat maps were created and they show the time and level of expression of examined proteins. The X-axis of the map is divided into 17 parts, and each of them represents one stage

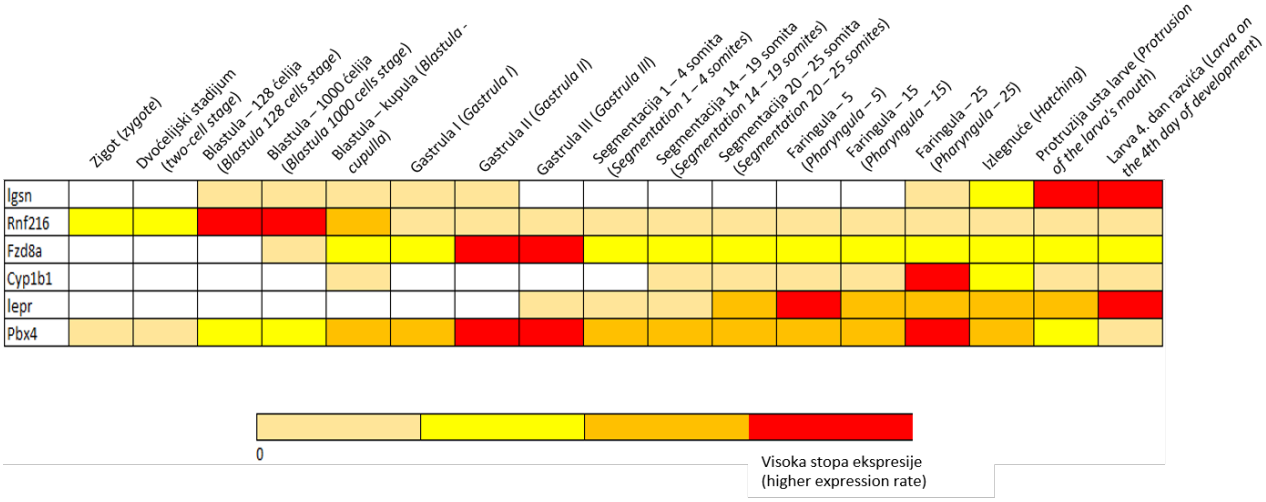
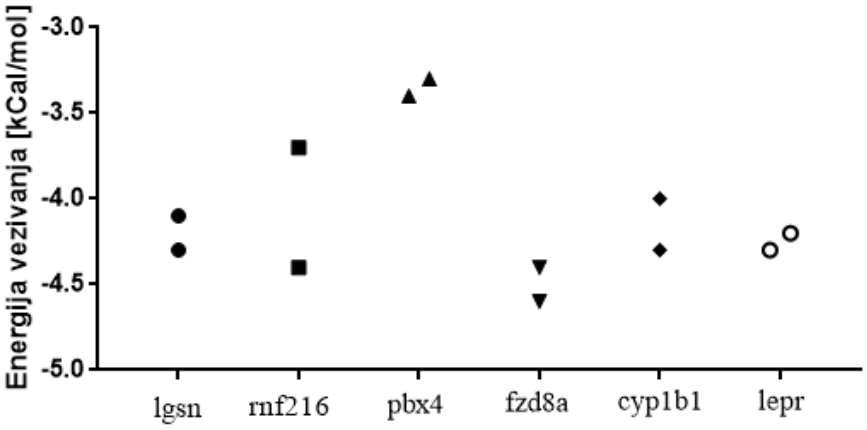


Figure 1. Heat map showing the expression levels of the examined proteins through different stages of embryogenesis (The scale shows the level of expression of the examined proteins through different stages of embryogenesis.)



Grafikon 2. Vrednosti Gibsove slobodne energije u interakcijama izmedju 2,4-DABA-e i ispitivanih proteina. Prikazane vrednosti za svaki protein imale su SKOAP niže od 3 (visoka preciznost dokinga), što je činilo po 2 interakcije po proteinu.

Interakcija *fzd8a* proteina i 2,4-DABA-e je imala najvišu vrednost Gibsove slobodne energije i iznosila je - 4,6 kCal/mol. Za protein *pbx4* je vrednost Gibsove slobodne energije bila najniža i iznosila je - 3,4 kCal/mol. Vrednosti energija ostalih interakcija su date u grafikonu 2.

Pored vrednosti energija, ispitivali smo i vezna mesta 2,4-DABA-e na ispitivanim proteinima. Iako međusobno različite funkcije proteina, sekvence za koje se vezivala 2,4-DABA su bile slične, odnosno, sadržale su aminokiseline koje u svom sastavu imaju –SH grupu (metionin, cistein) (Tabela 2).

Diskusija

2,4-DABA je neproteinska aminokiselina, koja ima dokazani neurotoksični, hepatotoksični efekat, dovodi do citolize ćelija, a ispituje se i njen kancerogeni efekat. Iako do sada slabo ispitana postavlja se pitanje mogućnosti uticaja pomenute aminokiseline na razvoj određenih tkiva ploda (16). Pored toga utvrđeno je da zbog difuzne raspoređenosti u stopi eksprimiranja ispitivanih proteina 2,4-diaminobuterne kiselina svoj efekat ostvaruje tokom čitave embriogeneze. To znači da ne postoji jedna kritična tačka u kojoj 2,4-DABA ostvaruje svoj efekat, već je njena toksičnost izražena tokom čitavog perioda, što povećava njen uticaj.

Pored standardnih animalnih modela danas se sve više koriste i kompjuterske simulacije (17) za ispitivanja mehanizama toksičnosti različitih supstanci. U izradi našeg rada koristili smo *AutoDock Vina*-u za ispitivanje uticaja 2,4-DABA-e na proteine uključene u razviće oka. Doking je metod koji omogućuje spoznaju orjentacije molekula koji su povezani u stabilni kompleks. Poznavanje orjentacije nam pokazuje afinitet vezivanja molekula koji grade kompleks. S obzirom da asocijacije između molekula imaju ulogu u prenosu signala, njihova orjentacija

Tabela 2. Aminokiselinske sekvence i njihova redna mesta u ispitivanim proteinima sa kojima je 2,4-DABA ostvarila veze

Oznaka proteina	Aminokiselina za koju se vezuje
Lgsn	Metionin135, cistein142, tirozin141
rnf216	Prolin611, arginin604, serin609
fzd8a	Serin268, metionin1,tirozin253
cyp1b1	Fenilalanin99, serin445, tirozin97
Lepr	Prolin389, Aspraginska kiselina370, valin392
Pbx4	Prolin389, Aspraginska kiselina370, valin392

Tabela sadrži oznake šest ispitivanih proteina, kao i aminokiselinske sekvence (sa rednim mestima) koje ostvaruju interrakciju sa 2,4 DABA-om.

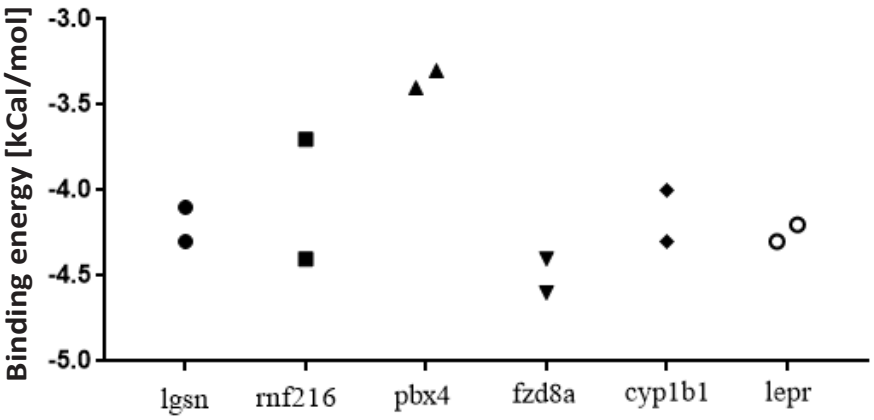


Figure 2. Gibbs free energy values in interactions between 2,4-DABA and the examined proteins. The displayed values for each protein had RMSD below 3 (high docking precision), resulting in 2 interactions per protein.

of development in which some characteristic changes occur. The examined proteins are on the Y-axis (Figure 1).

According to the obtained results, a diffuse distribution in the rate of expression of examined proteins was found. In this regard, it can be concluded that due to the continuous exposure during the period of embryogenesis, 2,4-DABA would show its effects throughout this period.

Molecular docking

Interactions, where RMSD values were lower than 3 (the precision of the analysis is considered low for higher values), were taken for comparison in molecular docking.

The interaction between fzd8a protein and 2,4-DABA had the highest Gibbs free energy value and it amounted to -4.6 kCal/mol. For protein pbx4, the Gibbs free energy value was the lowest and it amounted to -3.4 kCal/mol. The energy values of other interactions are presented in Figure 2.

In addition to energy values, we also examined the binding sites of 2,4-DABA on the examined proteins. Although the functions of proteins are different, the sequences to which 2,4-DABA bound were similar, that is, they included amino acids that contained an –SH group (methionine, cysteine) (Table 2).

Discussion

2,4-DABA is a non-protein amino acid, which has a proven neurotoxic, hepatotoxic effect, leads to cytolysis of cells, and its carcinogenic effect is also being investigated. Although it has been poorly investigated so far, the question relating to the possibility of impact of the above mentioned amino acid on the development of certain tissues of the fetus is raised (16).

Table 2. Amino acid sequences and their positions in the examined proteins with which 2,4-DABA formed interactions

Protein label	Amino acid to which procaine binds
Lgsn	Methionine 135, Cysteine 142, Tyrosine 141
rnf216	Proline 611, Arginine 604, Serine 609
fzd8a	Serine 268, Methionine 1, Tyrosine 253
cyp1b1	Phenylalanine 99, Serine 445, Tyrosine 97
Lepr	Proline 389, Aspartic acid 370, Valine 392
Pbx4	Glycine 117, Serine 116, Arginine 204

The table contains the labels of six examined proteins, as well as the amino acid sequences (with positions) that interact with 2,4-DABA.

može imati ulogu u tipu signala koji će se proizvesti, tako da možemo reći da je doking koristan za predviđanje jačine i tipa proizvedenog siganala (18).

Kod zebrica sa uklonjenim *fzd8a* genom (19) koji je našom doking analizom utvrđen da ima najjači afinitet vezivanja za 2,4-DABA-u, utvrđeno je da postoje brojne promene u odnosu na one sa funkcionalnim genom i njegovim proteinskim produktima. Međutim on ne deluje izolovano već u sadejstvu sa drugim proteinima iz ove familije (*fzd3*, *fzd9*, *fzd10*). Promene se pre svega odnose na razvoj nervnih struktura, kao što je dorzalni deo nervne cevi, ali i struktura oka pre svega pigmentnih ćelija-melanocita, a takođe je utvrđena nemogućnost migracije ćelija iz nervne kreste, što je u embriogenezi ključno za pravilno topografsko mapiranje.

U radu *Ferraiulo* i saradnika (20) pokazano je da 2,4-DABA ostvaruje dozno-zavisnu toksičnost. Na osnovu rezultata ovog rada utvrđen je uticaj na vijabilnost, kako embriona tako i odraslih jedinki, ali i na njihovu sposobnost plivanja, tj. na njihovu motoričku aktivnost. S obzirom na to možemo reći da ova aminokiselina nesumnjivo ima neurotoksični efekat na embrione. U našem radu potvrđene su interakcije ove aminokiselina i proteina ključnih za razvoj struktura oka embriona. Promene koje su uočene u pomenutom radu, pogotovo one koje se tiču motornih aktivnosti, mogle bi se objasniti uticajem 2,4- DABA, s obzirom na činjenicu da su strukture oka i strukture centralnog nervnog sistema istog embrionalnog porekla.

Purdie i saradnici su u svom radu (21) istraživali uticaj β-N-metilamino-L-alanin (BMAA) na razviće zebrica. Ova aminokiselina ima sličnu strukturu i dejstvo kao 2,4-DABA i generalno u prirodi najčešće sinergistički deluju. Ovo istraživanje potvrdilo je dozno-zavisne efekte na razvoj različitih struktura. Između ostalog nesumnjiv je uticaj na razvoj nervnih ćelija ključnih za inervaciju mišićnih strukuta, što se ispoljilo poremećajima u mišićnim aktivnostima u vidu klonusa sličnim konvulzijama. Navedeni rezultati mogli bi se objasniti interakcijama proteina koje smo ispitivali, a koji imaju bitnu ulogu u razvoju nervnih struktura i mišića.

Zaključak

Na osnovu dobijenih rezultata pokazano je da 2,4-DABA može ostvarivati svoje efekte tokom celog perioda razvića oka, ostvarujući značajne interakcije sa proteinima koji učestvuju u razviću oka. Rezultati *in silico* analiza se ne treba posmatrati izolovano, već kao početni korak i smernice za istraživanja u in vivo uslovima. Stoga i naša studija treba biti dopunjena rezultatima ispitivanja na živim embrionima.

Konflikt interesa

Autori su izjavili da nema konflikta interesa.

Reference

1. Krüger T, Mönch B, Oppenhäuser S, Luckas B. LC–MS/MS determination of the isomeric neurotoxins BMAA (β-N-methylamino-l-alanine) and DAB (2, 4-diaminobutyric acid) in cyanobacteria and seeds of Cycas revoluta and Lathyrus latifolius. Toxicon. 2010;55(2-3):547-57. doi: 10.1016/j.toxicon.2009.10.009
2. Collins MD, Jones D. Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2, 4-diaminobutyric acid. Journal of Applied Bacteriology. 1980;48(3):459-70. doi: 10.1111/j.1365-2672.1980.tb01036.x
3. Fan H, Qiu J, Fan L, Li A. Effects of growth conditions on the production of neurotoxin 2, 4-diaminobutyric acid (DAB) in Microcystis aeruginosa and its universal presence in diverse cyanobacteria isolated from freshwater in China. Environmental Science and Pollution Research. 2015;22(8):5943-51. doi: 10.1007/s11356-014-3766-y
4. Abe M, Matsuda M. On the existence of two GABA pools associated with newly synthesized GABA and with newly taken up GABA in nerve terminals. Neurochemical Research. 1983;8(5):563-73. doi: 10.1007/BF00964697
5. Tominaga M, Iwashita Y, Ohta M, Shibata K, Ishio T, et al. Antitumor effects of the MIG and IP-10 genes transferred with poly [D, L-2, 4-diaminobutyric acid] on murine neuroblastoma. Cancer gene therapy. 2007;14(8):696-705. doi: 10.1038/sj.cgt.7701059
6. Nishimura Y, Inoue A, Sasagawa S, Koiwa J, Kawaguchi K, Kawase R, et al. Using zebrafish in systems toxicology for developmental toxicity testing. Congenit Anom (Kyoto). 2016;56(1):18-27. doi: 10.1111/cga.12142
7. MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. Nat Rev Drug Discov. 2015;14(10):721-31. doi: 10.1038/nrd4627
8. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013;496(7446):498-503. doi: 10.1038/nature12111
9. Kronfeld PC. The gross anatomy and embryology of the eye. InVegetative Physiology and Biochemistry.Academic Press.1962 Jan 1; (pp. 1-62) doi: 10.1016/B978-1-4832-3090-0.50007-1
10. de Oliveira Poswar F, Farias LC, de Carvalho Fraga CA, Bambirra Jr W, Brito-Júnior M,et al.. Bioinformatics, interaction network analysis, and neural networks

In addition, it was found that due to the diffuse distribution in the rate of expression of examined proteins, 2,4-diaminobutyric acid achieves its effect during the entire embryogenesis. This means that there is no single critical point at which 2,4-DABA achieves its effect, but that its toxicity is pronounced over the entire period, which increases its impact.

In addition to standard animal models, computer simulations (17) are increasingly used today for the examination of the mechanisms of toxicity of various substances. In our study, we used AutoDock Vina to examine the impact of 2,4-DABA on proteins involved in eye development. Docking is a method that enables the realization of the orientation of molecules that are connected in a stable complex. Knowledge of the orientation shows us the binding affinity of molecules that make up the complex. Considering the fact that associations between molecules have a role in signal transmission, their orientation can have a role in the type of signal that will be produced, and therefore, it can be said that docking is useful for predicting the strength and type of produced signals (18).

In zebrafish with the removed fzd8 gene (19), which was determined to have the strongest binding affinity for 2,4-DABA in our docking analysis, it was found that there are numerous changes compared to those with a functional gene and its protein products. However, it does not act in isolation, but in cooperation with other proteins from this family (fzd3, fzd9, fzd10). These changes primarily relate to the development of neural structures, such as the dorsal part of the neural tube, and eye structures, first of all, pigment cells - melanocytes, and the impossibility of cell migration from the neural crest, which is crucial in embryogenesis for proper topographic mapping, was also established.

In a study by Ferraiulo et al. (20), it has been shown that 2,4-DABA achieves dose-dependent toxicity. Based on the results of this study, the impact on the viability of embryos and adults was found, as well as on their ability to swim, that is, their motor activity. In regard to this, we can say that this amino acid undoubtedly has a neurotoxic effect on embryos. The interactions between these amino acids and proteins key for the development of embryonic eye structures were confirmed in our study. The changes, which were observed in the above mentioned study, especially those concerning motor activities, could be explained by the influence of 2,4-DABA, considering the fact that eye structures and the structures of the central nervous system are of the same embryonic origin.

Purdie et al. (21) investigated the influence of ß-N-methylamino-L-alanine (BMAA) on the development of zebrafish in their study. This amino acid has a similar structure and effect as 2,4-DABA and they generally act synergistically in nature. This study confirmed dose-dependent effects on the development of different structures. In addition, there is an undoubted influence on the development of nerve cells key for the innervations of muscle structures, which is manifested as disorders in muscle activities in the form of convulsions similar to clonic seizures. The mentioned results could be explained by the interactions of examined proteins, which have an important role in the development of neural structures and muscles.

Conclusion

Based on the obtained results, it was shown that 2,4-DABA can achieve its effects throughout the entire period of eye development, thus achieving significant interactions with proteins that participate in eye development. The results of *in silico* analysis should not be considered in isolation, but as a starting point and guidelines for *in vivo* research. Therefore, our study should be supplemented with the results of research conducted on live embryos.

Competing interests

The author declared no competing interests.

References

1. Krüger T, Mönch B, Oppenhäuser S, Luckas B. LC–MS/MS determination of the isomeric neurotoxins BMAA (β-N-methylamino-l-alanine) and DAB (2, 4-diaminobutyric acid) in cyanobacteria and seeds of Cycas revoluta and Lathyrus latifolius. Toxicon. 2010;55(2-3):547-57. doi: 10.1016/j.toxicon.2009.10.009
2. Collins MD, Jones D. Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2, 4-diaminobutyric acid. Journal of Applied Bacteriology. 1980;48(3):459-70. doi: 10.1111/j.1365-2672.1980.tb01036.x
3. Fan H, Qiu J, Fan L, Li A. Effects of growth conditions on the production of neurotoxin 2, 4-diaminobutyric acid

to characterize gene expression of radicular cyst and periapical granuloma. Journal of endodontics. 2015; 41(6):877-83. doi: 10.1016/j.joen.2015.02.004

11. Arnold R, Boonen K, Sun MG, Kim PM. Computational analysis of interactomes: Current and future perspectives for bioinformatics approaches to model the host–pathogen interaction space. Methods. 2012;57(4):508-18. doi: 10.1016/j.ymeth.2012.06.011
12. Zhou H, Cao H and Skolnick J. FINDSITEcomb2. 0: A new approach for virtual ligand screening of proteins and virtual target screening of biomolecules. Journal of chemical information and modeling. 2018;58(11), 2343-2354. doi: 10.1021/acs.jcim.8b00309
13. Ruzicka L et al. ZFIN, The zebrafish model organism database: Updates and new directions. Genesis. 2015;53(8), 498-509. doi: 10.1002/dvg.22868
14. Lehrer S and Rheinstein PH. Ivermectin docks to the SARS-CoV-2 spike receptor-binding domain attached to ACE2. In vivo. 2020;34(5), 3023-3026. doi: 10.21873/invivo.12134
15. Yu R, Chen L, Lan R, Shen R and Li P. Computational screening of antagonists against the SARS-CoV-2 (COVID-19) coronavirus by molecular docking. International Journal of Antimicrobial Agents. 2020;56(2), 106012. doi: 10.1016/j.ijantimicag.2020.106012)
16. O'Neal RM, Chen CH, Reynolds CS, Meghal SK, Koeppe RE. The 'neurotoxicity'of L-2, 4-diaminobutyric acid. Biochemical Journal. 1968 (1;106(3):699-706). doi: 10.1042/bj1060699
17. Takahashi N, Smithies O. Human genetics, animal models and computer simulations for studying hypertension. TRENDS in Genetics. 2004;20(3):136-45. doi: 10.1016/j.tig.2004.01.004
18. Morris GM, Lim-Wilby M. Molecular docking. InMolecular modeling of proteins. 2008 (pp. 365-382). doi: 10.1007/978-1-59745-177-2_19
19. Nikaido M, Law EW, Kelsh RN. A systematic survey of expression and function of zebrafish frizzled genes. PloS one. 2013;8(1):e54833. doi: 10.1371/journal.pone.0054833
20. Ferraiuolo RM, Meister D, Leckie D, Dashti M, Franke J, et al. Neuro-and hepatic toxicological profile of (S)-2, 4-diaminobutanoic acid in embryonic, adolescent and adult zebrafish. Journal of Applied Toxicology. 2019;39(11):1568-77. doi: 10.1002/jat.3840
21. Purdie EL, Samsudin S, Eddy FB, Codd GA. Effects of the cyanobacterial neurotoxin β-N-methylamino-L-alanine on the early-life stage development of zebrafish (Danio rerio). Aquatic toxicology. 2009;95(4):279-84. doi: 10.1016/j.aquatox.2009.02.009

Primljen: 21.08.2024. **Revizija:** 09.09.2024. **Prihvaćen:** 09.09.2024.

(DAB) in Microcystis aeruginosa and its universal presence in diverse cyanobacteria isolated from freshwater in China. Environmental Science and Pollution Research. 2015;22(8):5943-51. doi: 10.1007/s11356-014-3766-y

4. Abe M, Matsuda M. On the existence of two GABA pools associated with newly synthesized GABA and with newly taken up GABA in nerve terminals. Neurochemical Research. 1983;8(5):563-73. doi: 10.1007/BF00964697
5. Tominaga M, Iwashita Y, Ohta M, Shibata K, Ishio T, et al. Antitumor effects of the MIG and IP-10 genes transferred with poly [D, L-2, 4-diaminobutyric acid] on murine neuroblastoma. Cancer gene therapy. 2007;14(8):696-705. doi: 10.1038/sj.cgt.7701059
6. Nishimura Y, Inoue A, Sasagawa S, Koiwa J, Kawaguchi K, Kawase R, et al. Using zebrafish in systems toxicology for developmental toxicity testing. Congenit Anom (Kyoto). 2016;56(1):18-27. doi: 10.1111/cga.12142
7. MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. Nat Rev Drug Discov. 2015;14(10):721-31. doi: 10.1038/nrd4627
8. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013;496(7446):498-503. doi: 10.1038/nature12111
9. Kronfeld PC. The gross anatomy and embryology of the eye. InVegetative Physiology and Biochemistry.Academic Press.1962 Jan 1; (pp. 1-62) doi: 10.1016/B978-1-4832-3090-0.50007-1
10. de Oliveira Poswar F, Farias LC, de Carvalho Fraga CA, Bambirra Jr W, Brito-Júnior M,et al.. Bioinformatics, interaction network analysis, and neural networks to characterize gene expression of radicular cyst and periapical granuloma. Journal of endodontics. 2015; 41(6):877-83. doi: 10.1016/j.joen.2015.02.004
11. Arnold R, Boonen K, Sun MG, Kim PM. Computational analysis of interactomes: Current and future perspectives for bioinformatics approaches to model the host–pathogen interaction space. Methods. 2012;57(4):508-18. doi: 10.1016/j.ymeth.2012.06.011
12. Zhou H, Cao H and Skolnick J. FINDSITEcomb2. 0: A new approach for virtual ligand screening of proteins and virtual target screening of biomolecules. Journal of chemical information and modeling. 2018;58(11), 2343-2354. doi: 10.1021/acs.jcim.8b00309
13. Ruzicka L et al. ZFIN, The zebrafish model organism database: Updates and new directions. Genesis. 2015;53(8), 498-509. doi: 10.1002/dvg.22868
14. Lehrer S and Rheinstein PH. Ivermectin docks to the SARS-CoV-2 spike receptor-binding domain attached to ACE2. In vivo. 2020;34(5), 3023-3026. doi: 10.21873/invivo.12134
15. Yu R, Chen L, Lan R, Shen R and Li P. Computational screening of antagonists against the SARS-CoV-2 (COVID-19) coronavirus by molecular docking. International Journal of Antimicrobial Agents. 2020;56(2), 106012. doi 10.1016/j.ijantimicag.2020.106012)
16. O'Neal RM, Chen CH, Reynolds CS, Meghal SK, Koeppe RE. The 'neurotoxicity'of L-2, 4-diaminobutyric acid. Biochemical Journal. 1968 (1;106(3):699-706). doi: 10.1042/bj1060699
17. Takahashi N, Smithies O. Human genetics, animal models and computer simulations for studying hypertension. TRENDS in Genetics. 2004;20(3):136-45. doi: 10.1016/j.tig.2004.01.004
18. Morris GM, Lim-Wilby M. Molecular docking. InMolecular modeling of proteins. 2008 (pp. 365-382). doi: 10.1007/978-1-59745-177-2_19
19. Nikaido M, Law EW, Kelsh RN. A systematic survey of expression and function of zebrafish frizzled genes. PloS one. 2013;8(1):e54833. doi: 10.1371/journal.pone.0054833
20. Ferraiuolo RM, Meister D, Leckie D, Dashti M, Franke J, et al. Neuro-and hepatic toxicological profile of (S)-2, 4-diaminobutanoic acid in embryonic, adolescent and adult zebrafish. Journal of Applied Toxicology. 2019;39(11):1568-77. doi: 10.1002/jat.3840
21. Purdie EL, Samsudin S, Eddy FB, Codd GA. Effects of the cyanobacterial neurotoxin β-N-methylamino-L-alanine on the early-life stage development of zebrafish (Danio rerio). Aquatic toxicology. 2009;95(4):279-84. doi: 10.1016/j.aquatox.2009.02.009

Received: 08/21/2024 **Revised:** 09/09/2024 **Accepted:** 09/09/2024