**SCIENTIFIC REPORT - Stage III – 2023 -**

"*In silico assist of experiments (part II).*"
Project: PN-III-P4-ID-PCE-2020-2444

## Scientific Description

The following **objectives** were realized:

(c1.1) Sampling conformations of the NS2-NS3 protein precleavage. (part II).

(c2.1) Study of NBARC conformational basins for the ATP/ADP binding site (part II).
(c2.2) Understanding the NB-ARC-mediated switch mechanism (part II).

(c3.1) Increasing the antigenicity of the YMD and EEY tyrosinase peptides in HLA-A0201 and HLA-B40:01 positive melanoma cells.
(c3.2) The expression of proposed tyrosinase mutations will be monitored through Western Blot, and their antigenic potential will be assessed through mass spectrometry using the pseudo-SRM method on the IBAR MS platform for quantification.
(c3.3) Confirmation of the antigenicity of proposed mutations for YMD and EEY using an IFNγ secretion test.

## Progress

All planned **deliverables** were solved:

(D-c1.1) The configuration basins in the pre-cleavage space of HCV NS2-NS3 and their relative abundance. Proposal for key amino acids at the interface.
(D-c2.2) The configuration landscape of RGA's CC 4/5 domains. Testing the hypothesis regarding the resistosome states.
(D-c3.2) The expression of proposed tyrosinase mutations, detection through Western Blot, and their antigenic potential through mass spectrometry.
(D-c3.3) Testing the secretion of IFNγ for YMD and EEY mutation.

**Indicators**: 1 article published.

Chiriţoiu GN, Munteanu CVA, Şulea TA, Spiridon L, Petrescu AJ, Jandus C, Romero P, Petrescu ŞM. "*Methionine oxidation selectively enhances T cell reactivity against a melanoma antigen. iScience*." 2023 Jun 25;26(7):107205. doi: 10.1016/j.isci.2023.107205. PMID: 37485346; PMCID: PMC10362274.

**Disemination**:

- Spiridon L, Sulea TA, Ungureanu VG, Martin EC, Minh DDL, "Robosample: A software using constrained molecular dynamics coupled with Gibbs sampling to investigate macromolecules.", Biophysical Journal, 122 (3), sup 1, 177A, February 10, 2023, 67th Biophysical Society Annual Meeting, San Diego, CA, USA

- Dr. Laurențiu Spiridon*, Drd. Teodor Asvadur Șulea, Victor Gabriel Ungureanu, Dr. Eliza Cristina Martin, Dr. Andrei Jose Petrescu, "**Explorarea complexelor biomoleculă-medicament prin prisma mecanicii roboților**", Farmacia azi: de la tradiție la interdisciplinaritate și inteligență artificială, Septembrie 2023, Cluj-Napoca, Romania.

- Șulea A. Teodor*, Nicolas Papadopoulos, Stefan N. Constantinescu, Laurentiu Spiridon, Petrescu J. Andrei, "*Unraveling molecular basis of myeloproliferative neoplasms (MPN) by experimentally constrained modelling and simulation*", **Studia Universitatis Babeș-Bolyai, Biologia, 68(2)**, December 2023, The Annual International Conference of the RSBMB, Cluj-Napoca, Romania

- Victor Gabriel Ungureanu*, Ioana Popa, Carmen Tănase, Laurențiu Spiridon, "*In-silico Study of RAGE-S100B Inhibitors*", **Studia Universitatis Babeș-Bolyai, Biologia, 68(2)**, December 2023, The Annual International Conference of the RSBMB, Cluj-Napoca, Romania

- Lia-Maria Cucoș*, Teodor Asvadur Șulea, Laurențiu Spiridon, Iuliana Caras, Irina Ionescu, Adriana Costache, Crina Stavaru, Norica Nichita, Costin-Ioan Popescu, "*Hepatitis C Virus Envelope Protein E2 Antigen Design and Characterization for Vaccine Development*", **Studia Universitatis Babeș-Bolyai, Biologia, 68(2)**, December 2023, The Annual International Conference of the RSBMB, Cluj-Napoca, Romania

- Laurentiu Spiridon*, Teodor Asvadur Șulea, Victor Gabriel Ungureanu, Eliza Cristina Martin, Andrei Jose Petrescu, "*Robosample: Harnessing the power of constrained molecular dynamics and Gibbs sampling to investigate macromolecules*", **Studia Universitatis Babeș-Bolyai, Biologia, 68(2)**, December 2023, The Annual International Conference of the RSBMB, Cluj-Napoca, Romania

- Juncu Andrei, Daniel Ion, Paula Florian, Gabi Chiritoiu, Teodor Sulea, Ana Borota, Luminita Crisan, Alina Bora, Sorin Avram, Ovidiu Vlaicu, Dan Otelea, Ursula Bilitewski, Laurentiu Spiridon, Liliana Pacureanu, Costin-Ioan Popescu*, "*Novel HTS cell based assay for cis acting protease activity identifies Hepatitis C Virus NS2 cysteine protease inhibitors with antiviral activity*", **Studia Universitatis Babeș-Bolyai, Biologia, 68(2)**, December 2023, The Annual International Conference of the RSBMB, Cluj-Napoca, Romania

**Activity 3.1. Proposal of key mutations for the HCV NS2-NS3 pre-cleaved form.**

Subactivity 3.1.1 Estimation of conformational basins of HCV NS2-NS3 pre-cleaved form and proposal of key mutations for their demonstration (part II).

**Results**

In this stage of the research, we investigated the conformational basins of the pre-cleaved form of the NS2-NS3 complex, considering the identification of potential mutations. For the efficient functioning of the Hepatitis C virus (HCV), autocleavage between the NS2 and NS3 domains must take place. To facilitate this autocleavage, the favorable positioning of the sequence to be cleaved near the cleavage site—triad H956, E976, C997—is crucial.
The two states of interest, namely "bound" and "unbound" (In-Groove/Out-Of-Groove), were generated and simulated using the Robosample program (Fig. 3.1.1 and 3.1.2). Notice in Figure 3.1.3 that, despite more severe steric hindrance, the NS3 domain occupied two distinct conformational basins. In the unbound state, the NS3 domain explored a single conformational basin. Additionally, the absence of conformational transitions between the two configurations during the simulation indicates the presence of a sharp energy barrier between them, and implicitly, a high free activation energy.

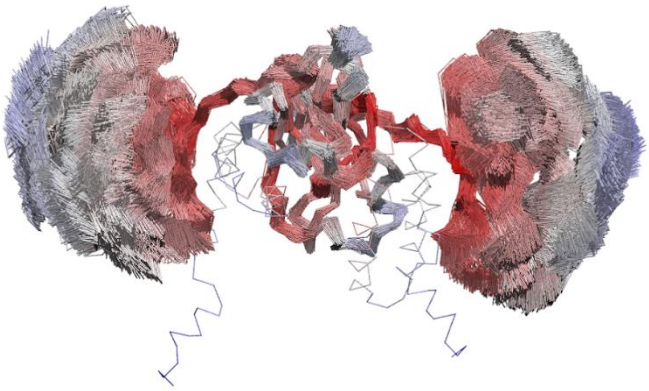


Fig 3.1.1. Sampling of the bound form (In Groove) using the Robosample program. Coloring based on the number of amino acid residues..

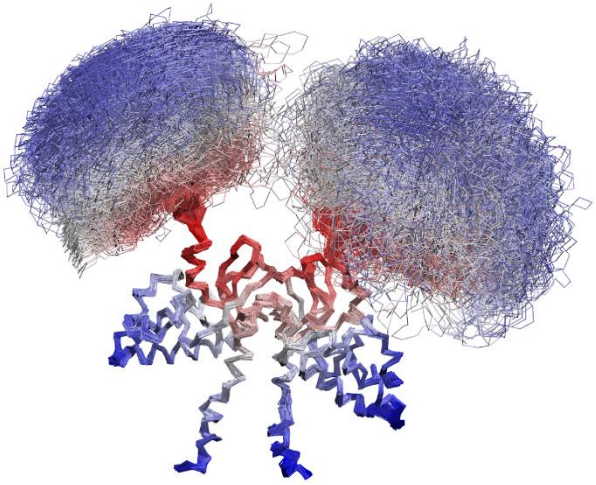


Fig 3.1.2. Representative sampling of the configurational space of the unbound form (Out-Of-Groove) using the Robosample program. Coloring based on the number of amino acid residues.

Following this analysis 5 mutants were proposed for validation.

**Act. 3.2. Validation of the predicted NS2-NS3 interface in activity 3.1.**
Subactivity 3.2.1 Directed mutagenesis of the interface and NS2/NS3 and quantification of the effect on expression.

**Results**

The mutations were tested with an inovative test for NS2-NS3.

**Activity 3.3. Sampling the NBARC configurational space to find the basins allowing the formation of the ADP/ATP binding pocket (Part II).**
Subactivity 3.3.1. Free energy surface difference between the conformational basins present in the NBARC domains upon ATP versus ADP binding.

**Introduction**
To achieve the objective c2.1, which aimed to sample the conformational space of NB-ARC1-ARC2 domains (NBARC) to discover the conformational basins allowing the binding of ADP and ATP, the Robosample program was employed. It facilitated more efficient sampling of the subspace of interest, specifically targeting the degrees of freedom in the NBD-ARC1 and ARC1-ARC2 binding regions. The use of classical molecular dynamics would not have allowed comprehensive real-time sampling.

According to Wang (Wang et al., 2019, Science), plant resistosome proteins (of which NBARC is a part) exhibit two distinct conformations: active (referred to here as "Open") and inactive ("Closed"). It has been observed that the inactive structure contains an adenosine diphosphate (ADP) molecule, while the active one contains an adenosine triphosphate (ATP) molecule. To find conformations that can accommodate these two molecules, the conformational spaces of both conformations were explored based on their resolved structures.

**Methods**
The closed and open structures of the NBARC domain of the ZAR1 protein from A. thaliana were extracted from the RCSB-PDB database (6J5T and 6J5W, respectively), subjected to potential energy minimization, followed by equilibration using OpenMM (Eastman et al., 2017), and then underwent simulations with Robosample (Spiridon and Minh, 2017; Spiridon et al., 2020). ADP and ATP molecules used parameters derived by Meagher et al (Meagher et al., J. Comput. Chem. 2003).
The sampling scheme for the NBARC protein in both forms used the following Gibbs blocks: (1) revolute joints along the side-chain bonds of amino acids and (2) singular revolute joints along the main chain of the link loops between the NBD/ARC1/ARC2 domains.

**Results**
The degree of solvent exposure is a direct indicator of the nucleotide-binding pocket formation as it is at the confluence of the three subdomains: NBD, ARC1, and ARC2. Therefore, the simulations

obtained in this phase were analyzed using average force potential surfaces to characterize the heterogeneity of the binding pocket (Figure 3.3.1).

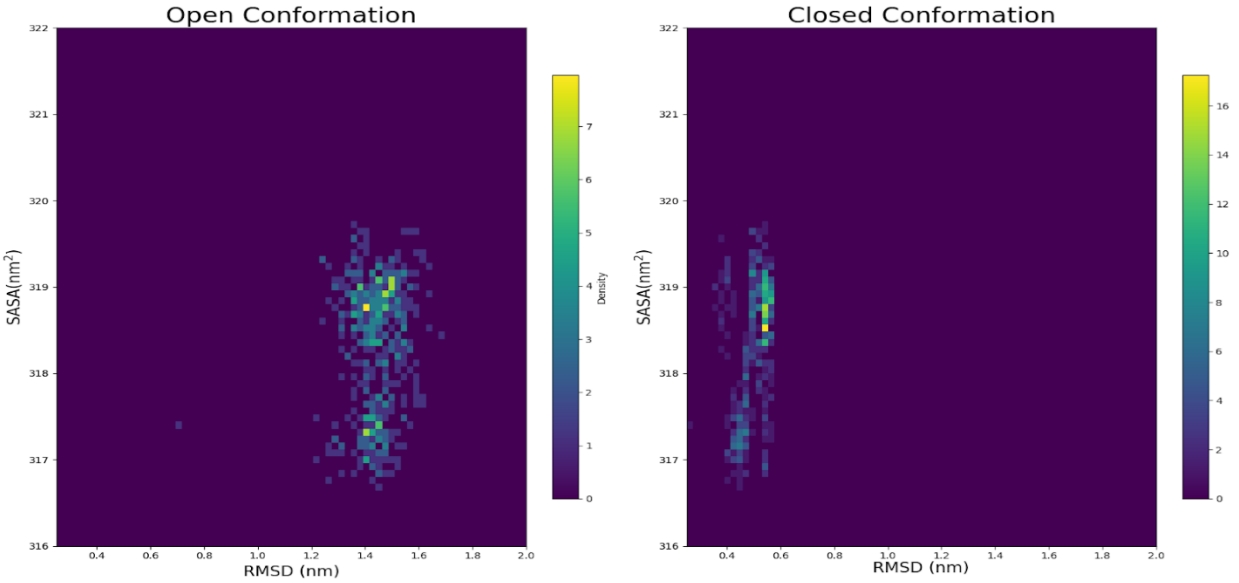



Fig. 3.3.1 - 2D Histogram, Root Mean Square Deviation (RMSD) vs Solvent Accessible Surface Area (SASA). These histograms comparatively show that the NB-ARC1-ARC2 (NBARC) domains are more stable in the closed conformation than in the open conformation (higher RMSD in the case of open form and the presence of two basins in that conformation).

**Act. 3.4. Explanation of the resistance gene analog (RGA) switch mechanism by studying the CC domains 4/5 in conjunction with NBARC.**

Subactivity 3.4.1: Proposals for thermodynamic states of the plant resistosome based on the conformational space of RGA 4/5 CC in Oryza, Brachypodium, Setaria, Sorghum, Hordeum, and validation through experimental data present in structural databases.

**Introduction.**

In this stage, the CC domains 4/5 in conjunction with NBARC were studied to propose thermodynamic states of the plant resistosome based on the conformational space of RGA 4/5 CC in Oryza, Brachypodium, Panicum, Setaria, Sorghum, and Triticum.

The significance of this study lies in the fact that RGA4 and RGA5 are genes encoding for CNL/TNL proteins, involved in the plant immune system, consisting of an NB (nucleotide-binding) - LRR (leucine-rich repeat) domain.

Plant resistance (R) proteins consist of a Toll-like receptor (TIR) / coiled-coil (CC) signaling domain, a nucleotide-binding and oligomerization (NB-ARC) switch domain, and a leucine-rich repeat (LRR) sensory domain. The switch domain comprises three subdomains, namely NB, ARC1, and ARC2, which align to accommodate ADP and ATP in the binding site.

**Results.**

Starting from the RGA4 and RGA5 sequences from Oryza sativa, a group of proteins was generated using the NLRscape atlas, available online at https://nlrscape.biochim.ro/. Sequences were selected based on identity percentage, resulting in 7 RGA4 and 3 RGA5 sequences from Oryza, Brachypodium, Panicum, Setaria, Sorghum, and Triticum. The 'Degree of Intrinsic

Disorder' indicated by the states of Disorder (D) or Order (O) within the analyzed sequences, specifically the regions within proteins incapable of forming stable three-dimensional structures under normal physiological conditions. These regions were considered for subsequent simulations to place the robotic joints.

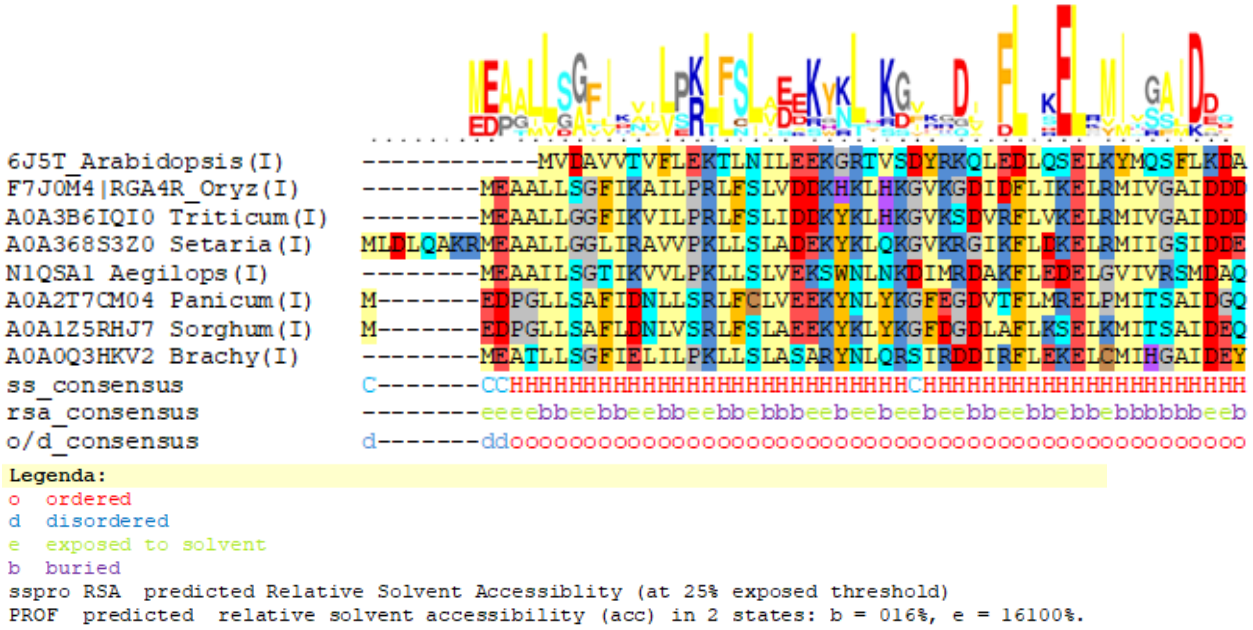
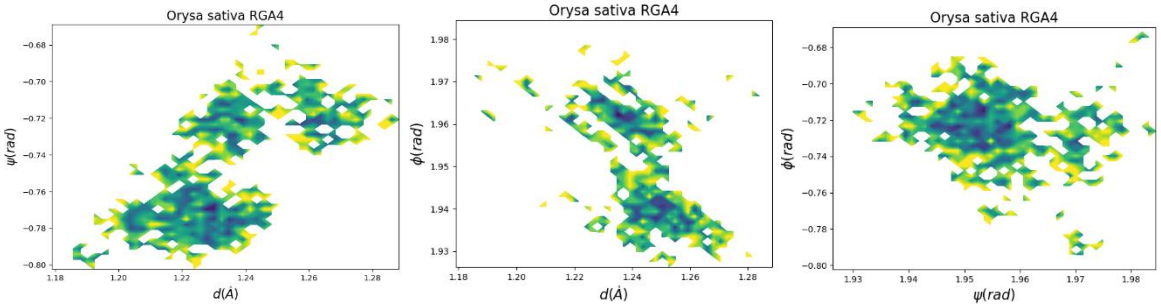



Fig.3.4.1. Fig. 3.4.1. Fragment from the sequence alignment for the RGA4 protein .

Subsequently, structural models were generated for each organism and subjected to potential energy minimization using the OpenMM program. To optimize and determine the most stable models, molecular dynamics simulations and GCHMC were performed with the Robosample application. The obtained trajectories from the simulations were analyzed for the extent of configurational space coverage through RMSD, RMSF, as well as free energy surfaces. To reduce dimensionality and bring the system closer to the transition function between the closed and open states in the formation of the resistosome, the degree of change in the distance between the N-terminal end of the first helix (H1) in the CC domain and the loop between H1 and H2, the angle formed by H1 and H2, as well as the dihedral angle formed with the linker between the NBS and LRR domains were analyzed (Fig. 3.4.2).




(a1) (b1) (c1)

Fig. 3.4.2. Free energy surface based on (a1) the degree of modification of the distance d (Å) between atoms 4 and 893 versus the dihedral angle φ (rad) formed by atoms 4 - 407 - 893 - 8221 of RGA4 from Oryza sativa. (b1) Degree of modification of the distance d (Å) between atoms 4 and 893 versus the angle ψ (rad)

formed by atoms 4 - 407 - 893 of RGA4 from Oryza sativa. (c1) Degree of modification of the dihedral angle φ (rad) format de atomii 4 - 407 - 893 - 8221 versus unghiul ψ (rad) format de atomii 4 - 407 - 893 al RGA4 de la Oryza sativa.

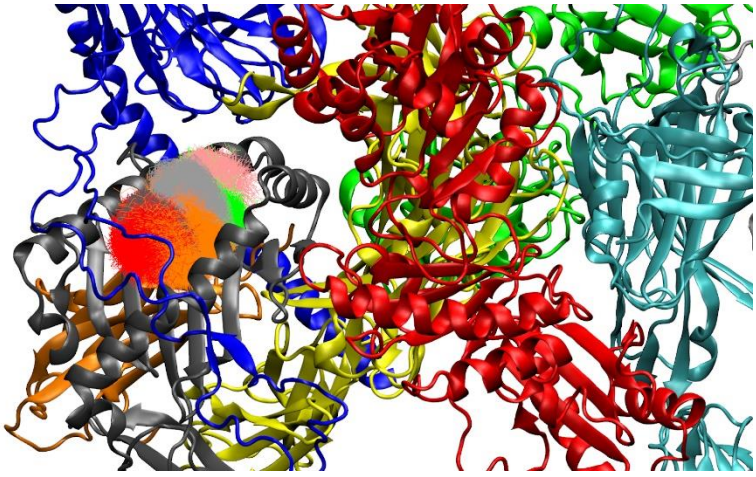
**Act. 3.5. Enhancing the antigenicity of the YMD and EEY peptides from tyrosinase in HLA-A0201 and HLA-B40:01 positive melanoma cells (part II).**

Subactivity 3.5.1 Proposal of mutations to increase the antigenicity of YMD and EEY from tyrosinase in HLA-A0201 and HLA-B40:01 positive melanoma cells (part II).Introduction.

The Major Histocompatibility Complex Class I (MHC-I) peptide-loading complex (PLC) is a critical multi-protein complex of the immune system composed of 5 proteins that form a dimerizing module in the endoplasmic reticulum. Each of the two modules of the PLC dimer consists of Tapasin, Calreticulin, ERp57, the Major Histocompatibility Complex Class I (MHC-I) composed of human leukocyte antigen (HLA), and β2-microglobulin. In this dimeric form, PLC controls the selection and loading of peptides onto MHC-I. After loading, the stable peptide-MHC-I complex is released and exits the endoplasmic reticulum (ER) through the secretory pathway to reach the cell surface for antigen presentation. Here, it is recognized by T cells, ensuring the detection of infected or mutant cells by the immune system. The processes occurring within the PLC during loading are poorly understood due to the complexity and size of this system, as well as its dynamics. For a more accurate assessment of the binding of the EEY peptide, and a mutant E2L of it, it is necessary to explore the conformational spaces of the two peptides before their binding to the PLC complex.

**Results.**

The aim of the study is to obtain a more precise image of the PLC complex based on HLA-B and to investigate the dynamic events that occur during the loading of the antigenic peptide E2L,



a native epitope of tyrosinase.

**Fig. 3.5.1. PLC** complex(albastru - Calreticulina, Rosu: ERp57, Gri: HLA-B, Portocaliu: beta-microglobulina, Galben: Tapasina) impreuna cu conformatiile peptidei EEY, obtinute prin esantionare cu programul Robosample.

**Conclusions**.
E1LE mutant was the most promising proposal for experimental validation.

**Activity 3.6 Expression of the proposed tyrosinase mutants followed by Western Blot and quantification of the antigenic potential through mass spectrometry.**
Subactivity 3.6.1 Expression of the proposed tyrosinase mutants followed by Western Blot and quantification of the antigenic potential through mass spectrometry using the pseudo-SRM method.

Experimental validation was conducted based on the previous mutant proposals. (Data not shown here)

**Act. 3.7. Confirmation of the antigenicity of the proposed YMD and EEY mutants using IFN-gamma secretion.**
Subactivity 3.7.1 Confirmation of the antigenicity of the proposed YMD and EEY mutants using IFN-gamma secretion.
Within this activity, the objective (c3.3) has been fully achieved – The antigenicity of the proposed mutants for YMD and EEY will be confirmed using the IFN-gamma secretion assay.Experimental validation was conducted based on the previous mutant proposals. (Data not shown here)

**Conclusions**.
Mutant proposal from 3.5 and 2.7. activities were validated experimentally

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