Optimization of IL-1Ra Structure to Achieve a Smaller Protein with a Higher Affinity to its Receptor

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Abstract

Background: Interleukine-1 family cytokines are key orchestrators of innate and adaptive immunity. In particular, up-regulation of IL-1R1 via its agonistic ligands consisted of IL-1β and IL-1α is implicated in a variety of human diseases, such as rheumatoid arthritis, psoriasis, type I diabetes, amyotrophic lateral sclerosis, and dry-eye disease. Until now, there are no small-molecule inhibitors of the IL-1R1 with increased antagonistic potency to be used for the treatment of peripheral inflammation. The objective of this study was to engineer a low-molecular-weight version of IL-1Ra with increased affinity and enhanced antagonistic activity for potential therapeutic use.

Methods: To develop a smaller protein-ligand with a better affinity to IL-1R, we used bioinformatics studies and in silico simulations to anticipate non-binding areas on IL-1Ra.

Results: We have successfully reduced the size IL-1Ra to a 110-amino-acid protein which at the same time confers a higher affinity for its receptor, IL-1R1, than its wild-type counterpart. This truncated antagonist displays structural stability and flexibility to the sequence alteration that can be exploited for other β-trefoil family proteins.

Conclusions: These findings should have an impact on the development of new treatments that block IL-1 signaling, although more research is needed *in vitro* and *in vivo*.

Background

Interleukine-1 (IL-1) is one of the first known interleukins involved in several immune responses [1] [2]. IL-1 family consists of 11 members: IL-1β, IL-1α, IL-18, IL-33, IL-1F5 to IL-1F10, and IL-1 receptor antagonist (IL-1Ra) [3]. Overproduction of IL-1α and IL-1β and consequently the up-regulation of IL-1R1 has been implicated in numerous chronic inflammatory and auto-immune disorders [4]. IL-1β, IL-1α, and IL-1Ra are composed of 12 β-strands and the linkers between them which form an anti-parallel β-barrel with a size of ~ 17 kDa [5] [6]. IL-1β and IL-1α exert pro-inflammatory effects through initially binding to the IL-1R1[7], ligand-recognition receptor subunit, which recruits a signaling subunit receptor termed Interleukin-1 Receptor Accessory Protein (IL-1RAcP) [8] [9]. Ectodomains of their receptors consisted of three immunoglobulin-like domains [10]. The juxtaposition of the intracellular Toll–IL-1 receptor domains of two subunit receptors after binding to agonistic ligands triggers intracellular signaling, which leads to activation of the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, therefore, a multitude of inflammatory mediators, such as cytokines and chemokines are expressed [11] [12] [13].

The pro-inflammatory activities of these cytokines can be tightly regulated through pathways that include both extracellularly and intracellularly levels. Naturally occurring inhibitors include protein receptor antagonist IL-1Ra, decoy receptor IL-1R2 [14], and soluble forms of all IL-1 receptors [15] [16] [17] [18]. Firstly, IL-1Ra competitively binds to the IL-1R1 with a high affinity to prevent its binding with agonistic

ligands. This complex is not able to recruit the accessory protein subunit (signaling subunit), therefore no signal transduction occurs [19] [4] [9].

Currently, a great number of anti-inflammatory drugs are actively used to inhibit the signal cascade of IL-1R1 to cure a broad spectrum of inflammatory diseases [20]. Rilonacept is a dimeric chimer protein consisting of Ig-like domains of IL-1R1 and IL-1RAcP along with the Fc-fragment of human IgG. It can capture the IL-1β/α proteins and inhibit their function [21] [22]. Canakinumab is the human IL-1β monoclonal antibody [23]. Anakinra is a non-glycosylated recombinant version of IL-1Ra by the presence of an additional N-methionine which competitively binds to IL-1R1 and blocks its actions [24]. They have already been approved for the treatment of autoinflammatory disorders [25], such as rheumatoid arthritis [26], type 2 diabetes mellitus [27], systemic-onset juvenile idiopathic arthritis [28], osteoarthritis [29], and adult-onset Still's disease [30] [31]. Despite the high efficacy of available drugs, they have some disadvantages, such as local injection reactions, on-target toxicity, the nonexistence of oral bioavailability. Moreover, daily injection is required because of short half-life so they have to be administered at high concentrations that give rise to toxic systemic effects [32]. Alternatively, the inefficiency of these agents could be due to an insufficient receptor affinity, thus a low-molecular-weight IL-1Ra with improved functional activity and receptor affinity could be expected for the aim of peripheral inflammation treatment.

Protein engineering technology is capable of generating macromolecules with enhanced therapeutic efficacy [33] [34]. Identifying the contact regions between a ligand and its binding receptor is essential for creating new therapeutic proteins that block the interaction [35] [36]. Unfortunately, the large ligand-receptor interface and hidden contact regions inside the binding interface of ligand-receptor poses a challenge in recognizing binding sites for low-molecular-weight antagonist development [37]. Here, we utilized bioinformatics' tests and *in silico* simulations to predict non-binding regions on IL-1Ra to design a smaller protein-ligand with a higher affinity to IL-1R. We kept interactive sites of the ligand with the IL-1RI subunit and truncated the protein from non-binding sites, without altering three-dimensional (3D) structures of IL-1Ra.

Methods

Protein selection

For this study, two natural IL-1 ligands (IL-1Ra/IL-1β) and a chimera derived from IL-1Ra and IL-1β were used (Fig. 1b,c). Considering EBI-005 chimer proteins, alongside a wide range of mutants and peptides derived from natural IL-1 ligands with increased antagonistic potency, it is suggested that β-trefoil family proteins in the IL-1 display structural stability and flexibility to the sequence alteration. IL-1Ra is composed of 12 β-strands that bind to its compatible receptor protein IL-1RI and is responsible to restrict signal cascade. Three crystal structures of IL-1 human origin presented in RCSB Brookhaven Protein Data Bank (PDB) were chosen with entry codes: 1ITB (IL-1β), 1ILR(IL-1Ra), and 4GAI(EBI-005), respectively with **2.5** Å resolution/152 amino acids, 2.1 Å resolution/152 amino acids and 1.49 Å resolution/153amino

acids. They represent pure IL-1/IL-1R1 complexes. the IL-1RAcP was detached from the 4GAF crystal structure.

Identifying truncating residues

IL-1 family proteins contain IL-1β/α/Ra, exhibit scant identity in sequence despite the high similarity in a three-dimensional structure. Protein 3D-superposition was performed using chimer software 25.42. 1611 to identify similarities of protein folds and superimpositions of proteins were displayed in Pymol (Fig. 1b,c). Protein sequence alignments could identify regions of similarity that may reflect biological relationships among the input sequences. Here we used the protein BLAST tool provided by NCBI for running BLAST of IL-1 β/Ra/EBI-005 sequence for comparing protein query sequence against a protein sequence subject (Fig. 2). IL-1 ligands bind the same receptor (IL-1R1) through overlapped β-sheets. The receptor-binding site can be subdivided into site A and B which site A is more affine for antagonist while the agonists bind receptor with higher affinity in site B. inter-protein interaction analysis of IL-1β, IL-1Ra, and EBI-005 in complex with their competitive receptor IL-1R1 were performed by Molegro Virtual Docker, delineated crucial overlapped β-sheets of three ligands involved and not involved in the interaction. The interacting residues are highlighted in figure 2.

Truncated IL-1Ra protein preparation

Random truncation was performed on specific selected nonbinding sites of IL-1Ra, afterward, homology models of the truncated proteins were constructed using the automated homology modeling software MODELLER6v2. According to structural similarity to the natural IL-1Ra, 100 models were selected for the docking. The molecular docking was performed by the Z-DOCK program, which applies a fast Fourier, transform to find all feasible binding modes of proteins. For each model, the top 2,000 predictions are given to the Rosetta Dock program to eliminate clashes and improve energies, and then the ZRANK program re-ranks all models. Visual analysis of the interactions between models and receptor were performed in Chimera. The modes of interactions of truncated IL-1Ra are displayed in figure 3.

Protein model validation

The PDB file of complexes (IL-1Ra/IL-1R1, T-IL-1Ra/IL-1R1) was converted to the topology files, adapted for Gromacs package using MDWeb (http://mmb.irbbarcelona.org/MDWeb/) server. Molecular Dynamic (MD) simulations were conducted in GROMACS 4.5.5 using OPLS-AA all-atom force field and TIP4P water model implemented on Intel Xeon 2x 6-Core W3530 2.8 8M 1366 Processor with Bio-LINUX 8 operation system. MD simulation was carried out in a dodecahedron box (> 1.2 nm between the protein edge and the box), filled by SPC216 water molecules up to at least 10.0 Å from the protein surface. Standard protonation states of the residues were used and the charges of the system were neutralized by replacing water molecules with Na+ and Cl- ions. The energy of the system was minimized using the steepest descent algorithm followed by the conjugate gradient procedure; afterward, all bonds were constrained by the LINCS algorithm. The solvent was equilibrated in position-restricted simulation at 200 ps. The temperature coupling was carried out using a Nose-Hoover thermostat in a coupling time constant, of

0.4ps. The pressure of the system was held around 1 bar using the Parrinello-Rahman barostat method with a coupling constant, of 2ps. Bond lengths were constrained using the LINCS algorithm in 2 fs time steps. Terminally, MD simulations were performed at 310K for 50 ns. In all simulations first 10 ns were ignored and the analysis starts from 10 ns to 50 ns. PyMOL, Chimera, and XMGrace software were used to analyze and prepare publication-quality figures.

RMSD and RMSF calculation

The backbone root means square deviation (RMSD), which indicates protein structure stability, is a crucial analysis to evaluate the MD simulations. Backbone RMSD was calculated using the Gromacs package included tool, g_rms. Adapted crystal structure used as a reference, time to reach stable RMSD was indicated and the first unstable stage was discarded for more analysis to ensure that calculated results reflect protein behavior in the given temperature (Fig. 5a). Protein backbone fluctuations were determined by computing RMSF values using the GROMACS package g_rmsf tool. The RMSF value was calculated in the different temperature trajectories for Cα atoms of all residues for the average structure as a reference (Fig. 5b).

Hydrogen Bonds, Electrostatic interactions and Salt Bridges

The g_hbond tool was utilized to compute the total number of protein-protein and protein-solvent hydrogen bonds. The g_hbond calculates the number of donor-acceptor pairs with appropriate angle and distance cutoff for hydrogen bond formation. The angle cutoff (angle formed by the hydrogen, donor, and acceptor atoms) has been set at 60° and the distance cutoff has been set to 0.35 nm. The electrostatic interactions have been computed by calculating the distances between all negatively charged groups and all positively charged groups in the trajectories. Salt bridges in trajectories were calculated between oppositely charged residues by the visual molecular dynamics (VMD) program. Salt bridge cut-off length was set in 0.4 nm distance and persistence for at least 20% of the frames (Tab. 1).

Results

Creating Truncated-IL-1Ra.

3D structures of IL-1 ligands show a similar structural fold, i.e., they are constituted by a 12-stranded beta-trefoil domain with the linkers between them [12] [38]. These ligands share only 22% sequence identity, but they are structural homologs [39]. This information can prove the flexibility of IL-1 family ligands for sequence alteration and their ability to preserve overall conformation. This idea encouraged us to design a series of truncated-IL-1Ra that preserve parental structure and contact regions to the receptor (IL-1R1). Structural superimposition of IL-1β/Ra/EBI-005 reveals several similarities that may account for the ligand-receptor binding sites (Fig. 1b,c).

Here we utilized Molegro Virtual Docker for *in silico* screening of ligands-non-conserved sequences, which they do not implicate in ligand-receptor interaction. This information led to the recognition of the identical

non-binding site at all three ligands (IL-1Ra/β/EBI-005). Subsequently, we mapped the deletion cluster of IL-1Ra, around residue 50-100 (β5-β6-β7) which is not implicated in ligands-receptor (IL-1R1) binding interface and is far from the core of protein structure (Fig. 1a). Altogether, the results demonstrated that most of the deletions in this site would not cause a considerable alteration in ligand conformation. Among the truncated IL-1Ra proteins, the 3D structure of T-IL-1Ra (57-98 residues) had a much greater construction similarity to the native protein than other truncations in the targeted site (β5-β6-β7), therefore, we selected this truncated form as our model (Fig. 2)

T-IL-1Ra/IL-1R1 binding interface in comparison with IL-1Ra/IL-1R1

As mentioned, the deletion clusters in the β5-β6-β7 strands, suggesting that this area is favorable for truncation. It is evident in the structure that this area is located far from the core of protein structure and it does not participate in the interaction with IL-1R1. Crystallography data analysis of IL-1Ra/IL-1R1complex (Fig. 3a, b) revealed that the interface between antagonist and IL-1R1 contains strong contacts between D1D2 domain of receptor and β1–β2, β2–β3, β3–β4, β10–β11 loops of ligand and a few interactions between IL-1R1-D3 domain and ligand. As it is demonstrated in figure 2, the binding interface of the engineered ligand is significantly similar to the native protein, besides few extra interactions made in the interface of the T-IL-1Ra/IL-1R1 complex (Fig. 3c). The Z-score for T-IL-1Ra and IL-1Ra were predicted to be -6.77 and -4.54, respectively by the ProsaWeb server (Fig. 3d, e). The higher negative score of T-IL-1Ra than the wild-type protein ensures the maximum quality of the modeled truncated protein.

T-IL-1Ra antagonistic feature survey via comparing of IL-1/IL-1R1/IL-1RAcP complexes

The crystallography structure studies of ligands in the complex with not only recognition receptor (IL-1RI (A001241)), but also co-receptor (IL-1RAcP (A003536)) suggested low affinity of IL-1Ra to the IL-1R1-D3 domain in the conformational basis of the antagonism. IL-1RAcP is a co-receptor that only can bind to the binary complex of the IL-1R1/IL-1α-β complex [40]. For the stabilization of ternary complex interactions (IL-1/IL-1R1/IL-1RAcP), the D3 domain of IL-1RAcP has to turn around the binary complex to bind the D3 domain of IL-1R1. The D3 domain of IL-1R1 in the complex with IL-1Ra stays far away from the D3 domain of IL-1RAcP, which is anticipated to disrupt the D3-D3 binding interface. As it is obvious in our protein model, the binding interface of T-IL-1Ra is similar to the parental protein which is important for antagonistic features, therefore the D3 domain of IL-1R1 stays far away from the complex conduces decreased affinity between D3-D3 domains of IL-1R1-IL-1RAcP (Fig. 4).

Protein structure validation

Molecular dynamic simulation

To investigate the structural changes in the protein-protein complex induced by ligand binding, several conformational properties were analyzed, such as root RMSD, root mean square fluctuations (RMSF), the radius of gyration (Rg), number of hydrogen bonds (NHBs), electrostatic interactions and salt bridges.

RMSD (nm) vs. time (ns) for all the backbone atoms of IL-1Ra/IL-1R1 and T-IL-1Ra/IL-1R1 complex simulations were calculated to survey the stability of complexes. As shown in figure 5a, early in the simulation of complexes, IL-1R1 domains turn around the ligands because of the flexibility of the linker between the D1D2 and D3 domain, causing an immediate ascent in the overall RMSD value. From 8.5 ns onwards truncated complex attained the approximate equilibrium phase with the RMSD value averaged around 4.6 Å, whereas, the native complex trajectory experienced an ascending trend, which suggested relatively higher stability of T-IL-1Ra complex than native complex. Both systems gradually tended to converge in the last 8 ns (Fig. 5a).

RMSF of T-IL-1Ra/IL-1R1 and IL-1Ra/IL-1R1 complexes were computed to investigate changes in protein flexibility of the complex upon ligand binding. RMSF fluctuation plot of Cα ca

rbon atoms vs time (50 ns) separately for two complexes is shown in figure 5b Residues in T-IL-1Ra/IL-1R1 complex experienced minor fluctuation and the overall RMSF of the truncated complex was lower than the native complex, which indicated this complex was relatively more stable during the simulation process. New interactions involved in stabilizing the truncated complex could play an important role in minimizing the fluctuations and maintaining the proteins in a rigid structure to simplify the formation of the complex (Fig. 5b).

The radius of gyration is a significant parameter to survey the compactness of protein. The radius of gyration for T-IL-1Ra/IL-1R1 and IL-1Ra/IL-1R1 complexes showed fluctuation in Rg value until 10 ns, afterward attained virtually stable Rg value. It was indicated that the Rg average values for truncated and native systems were around 3.11 nm and 3.13 nm, respectively. The lower Rg value of truncated ligand bond to the receptor than the native complex can be attributed to the elimination of ligand-space-barrier which lets the IL-1R1 domains encompass the ligand more tightly. Therefore, T-IL-1Ra/IL-1R1 complex showed higher compact than the native ligand (fig. 5c).

Interaction energetic feature

MM-GBSA method was used to calculate the binding free energy of systems. The average binding free energies and detailed energetic contribution components of 50 ns were shown in table 1. Interestingly, the free energy of the truncated system (-1087.037 KJ/mol) is significantly lower than the native system (-836.819 KJ/mol) which could demonstrate a higher binding affinity of the truncated ligand with its receptor than the native protein. This result conforms to the outcomes obtained from RMSF analysis. Furthermore, dissecting the binding free energy into contributing components showed that the electrostatic interaction in truncated complex (-2214.386 KJ/mol) had a major role in the low free energy of the truncated system and the system stability.

Discussion

IL1-Ra is one of the latest therapeutic targets to block IL-1 activity. Minimizing adverse effects while maintaining the efficiency of current drugs is under development [41] [42]. We suppose that a smaller

form of IL-1Ra with a higher affinity to the receptor would be advantageous in respect of longer administration intervals due to prolonged half-life compared with IL1-Ra. This truncated molecule [43], could be more effective trans epithelial[44], presumably lacks adverse effects caused by the need for frequent injections [45], and would be less costly[46] while having an increased efficacy. The main aim of the current study was to develop a truncated and more effective form of IL-1R1 antagonist for the treatment of peripheral inflammation or any other possible complications.

Crystallographic and bioinformatics studies on the 3D structure of IL-1Ra and IL-1β/α demonstrate structural similarity despite diverse amino acid sequences (Fig. 1, 2). All three molecules can efficiently bind the same receptor (IL-1R1) through overlapped binding sites [47] [48]. Recently, the crystal structure of an antagonist chimer protein (EBI) derived from the IL-1Ra/IL-1β was determined and showed that its architecture is nearly unchanged compared with parental protein structures [49]. This chimer protein had a dramatically different protein sequence from its parental proteins but preserved the same secondary structure of original proteins [50]. This data revealed the structural stability of IL-1 ligands in response to sequence alterations. Moreover, the IL-1R1 receptor can bind to different ligands (IL-1β/α/Ra/ EBI-005) with high flexibility. Based on the substantially conformational change of IL-1R1 domains upon interacting with various ligands beheld in crystal structures, we hypothesized that transient low-molecular-weight binding sites may exist when IL-1R1 domains undergo conformational rotation and therefore suitable for inhibitor development. Here we mapped the non-binding sites of all three ligand to achieve possible regions for truncation (Fig. 2)

Based on these hypotheses and following a wide range of IL-1 ligands-receptor sequence mapping, we identified a stretch of 40–50 amino acids of three-targeted ligand (IL-1β/Ra/ EBI-005) with low primary sequence homology, which has no interaction with IL1R1 with positions far from the protein core (Fig. 2). Therefore, we suggested truncating this site from IL-1Ra, would not dramatically alter the secondary as well as tertiary structure of the protein compared with wild-type IL-1Ra (Fig. 1). To gain insight into the mechanism by which truncated-IL-1Ra binds to its receptor, 3D structural models were constructed by homology modeling based on known structures of human IL-1RI and IL-1Ra (Fig. 3). We used this structure as the template for our *in silico* docking and molecular dynamics simulations to examine the interface between engineered protein and its receptor. Unexpectedly, this deletion displayed significantly increased IL-1Ra/IL-1R1 affinity (Table 1). For the RMSD value, truncated complex trajectory attained the proximate equilibrium from 8.5 ns onward (Fig. 5a) and RMSF value of native complex was higher than truncated complex (Fig. 5b) which indicated lower fluctuation as a result of new interactions and higher stability of T-IL-1Ra/IL-1R1 complex than IL-1Ra/IL-1R1. Higher Rg value of T-IL-1Ra/IL-1R1 showed that truncated area is somehow a space barrier for interacting ligand to the receptor, therefore, deleting this site lets IL-1R1 turn more tightly around the ligand (Fig. 5c).

In this study, we proposed the possible critical sites on ligands implicated in interaction with IL-1RAcP to prevent turning antagonist into agonist during protein engineering processes, therefore, the interacting angle of T-IL-1Ra, which was similar to the native protein antagonist enabled the binary complex to recruit the signaling subunit IL-1RAcP and retained its antagonistic feature (Fig. 4)

Conclusions

In conclusion, our *in silico* simulations resulted in a novel 110–amino acid antagonist of IL-1, that properly binds to all three domains of IL-1RI with higher affinity. These findings should impact the development of new therapeutics that neutralizes IL-1 signaling but it needs to be examined *in vitro* and *in vivo* examined.

Abbreviations

IL-1: Interleukine-1

IL-1R: Interleukine-1 Receptor

IL-Ra: Interleukine-1 Receptor antagonist

IL-1RAcP: Interleukin-1 Receptor Accessory Protein

NF-κB: Nuclear Factor κB

MAPK: Mitogen-Activated Protein Kinase

PDB: Protein Data Bank

BLAST: *Basic Local Alignment Search Tool*

MD: Molecular Dynamic

RMSD: Root Means Square Deviation

VMD: Visual Molecular Dynamics

RMSF: Root Mean Square Fluctuations

Rg: radius of gyration

NHBs: Number of Hydrogen Bonds

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

B.B, S.SH and M.N.B conceived the study. M.N.B, E.KH, S.N, and S.SH wrote and revised the manuscript. M.N.B, E.KH, and J.KH performed the experiments; M.N.B, S.SH, S.N analyzed the data.

All authors read and approved the final manuscript.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

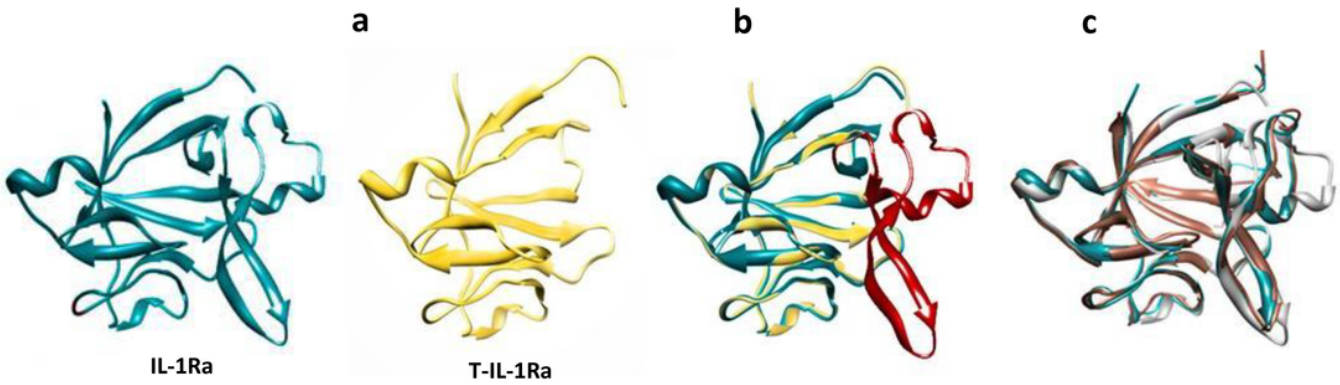

Fig 1: 3D structure of IL-1 ligands. (a) 3D structure of IL-1Ra in comparison with T-IL-1Ra model. (b) superimposition of IL-1Ra and T-IL-1Ra (superimposed RMSD 0.407 Å). (c) superimposition of IL-1Ra (blue ribbon), IL-1β (pink ribbon), and EBI-005 (white ribbon) (superimposed RMSD 0.407 Å).

Figure 1

See image above for figure legend

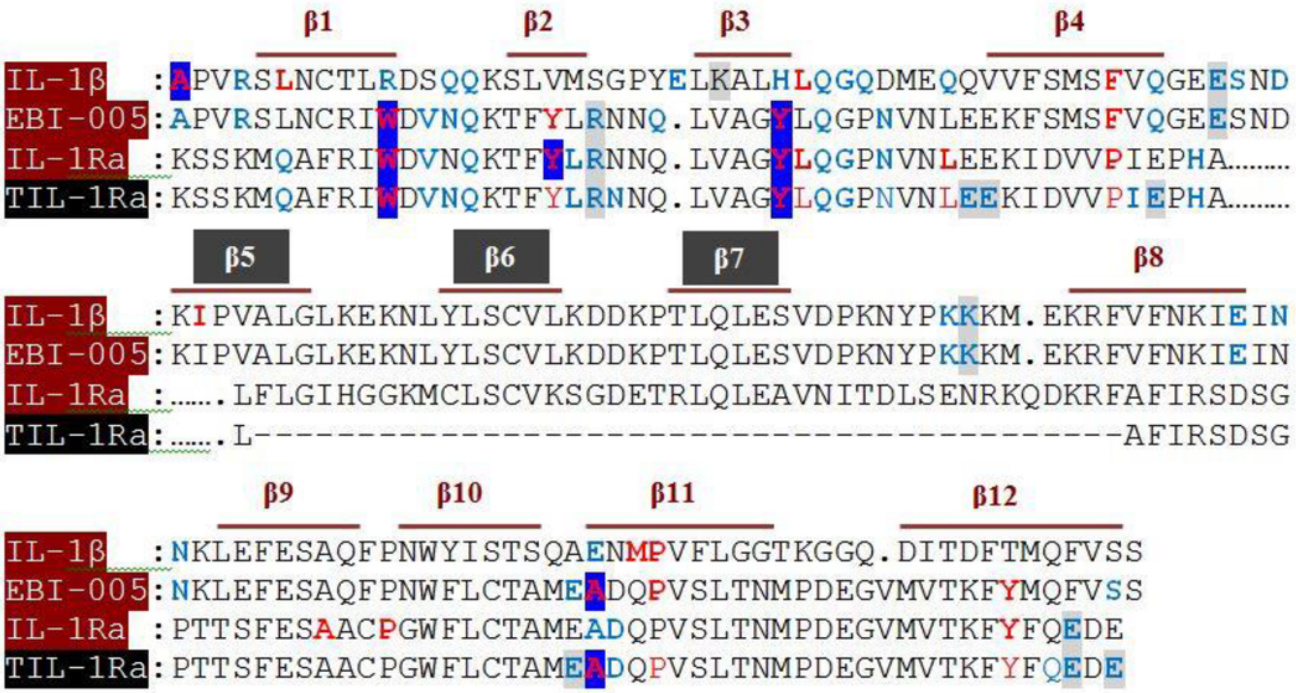

Fig 2: Identification and characterization of a novel truncated IL-1Ra that shares homology with IL-1 family proteins. Sequence alignment of IL-1 ligands with colorful residues implicated in interaction with IL-1R1, identified by ZDOCK and Molegro Virtual Docker within 4Å. Hydrophobic interaction: red, Hydrogen bond: blue and Ionic interaction: gray.

Figure 2

See image above for figure legend

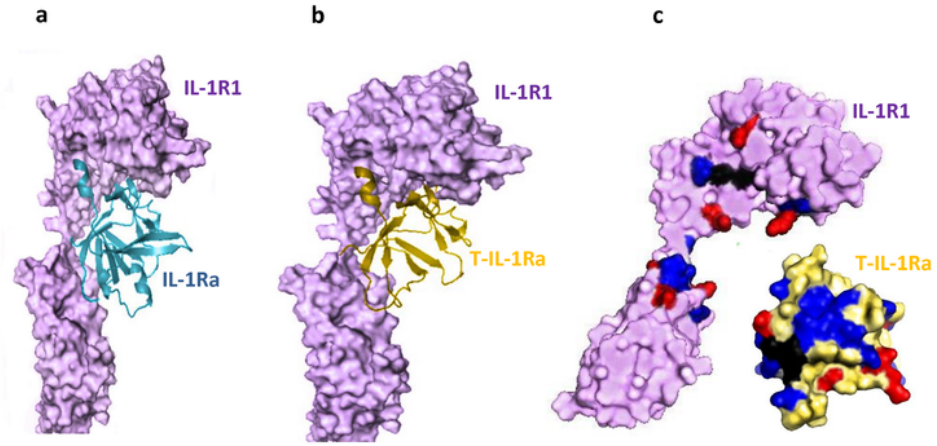

Fig3 a, b and c.

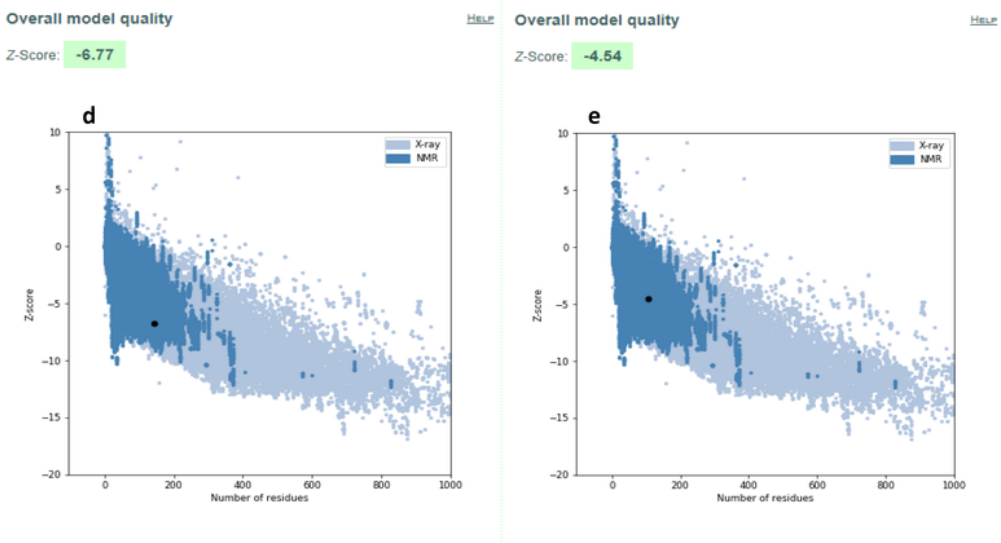

Fig3: 3D structure comparison of IL-1Ra and T-IL-1Ra in complex with their receptor IL-1R1. (a) 3D structure of IL-1Ra in complex with IL-1R1. (b) 3D structure of T-IL-1Ra in complex with IL-1R1. (c) New residues of truncated ligand involved in interaction with IL-1R1 which do not make contact with receptor in native ligand/receptor complex. Hydrophobic interaction (red), Hydrogen bond (blue), and Ionic interaction (black) structure model validation of native (d) and truncated (e) ligands, using ZDOCK server.

Figure 3

See image above for figure legend

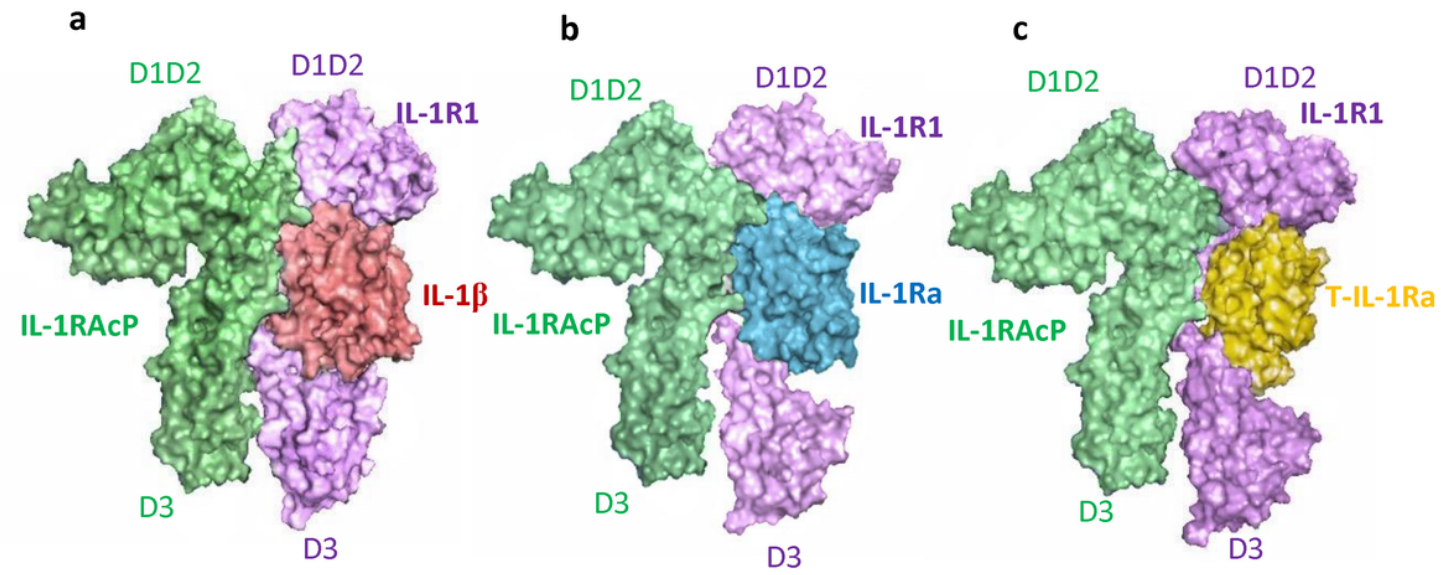

Fig 4: comparison natural and truncated IL-1 ligand in the complexes with IL-1R1/IL-1RAcP (a) surface representation of IL-1β/IL-1R1/IL-1RAcP structure. (b) surface representation of IL-1Ra/IL-1R1 in complex with IL-1RAcP. (c) surface representation of T-IL-1Ra in complex with T-IL-1Ra.

Figure 4

See image above for figure legend

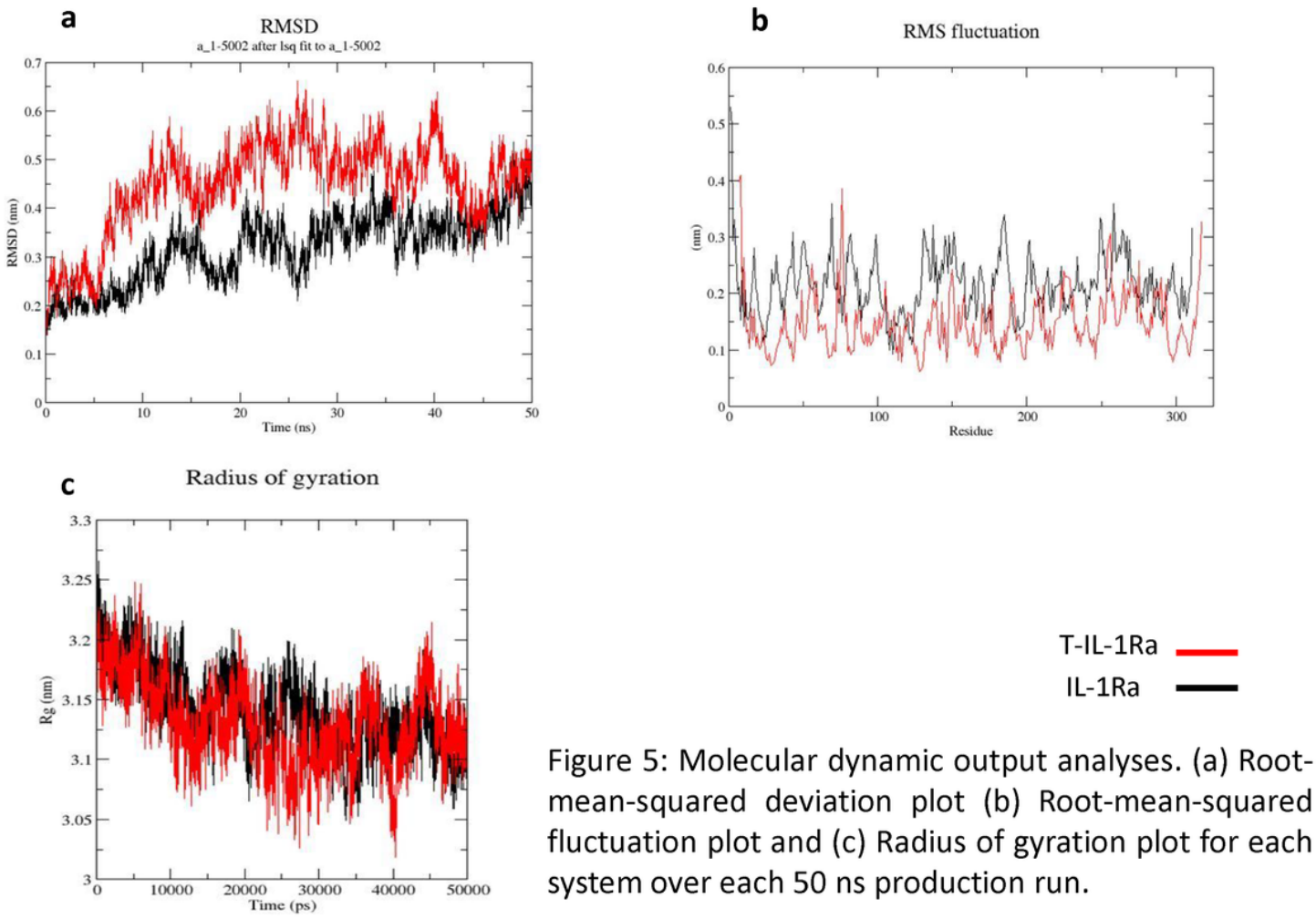

Figure 5: Molecular dynamic output analyses. (a) Root-mean-squared deviation plot (b) Root-mean-squared fluctuation plot and (c) Radius of gyration plot for each system over each 50 ns production run.

Figure 5

See image above for figure legend

Supplementary Files

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- Table1.tiff