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Recombinant Production and One-Step Purification of IL-1Ra in *Escherichia coli* and Evaluation of its IL-1 Antagonizing Efficacy

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ABSTRACT

Background: Anakinra (Kineret®), an IL-1 receptor antagonist, is the first FDA-approved biologic drug for antagonizing IL-1 effects in patients with Rheumatoid arthritis. Notably, the less expensive production of this drug might help reduce the final therapeutic costs.
Objectives: This study aimed to evaluate the possibility of producing biologically active recombinant IL-1Ra by a single-step purification procedure mediated by a self-cleavable intein.
Methods: Soluble expression of the rIL-1Ra was performed in *E. coli* BL21 (DE3) infusion to intein1 of pTWIN-1 vector and its cleavage induction using an elution buffer (pH 6.8) at room temperature. Evaluation of the antagonizing efficacy of this protein in various concentrations was performed on A375 and HEK293 cells treated by a constant concentration of IL-1β (2 ng/mL).
Results: IPTG induction of *E. coli* BL21 (DE3) transformed with the recombinant pTWIN-1, revealed a band approximately in 45 kDa, which is related to the intein1-rIL-1Ra fusion protein in the SDS-PAGE. Moreover, protein purification was confirmed by observing a band in 18 kDa. Finally, the percentage of inhibition effects of rIL-1Ra and Kineret® against IL-1β was not statistically significant in IL-1-responsive A375 cells. The inhibition percentage was calculated as 86% in cells treated with 15µg/mL of rIL-1Ra, which was 96% for the inhibitory effects of the standard drug.
Conclusion: In this study, biologically active soluble rIL1-Ra was successfully produced with high purity through a one-step procedure. This method can reduce the cost and time of production for this protein and might be applicable for producing other biologics.
Keywords: rIL-1Ra, Anakinra, Purification, IMPACT, Rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is the most common inflammatory arthritis in adults such that it affects 0.5-1% of the world's population. RA can lead to pain and stiffness of the joints and decreasing the patient's quality of life (1). Although the main reason

causing RA is still, it has been evidenced that IL-1 has a role in joints and bones erosion (2). *In vitro* studies have shown that IL-1 can lead to cartilage destruction by releasing matrix metalloproteinase and other destructive agents. It can also cause bone erosion by stimulating osteoclasts differentiation and activation (2). In RA patients, the IL-1 level increases in synovial fluid and plasma while there is not sufficient production of endogenous IL-1Ra (3). In experimental models, IL-1 blocking agents such as IL-1 receptor antagonist (IL-1Ra), significantly decrease clinical and histological symptoms of the disease. Thus inhibiting the activity of IL-1 using the human recombinant form of IL-1Ra (Anakinra) is useful in the treatment of RA (4).

Today, biotechnological drugs have become an important part of modern pharmacotherapy such that they are expected to affect 50% of the pharmaceutical market in the next few years. The expiration of the patent form of many biological drugs has made the companies to produce them with maximum similarity with the reference drug called Biosimilar (5).

Anakinra (Kineret®), the recombinant non-glycosylated form of human IL-1Ra, is the first biologic drug that was specially designed and produced (by Amgen company) for antagonizing IL-1 activity in RA patients. Anakinra was approved by FDA in 2001 and by Europe in 2002 for the treatment of RA (6). This protein, with 153 amino acid residues and a molecular weight of 17.3 kDa, is synthesized in *Escherchia coli* expression system. The only difference of this protein with the human IL-1Ra is the presence of a methionine residue at its N-terminus.

Several studies have considered the efficient expression of Anakinra recombinant production using inclusion bodies refolding or auto-induced systems. The amount of total protein has reached 2 and 0.3 g/L of the medium culture, respectively (7, 8).

Producing a soluble form of the protein instead of inclusion bodies can lead to bypassing difficult and expensive steps of inclusion bodies solubilizing and refolding. Also, the use of intein-based purification methods reduces the steps and final cost of the purification of the recombinant protein (9).

As mentioned before, Anakinra is one of the drugs used to treat RA. The patent form of the Anakinra expired in May 2009 in Europe and in February 2022 in the US. Therefore, with the expiration of the patent form of this drug, the production of its Biosimilar will lower the costs so that patients can have easy access to it (5). Accordingly, the aim of the present study was the recombinant production of the IL-1 receptor antagonist in *Escherichia coli* and its intein-mediated purification, followed by evaluating its IL-1 antagonizing ability compared to the commercial form of this drug (i.e., Kineret®).

MATERIALS AND METHODS

Plasmid and Gene, Cell Lines, Bacterial Strain and Reagents

Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT™) Kit was purchased from New England Biolabs (Ipswich, MA, USA). Ampicillin was obtained from Sigma (San Diego, California, USA) and used for selecting transformed bacteria with plasmid. The coding sequence of IL-1Ra was obtained from DrugBank (https://go.drugbank.com) with an accession number DBCAT002727. Next, it was synthesized and cloned to pTWIN-1 vector using Seamless Cloning and Assembly technique (Iranian Institute of Cell & Gene Therapy (IICGT), Tehran, Iran). The expression procedures were performed in *E. coli* BL21 (DE3). The A375 and HEK293 cell lines were purchased from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). Anakinra was obtained from Amgen (US) andIL-1β was purchased from PeproTech. All other chemicals were obtained from other commercial sources and were of molecular biology grade. All molecular biology experiments were

performed according to standard procedures (10) unless otherwise mentioned.

Expression of Intein1-IL-1Ra Fusion Protein and Its Establishment by Western Blotting

The pTWIN-IL-1Ra plasmid was used to transform the *E. coli* BL21 (DE3) cells, followed by selecting recombinant colonies on LB-agar plates containing 100 µg/mL of ampicillin. The selected colonies were then cultivated overnight and used to inoculate fresh cultures to reach an OD_{600} of 0.4 to 0.6. Next, the soluble expression of Intein1-IL-1Ra was induced by 0.4 mM IPTG for 16 hrs. at 15 °C. Then, the cells were harvested via centrifuging at 7000×*g* for 10 min at 4 °C. Finally, the protein expression was evaluated by 12% SDS-PAGE. The band accuracy was confirmed by western blot analysis using an anti-CBD monoclonal antibody (New England Biolabs, USA).

Purification of the Recombinant Protein

As mentioned before, on-column cleavage and purification of IL-1Ra were mediated by the IMPACT™ purification system. Briefly, the cell pellets were re-suspended in B1 buffer (Tris–HCl 20 mM, NaCl 500 mM, and EDTA 1 mM, pH 8.5), followed by sonicating for the cell disruption. The sample was then centrifuged at 7000×*g* and 10 °C for 15 min and finally, the supernatant was loaded on the chitin column. The flow-through was discarded and replaced with B2 buffer (Tris–HCl 20 mM, NaCl 500 mM, and EDTA 1 mM, pH 6.5). The column was incubated for 24 hrs. at 25 °C. Eventually, various elutions were collected and analyzed by 15% SDS-PAGE. All elutions were mixed and subjected to dialysis against phosphate-buffered saline (PBS) pH 7.4 for 24 hrs. at 4 °C. Finally, the protein concentration was determined using the Bradford method against various concentrations of human serum albumin (HSA, Octapharma, Sweden).

Biological Assay

MTT assay was performed to evaluate the proliferative effects of IL-1Ra protein on the A375 cell line (as IL-1 receptor-positive cells). HEK293 (as IL-1 receptor-negative cells) was cultured in Dulbecco's modified eagle medium (DMEM) and RPMI-1640 containing 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) at 37 ºC in a 5% CO_2 incubator. To determine the effective anti-proliferative concentration of IL-1β, first, 180 µL of the medium containing 3×10^4 cells/mL of the A375 cells was poured into each 96 wells of microplates and was incubated for 24 hrs. at 37 ºC. Afterward, 20 µL of the IL-1β with the various concentrations (0, 0.1, 1, 10, 100, 1000, and 10000 pg/mL was added to each row of the plate except for blank. After 48 hrs. of incubation at 37 °C in 5% CO2, 20 µL of MTT (5 mg/mL) was added to each well for the other 3 hrs. of incubation. All well contents were replaced with 150 µL of dimethyl sulfoxide (DMSO) to dissolve formazan crystals. Finally, the plates were subjected to absorbance read at 570 nm by a microplate reader (Bio-Rad, USA).

At the next stage, to determine the antagonizing properties of recombinant IL-1Ra, 160 µL of the cell suspension (3×10^4 cells/mL of each cell line) was used. Next, after 24 hrs, 20 µL of the IL-1β with the final concentration of 2 ng/mL was added to the upper half of the plate except for blank and negative control. After 1 hr, 20 µL of the purified rIL-1Ra with the final concentrations of 15, 7.5, 3.75, 1.8, and 0.94 µg/mL was added. The first row, which was used as blank, contained only 200 µL of the culture and the second row contained 20 µL PBS (as a negative control). Afterward, they were incubated for the other 48 hrs. at 37 °C in a CO2 incubator. Finally, MTT (5 mg/mL) treatment was performed and absorbance was read at 570 nm by a microplate reader. This experiment was repeated for Kineret® (Amgen, US) instead of rIL-1Ra, for both cell lines.

Statistical Analysis

To ensure the accuracy and reproducibility of data, the MTT test was performed for each

cell line as a triple-independent experiment. Cell culture media used as blank, PBS treated cells considered as a negative control, and cells treated with 2 ng/mL of IL-1β, were assumed as the positive control. SPSS 25 software was used for statistical analysis. Analysis of variance (ANOVA) followed by Tukey's post hoc test were used to determine the differences between groups. $P<0.05$ was considered the statistical significance of the analyses.

RESULTS

Expression of rIL-1Ra and Western Blot Analysis

Expression of the Intein1-IL-1Ra fusion protein was evaluated by 12% SDS PAGE. Expression was done in two various conditions. Figure 1a represents the expression at 37 °C after the addition of 1 mM IPTG. As shown, expression induction using 1mM IPTG, revealed a band approximately in 55 kDa, for cells transformed with a non-recombinant pTWIN-1 plasmid which corresponds to the molecular weight of intein 1 and 2 fusion protein. For *E. coli* BL21 (DE3) cells containing recombinant pTWIN1-IL-1Ra, on the other hand, induction with 1 mM IPTG, represents a band approximately in 45 kDa which represented the expression of IL-1Ra in fusion to intein 1 which was confirmed by Western blot analysis as shown in Figure 1b. Figure 1c represents the protein expression in 15 °C. Expression in the mentioned condition revealed the correct band after cell lysis and centrifuge in 7000 ×g for 10 min. According to the Bradford method and using the GelAnalyzer software, the amounts of expressed protein in soluble form were calculated as 175 µg/ml toward the 383 µg/ml of total expressed protein.

Purification of the rIL-1Ra

The collected fraction from the chitin column was incubated for 24 hrs. at room temperature and was analyzed on the 15% SDS-PAGE. In Figure 2, a band of approximately 18 kDa represents the recombinant IL-1Ra. The final yield of recombinant protein production was calculated to be 2.1 mg per

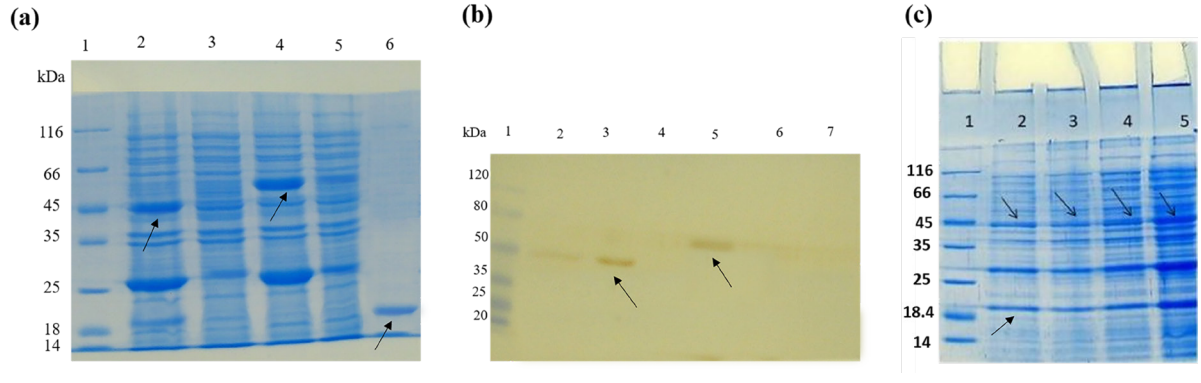

Figure 1. a. 12% SDS-PAGE for analysis of the Intein1-IL-1Ra expression at 37 °C for 4 hrs. and with 1 mM IPTG. Lane 1: protein marker, Lane 2: induced *E-coli* BL21(DE3) cells containing pTWIN1-IL-1Ra, Lane 3: un-induced *E. coli* BL21(DE3) cells containing pTWIN1-IL-1Ra, Lane 4: induced *E. coli* BL21(DE3) cells containing non-recombinant pTWIN-1, Lane 5: un-induced *E. coli* BL21(DE3) cells containing non-recombinant pTWIN-1, Lane 6: the cleaved IL-1Ra from the intein 1. **b.** western blot analysis of expressed proteins. Lane 1: protein marker, Lane 2: un-induced *E. coli* BL21(DE3) cells containing pTWIN1-IL-1Ra, Lane 3: induced *E. coli* BL21(DE3) cells containing pTWIN1-IL-1Ra, Lane 4: un-induced *E. coli* BL21(DE3) cells containing non-recombinant pTWIN-1, Lane 5: induced *E. coli* BL21(DE3) cells containing non-recombinant pTWIN-1, Lane 6: un-induced non-recombinant *E. coli* BL21(DE3) cells, Lane 7: induced non-recombinant *E. coli* BL21(DE3) cells. **c.** 12% SDS-PAGE for analysis of the soluble expression of recombinant IL-1Ra, following 16 hrs. incubation at 15 °C and induction with 0.4 mM IPTG. Lane 1: protein marker, Lane 2: induced *E. coli* BL21 (DE3) cells containing pTWIN1-IL-1Ra after sonication, Lane 3: cell lysate supernatant of the *E. coli* BL21 (DE3) cells containing pTWIN1-IL-1Ra after centrifuging, Lane 4: the remained cell pellet of the *E. coli* BL21 (DE3) cells containing pTWIN1-IL-1Ra after centrifuging Lane 5: the total cell pellets of *E. coli* BL21(DE3) cells containing pTWIN1-IL-1Ra suspending in PBS.

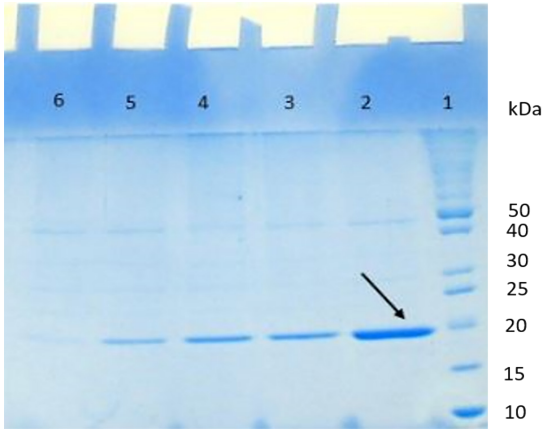

Figure 2. 15% SDS-PAGE for the evaluation of the purification procedure. Lane 1: protein marker, Lane 2: purified recombinant IL-1Ra after the first elution of the column with B_2 buffer, Lane 3: the second elution of the column, Lane 4: the third elution of the column, Lane 5: the fourth elution of the column.

liter of bacterial culture medium.

In vitro Assays

Evaluation of the anti-proliferative effects of IL-1β showed that increasing the concentration of this cytokine lead to a decrease in A375 cell survival. This effect was statistically significant in the concentration of 10 pg/mL (P=0.03) and above (P value for the concentration of 100 pg/mL was equal to 0.007 and for lower concentration was <0.001) compared to the cell treated by PBS (Figure 3). The A375 cells treated with various concentrations of IL-1Ra, after the incubation with a fixed concentration

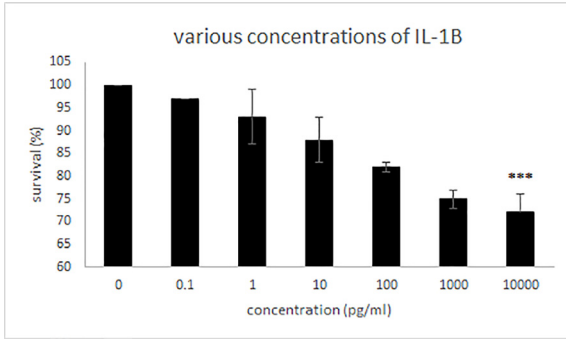

Figure 3. Effect of different concentrations of IL-1β on A375 cell survival. Error bars represent SD. n=3. Stars show statistical differences of each concentration in comparison to the negative control. *P<0.05, **P<0.01 and ***P<0.001

of IL-1β (2 ng/mL), the results showed that, in the similar concentration of the antagonist (15 µg/mL), there were no significant differences between the effects of our recombinant protein and Anakinra (Kineret®) (P=0.297). However, there were significant differences between the A375 survival after treatment with the recombinant IL-1Ra in the presence of IL-1β or its absence (P=0.013). Overall, it is concluded that the produced rIL-1Ra was able to inhibit the interaction of IL-1β to its receptor. These results were repeated for these cells treated by Kineret® (P=0.000). For HEK293, on the other hand, IL-1β led to less survival percent of these cells and both types of antagonists (rIL-1Ra and Kineret®) could increase this survival; however, these effects were significantly less than A375 cells (P=0.043) (Figure 4).

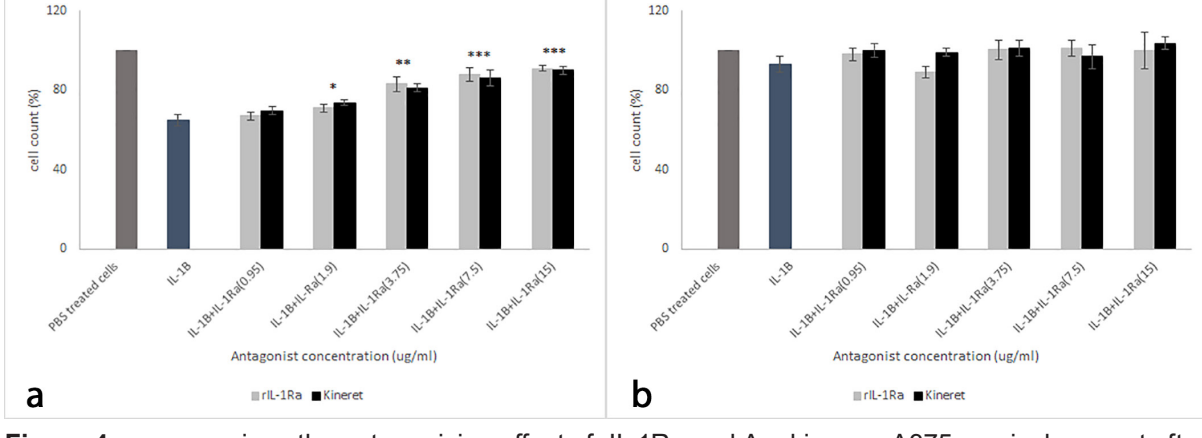

Figure 4. a. comparison the antagonizing effect of rIL-1Ra and Anakinra on A375 survival percent after the treatment with IL-1β. **b.** comparison of the antagonizing effect of rIL-1Ra and Anakinra on HEK293 survival percent after the treatment with IL-1β. Error bars represent SD, n=3. Stars show the significant differences between groups and the positive control (cells treated with IL-1β) in the same concentrations. *P<0.05, **P<0.01 and ***P<0.001.

DISCUSSION

In the present study, the recombinant IL-1Ra was produced by an *E. coli* expression system and purified using intein mediated procedure. Finally, antagonistic activity of the recombinant IL-1Ra was evaluated against A375 and HEK293 cell lines in the presence of IL-1β.

Anakinra, as a non-glycosylated protein, has been produced with the recombinant approach using the *E. coli* expression system. For instance, in (11), the human U937 cells produced IL-1Ra, which was produced in this expression system (12).

The *E. coli* expression system has many advantages such as rapid growth in large volumes without needing expensive substrates, known genetics, and the presence of a large number of cloning vectors (13). Nevertheless, there are several drawbacks related to this expression system including lack of post-translational modifications, low solubility, recombinant protein misfolding, the formation of inclusion bodies, endotoxin problems, and inability to secrete the produced protein (14).

Recombinant production of proteins as inclusion bodies is one of the most important problems related to the *E. coli* expression system. However, there are several solutions to prevent insoluble protein production. These strategies include the co-expression with molecular chaperones, increasing the time of protein synthesis by lowering the temperature as well as inducer concentration, as well as using highly soluble polypeptides as fusion tags (14, 15). Despite these solutions, some tags such as glutathione S-transferase, MBP, and thioredoxin cannot perform the purification process alone and the presence of some affinity tags along with thioredoxin is required for their separation after the purification. In this respect, inteins can cleave themselves from the protein precursor alone and bind the two deflected parts with a peptide band (16).

Expression of protein at lower inducer concentrations leads to a significant reduction in the expression of recombinant protein. For example, in one study, the effect of increasing concentrations of IPTG on the expression of DT386-BR2 protein in *E. coli* was evaluated. The results showed that with the concentration of 1 mM IPTG, the highest amount of recombinant protein production occurs but as an inclusion body. In the current study, to produce the soluble protein, IPTG was used in 0.4 mM concentration.

Another effective factor in the expression of recombinant proteins is the incubation temperature after induction. Generally, the formation of inclusion bodies decreases in lower temperatures (17). For example, in one study for the synthesis of recombinant human G-CSF, decreasing the temperature to 15 °C and increasing the incubation time to 6 hrs., led to the soluble expression of desired protein with the final yield as 5 mg/L of purified soluble protein, comparable with the amount of soluble protein eared in our study as about 2.1 mg/L with yield purity of 95%.(18). Although the amounts of total protein were lower than similar studies such as Bashir et al, (2 g/L as an inclusion body), the final protein was soluble with appropriate folding for biological activity.

For industrial scales, intein usage can significantly lower the costs of producing recombinant proteins by providing a highly specific purification system and inexpensive substrates such as chitin columns. The conventional intein system raises costs due to the need for reducing agents such as dithiothreitol (DTT). Nevertheless, the methods used today are completely optimized and they can reduce all the costs (19). Using intein1 (ssp DnaB) of the pTWIN-1 plasmid is more convenient owing to induction of its self-splicing activity by changing pH, same as the previous study for purifying the recombinant human G-CSF(18).

Another study using the IMPACT system is about the recombinant production and purification of the human brain natriuretic hormone using ssp DnaB intein in 37 °C, with 0.4 mM IPTG, and for 4 hrs. After refolding, the protein was purified on the

chitin column. This purification was done by changing the pH to 7 at 25 ° C for 16 hrs. with high efficiency (20). The yield of purified recombinant protein was calculated as 2.44 mg/L which was equal to our study output but as refolded inclusion body.

The cytotoxic and apoptotic effects of IL-1β against cells with highly expressed IL-1R were established (21). Also, it has been shown in several studies that IL-1Ra can effectively bind to the receptor on the cell surface and inhibit the signaling pathway activation of IL-1R (22). Therefore, based on the previous studies in the evaluation of the effects of Anakinra in preventing the cytotoxic effects of IL-1β, the protocol mentioned in the Methods section was used. The biological results showed that an increase in the concentration of rIL-1Ra led to a corresponding increase in inhibitory effects of IL-1Ra on IL-1β; exactly, this inhibition effects in the lower concentration of IL-1Ra was calculated as 25 %, while this percent for the higher investigated concentration was about 86. The inhibition percentage was calculated with the following formula: Inhibition (%) = $[[A570_M - A570_I]/[A570_N - A570_I]] \times 100$; Here, $A570_I$ is the mean absorbance of wells with the addition of only IL-1β; $A570_M$ is the mean absorbance of wells with the addition of the mixture solution of IL-1β and IL-1Ra, and $A570_N$ is the absorbance of wells without the addition of both IL-1β and IL-1Ra (22). The study of Lui et al used this method for evaluating the inhibitory effects of a fusion protein of IL-1Ra with an extended half-life. the results showed that 32 nM of IL-1Ra led to 100 % inhibition effects of 1 ng/mL IL-1β (21). The concentrations of rIL-1Ra used in the present study were higher than those investigated by Lui et al. In another study, Yu-Xin produced several mutated forms of IL-1Ra and compared their biological activities with those of native IL-1Ra. The results showed that at higher investigated concentrations (25 µg/mL), IL-1Ra can inhibit the effects of IL-1 (23).

The antagonizing efficacy of rIL-1Ra was not statistically significant compared to Anakinra for highly expressed IL-1 receptor cells, A375. The strength of the produced recombinant protein was about 96% of the drug in the market.

The IL-1 antagonizing efficacy of IL-1Ra using A375 was evaluated in two studies (22, 24). Lui et al. used the *Pichia pastoris* expression system for the recombinant production of IL-1Ra. the results showed that this protein could antagonize the cytolytic activity of IL-1β used in a concentration of 1 ng/mL. IL-1Ra created 50 % inhibition of IL-1β in a concentration of about 0.5 nM. In this study, the antagonist and IL-1β were added without any time interval (24). In the second study, it was revealed that IL-1β led to apoptosis in the A375 cells. IL-1Ra binds to the IL-1 receptor without triggering a signaling cascade and competitively inhibits IL-1β binding, preventing its apoptosis-inducing effect. Thus to investigate the inhibitory effects of rIL-1Ra against IL-1β, the antagonist protein was used in the final concentration of 50 ng/mL and IL-1β was used in the final concentration of 2 ng/mL. This article provided a standard method for evaluating the effects of IL-1Ra.

In the present study, following a previous study, the time interval for adding IL-1Ra protein was 4 hrs. after the treatment of cells with IL-1β (23). However, the results showed that the survival of cells treated by antagonist after 4 hrs. of IL-1β addition, was not significantly more than un-treated cells by the recombinant antagonist. Therefore, a time interval of 30 and 60 min was investigated. The results showed that rIL-1Ra could successfully act in antagonizing the toxic effects of IL-1β in a time interval of 1 hr.

Gao et al. investigated time intervals of 0.5, 1, and 2 h between the addition of IL-1β and rIL-1Ra and, finally, selected 1 h as the best interval to fully antagonize the anti-proliferative effects of IL-1β (22).

Another study investigated the role of p53 following the addition of IL-1, which caused cell growth arrest in the G1 phase. After the

treatment of A375-C6 cells with IL-1 at time intervals of 2, 3, and 5 hrs. and the amount of p53 protein in the nucleus was measured as a criterion for cell growth inhibition of IL-1. The results of this project showed that the amounts of P53 protein increased by the end of 5 hrs. (25). Therefore, it seems that the addition of IL-1Ra after 4 hrs. of IL-1β treatment, is ineffective. The reason is that it has been already internalized to cells and it has activated the receptor-mediated signaling such that antagonizing the effects of IL-1 will be ineffective in this time interval.

In the end, Powers et al. used the IL-1-responsive A549 cell line for evaluating the competitive antagonizing effects of IL-1Ra (26). In this study, Anakinra or the Pasylated form of IL-1Ra was added to cells and 1 hr later, IL-1α was added to the cells. The efficacy of IL-1α (in the final concentration of 10 ng/mL), in IL-6 release, was evaluated, and found that both Anakinra and Pasylated form of IL-1Ra antagonized the IL-6 secretion (26). The results of this study, also confirmed the Anakinra antagonizing effects on IL-1, only before its internalizing to the cells with highly expressed receptors on their surface.

CONCLUSION

In this project, we successfully produced and purified the recombinant IL-1Ra in soluble form. The antagonizing effects of this protein were statistically equal to its commercial counterpart, Anakinra (Kineret®). Given that this protein was produced in considerable amounts, the intein mediated purification system might be applicable for its recombinant production in industrial scales as well. However, further structural analysis, as well as *in vitro* and *in vivo* biological evaluations are required for this protein to act as a Biosimilar.

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AUTHORSHIP CRITERIA

F Sh, designed the study and conducted the experiments, performed the statistical analysis, and revised the manuscript. R AN performed the experimental test and prepared the manuscript.

Conflicts of Interest: None declared.

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