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Curpocket as a Tool for Studying Cytokine Activity under Experimental Conditions

Yakubkhodjaeva Malika Razakovna*

Senior Lecturer, Tashkent State Medical University, Tashkent, Uzbekistan

**Corresponding author*

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A B S T R A C T

Cytokines are key regulators of intercellular signaling that play an essential role in immune regulation and the maintenance of homeostasis. Conventional quantitative analytical approaches are limited in their ability to evaluate the spatiotemporal dynamics of cytokine activity and the complexity of their functional interactions. The present study applies the CurPocket method, which combines the analysis of protein conformational pockets with computational modeling, to identify active sites of cytokines and to assess their contribution to intercellular signaling mechanisms. The obtained findings demonstrate the potential of the CurPocket approach for the systematic analysis of cytokine networks and for the development of new strategies in the diagnosis and treatment of immune disorders.

Introduction

Cytokines represent key regulators of intercellular communication involved in the maintenance of homeostasis, modulation of immune responses, and regulation of regenerative processes in the organism (1, 4). These low-molecular-weight protein mediators ensure the dynamic adaptation of cells to external and internal stimuli, which makes them central targets in studies of the pathogenesis of inflammatory, autoimmune, and oncological diseases (1, 2).

Despite significant progress in elucidating the functional activity of individual cytokines, a comprehensive assessment of the cytokine system and its active sites remains a challenging task that requires the application of modern analytical and computational approaches (3).

Traditional methods for the quantitative assessment of cytokines, such as enzyme-linked immunosorbent assay (ELISA) and multiplex analytical techniques, allow accurate measurement of cytokine concentrations in biological media. However, these methods are limited in their ability to characterize the spatiotemporal dynamics of cytokine signaling and to identify functionally relevant active sites responsible for biological activity (1, 3). In this regard, increasing attention is being paid to the development and implementation of novel approaches capable of integrating high-precision experimental measurements with computational modeling algorithms,

providing a deeper understanding of the systemic regulation of cytokine responses (3, 6).One of the promising methods for evaluating complex biomolecular interactions is the CurPocket approach, an analytical tool based on the identification of conformational pockets and structural features of active sites in protein molecules combined with computational modeling techniques (3, 6, 8). Initially developed for the analysis of active regions of enzymes and receptors, CurPocket has demonstrated high sensitivity in detecting functional domains responsible for biochemical activity (6, 12). Application of this method to the cytokine landscape enables not only the identification of structural and functional features but also the assessment of the potential of cytokine molecules in mediating intercellular signaling processes. CurPocket-based strategies provide a multidimensional approach to the study of biomolecular interactions by integrating structural analysis, computational optimization, and experimental validation. This is particularly important for the investigation of flexible and dynamic proteins such as cytokines, whose biological activity is closely related to conformational variability (8, 12). In recent years, interest in integrating structural data with biological function has increased due to advances in X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and cryo-electron microscopy, which make it possible to obtain high-resolution three-dimensional structures of cytokines and their complexes (15). These data, in combination with CurPocket-based analysis, open new perspectives for studying active sites within cytokine networks and for predicting their responses to external stimuli.

One of the key challenges in modern immunology is the interpretation of the functional significance of changes in cytokine levels under physiological and pathological conditions (1, 4). Given that cytokine responses are characterized by nonlinear dynamics and considerable interindividual variability, conventional quantitative approaches are often insufficient for a comprehensive understanding of regulatory mechanisms (2). The CurPocket method, owing to its ability to identify active functional regions at the molecular level, offers a promising alternative for assessing correlations between the structural properties of cytokines and their biological activity.

Experimental application of CurPocket to the study of cytokine systems requires adaptation of the methodology to the specific features of immune mediators. An important aspect is the consideration of protein conformational variability caused by interactions with receptors, cofactors, and other components of the cellular environment (6, 12). Recent studies emphasize the role of structural rearrangements in regulating cytokine activity and their capacity to form functional signaling networks (15). This makes it necessary to employ analytical tools capable of accounting for dynamic structural changes, which constitutes the methodological basis and analytical strength of the CurPocket approach. Thus, integration of the CurPocket method into experimental studies of cytokine systems represents a significant step toward a systemic understanding of the molecular mechanisms underlying immune regulation. The combination of structural-functional analysis with experimental biology enables identification of active sites critical for cytokine mediator functions and allows prediction of their behavior under changing physiological conditions (3, 6). The present study is aimed at evaluating the effectiveness of the CurPocket method in identifying and characterizing active sites within cytokine networks, which may contribute to the development of novel approaches for the diagnosis and treatment of immune-related disorders.

The aim of the present study was to experimentally assess the state of the cytokine system and to characterize the active sites of cytokines using the CurPocket method in order to identify the structural and functional features of cytokine molecules and to evaluate their role in the regulation of intercellular signaling interactions.

Materials and Methods

The experiments were performed on 126 adult male rats weighing 180–220 g. The anti-inflammatory activity of the tested compounds was evaluated based on the difference in paw volume between control and experimental groups.MP-7 is a novel 1,2,3-triazole compound based on carbamate derivatives containing an acetylene group. The compound was synthesized and purified according to established procedures and used in the experimental study. Diclofenac sodium, a widely used nonsteroidal anti-inflammatory drug applied in the treatment of rheumatoid joint disorders and other inflammatory conditions, was used as a reference drug. The investigated compounds were administered orally at doses of 25, 50, and 75 mg/kg once daily for 7 consecutive days. Given the broad application of anti-inflammatory agents in the treatment of rheumatoid joint diseases, the effect of MP-7 on the course of

experimental arthritis in animals was evaluated. The study was conducted using a rat model of adjuvant arthritis, which is considered one of the most adequate experimental models of rheumatoid arthritis in humans. Under ether anesthesia, 0.1 mL of complete Freund's adjuvant (CFA) was injected intradermally at the base of the rat tail. The day of adjuvant administration was designated as day 0 of the experiment, with subsequent days counted sequentially. From the day of induction, the animals were observed daily for a period of 30 days. Paw volume was measured using a water plethysmometer at baseline and, starting from day 2 of the experiment, at 4-day intervals. In addition, during the observation period, the number of affected limbs and the presence of lesions in the intervertebral joints were recorded. The severity of joint damage was assessed using a comprehensive scoring system. An increase in paw volume relative to baseline by 1–20% was scored as 1 point; 21–40% as 2 points; 41–60% as 3 points; 61–80% as 4 points; 81–100% as 5 points; 101–120% as 6 points; 121–140% as 7 points; 141–160% as 8 points; 161–180% as 9 points; and 181–200% as 10 points. In addition to these scores, one extra point was added for each affected limb, and one additional point was assigned in the presence of lesions in the intervertebral joints of the rat tail. The investigated compounds were administered daily at doses of 100–200 mg/kg once per day. In the evaluation of prophylactic effects, drug administration was carried out from the day of complete Freund's adjuvant (CFA) inoculation through day 14 of the experiment. In the assessment of therapeutic effects, the compounds were administered from day 10 to day 22 after induction of arthritis.

The effects of MP-7 and diclofenac were evaluated on day 14 (prophylactic regimen) and on day 22 (therapeutic regimen).Parameters of the cytokine system, including pro- and anti-inflammatory cytokines (IL-1β, IL-6, TNF-α, and IL-8), were determined in blood serum using the enzyme-linked immunosorbent assay (ELISA). Commercial reagent kits produced by Cytokine LLC (Saint Petersburg, Russia) were used in strict accordance with the protocols provided by the manufacturer. The obtained data were subjected to statistical analysis using the Statistica 6.0 software package for Windows. Mean values and standard errors of the mean were calculated. Intergroup differences were evaluated using Student's t-test and the Mann–Whitney U test. Correlation analysis was performed using Pearson's correlation coefficient, and differences were considered statistically significant at the accepted confidence level.

Results and Discussion

During modeling of adjuvant arthritis, an increase in the concentrations of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 was recorded in the control group. Administration of complete Freund's adjuvant resulted in a statistically significant elevation of serum TNF-α and IL-1β levels starting from day 10 of the experiment, and by the end of the observation period their concentrations increased by more than threefold and fourfold, respectively, compared with baseline values. A significant adjuvant-induced increase in IL-6 concentration, reaching approximately a twofold elevation relative to the basal level, was observed on day 25 of the experiment.

Tumor necrosis factor alpha (TNF-α) is a cytokine that plays a key role in inflammatory processes and in the regulation of immune responses. This molecule regulates various aspects of immune activity, including inflammation, apoptosis (programmed cell death), and host defense against infections. TNF-α is a potent pro-inflammatory cytokine that stimulates inflammatory reactions and activates multiple immune cell types, such as macrophages, neutrophils, and endothelial cells, thereby enhancing inflammatory responses required for protection against infections or tissue damage. While TNF-α is essential for effective immune defense, its excessive or prolonged activity may contribute to the development of inflammatory diseases and tissue injury. In view of these considerations, the effects of the studied compounds on TNF-α activity were evaluated in experimental animals. Repeated administration of MP-7 resulted in a 69% reduction in TNF-α levels, reaching 28.4 ± 0.94 pg/mL compared with 54.5 ± 2.54 pg/mL in the control group. In the serum of animals receiving combined therapy with both drugs, a statistically significant decrease in TNF-α levels of 78% was observed relative to the control group (Fig. 1).

Interleukin-1 (IL-1) is one of the key cytokines involved in inflammation and immune responses. It plays an important role in the activation of inflammatory processes, particularly in response to infection, trauma, or other forms of tissue injury. IL-1 belongs to the group of pro-inflammatory molecules that regulate interactions between immune cells and tissue cells (1–3). It activates various immune cell types, including macrophages, neutrophils, and endothelial cells, thereby enhancing inflammatory responses and stimulating the production of other cytokines such as IL-6, IL-8, and TNF-α, which

contributes to the amplification and maintenance of inflammation (2–4).As shown in Figures 1 and 2, repeated administration of diclofenac sodium did not result in a statistically significant effect on the levels of the studied cytokines compared with the control group. In contrast, administration of MP-7 led to a reduction in TNF-α levels by 69%, IL-1β levels by 64%, and IL-6 levels by 64%. In animals receiving combined therapy with MP-7 and diclofenac sodium, a more pronounced decrease was observed, with TNF-α reduced by 78%, IL-1β by 58%, and IL-6 by 70% relative to control values.

Interleukin-8 (IL-8) is a chemokine responsible for neutrophil chemotaxis to the site of inflammation. IL-8 is synthesized by macrophages, lymphocytes, fibroblasts, epithelial, and epidermal cells. The production of IL-8 can be induced by interleukins IL-1 and IL-3, tumor necrosis factor alpha (TNF-α), and other inflammatory mediators. IL-8 exhibits pronounced pro-inflammatory properties by inducing the expression of intercellular adhesion molecules and enhancing the adhesion of neutrophils to endothelial cells and subendothelial matrix proteins. In rheumatoid arthritis, a significant increase in IL-8 concentration in synovial fluid has been reported due to its hyperproduction by neutrophils (16, 17).

IL-8 does not act in isolation but interacts with other cytokines and inflammatory mediators. In particular, IL-8 is known to interact with interleukins IL-1 and IL-6, as well as with TNF-α, thereby enhancing inflammatory responses.

In addition, IL-8 can stimulate the hyperproduction of other chemokines and inflammatory mediators, leading to persistent inflammation and progressive joint tissue damage.

In one experimental study comparing IL-8 levels with those of other cytokines in animals with adjuvant arthritis, IL-8 was shown to exhibit levels comparable to IL-1β; however, unlike IL-1β, IL-8 plays a more active role in neutrophil chemotaxis, which makes it particularly important during the early stages of inflammation.

Following adjuvant injection, animals develop chronic joint inflammation accompanied by elevated levels of various cytokines, including IL-8. Studies of serum IL-8 levels in adjuvant arthritis have demonstrated an approximately 8.5-fold increase compared with intact animals, confirming the role of this cytokine in inflammatory and pathological responses. Mean values indicate that in healthy animals, serum IL-8 levels are 8.54 ± 0.34 pg/mL, whereas in adjuvant arthritis, IL-8 concentrations reach 72.6 ± 4.64 pg/mL.

Administration of diclofenac sodium resulted in serum IL-8 levels of 54.3 ± 1.45 pg/mL in experimental animals, whereas treatment with MP-7 reduced IL-8 levels to 48.6 ± 1.47 pg/mL. Combined administration of both compounds led to a more pronounced decrease in IL-8 concentration, with a reduction of 52.6% relative to control values and a final level of 35.2 ± 0.87 pg/mL.

Thus, IL-8 represents an important mediator of the inflammatory process in adjuvant arthritis. Elevated serum IL-8 levels correlate with disease severity and inflammatory activity, suggesting that this cytokine may serve as a reliable biomarker of inflammation and may be used in clinical practice to assess disease progression and treatment efficacy.

According to the results of structural analysis, pocket C1 demonstrated the highest pharmacological relevance and was characterized by the most favorable structural organization for binding drug molecules or substrates. Pocket C2, despite its smaller size, may also represent a site of considerable interest as a potential binding region for secondary ligands or cofactors. In contrast, pockets C3, C4, and C5 appear to be more suitable for the localization of low-molecular-weight compounds, ions, or water molecules and are likely involved in maintaining protein structural stability or in auxiliary interactions with other biomolecules.

Based on the obtained data, further molecular docking, molecular dynamics, and biochemical studies are warranted, with particular focus on pockets C1 and C2 as the most promising targets for functional and pharmacological modulation.

In conclucions, Biochemical modeling studies employing molecular docking methods made it possible to characterize in detail the mechanisms of interaction between propargyl carbamate–triazole compounds and the receptors of pro-inflammatory cytokines IL-1, IL-6, and IL-8.

It was established that the active sites of these interleukins possess favorable spatial, geometric, and electronic characteristics that enable high-affinity binding of 1,2,3-triazole carbamate derivatives.

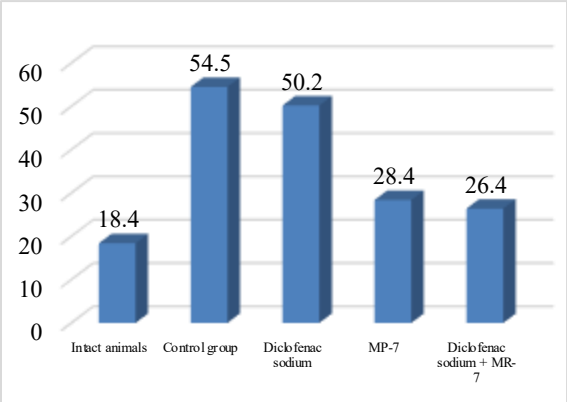

Fig.1 Effect of repeated administration of diclofenac sodium, MP-7, and their combination on serum TNF-α levels in rats with experimentally induced adjuvant arthritis on day 25 of the experiment.

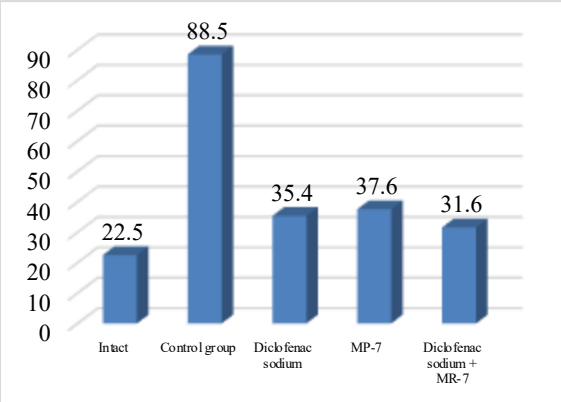

Fig.2 Effect of repeated administration of diclofenac sodium, MP-7, and their combination on serum IL-1β levels (pg/mL) in rats with experimentally induced adjuvant arthritis on day 25 of the experiment.

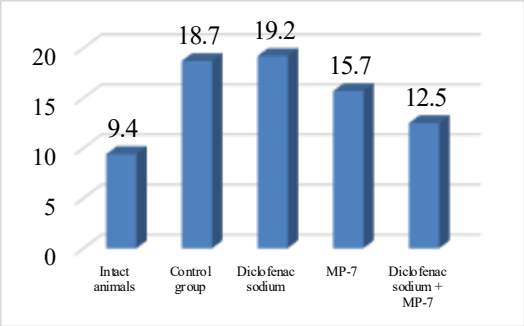

Fig.3 Effect of repeated administration of diclofenac sodium, MP-7, and their combination on serum IL-6 levels in rats with experimentally induced adjuvant arthritis on day 25 of the experiment.

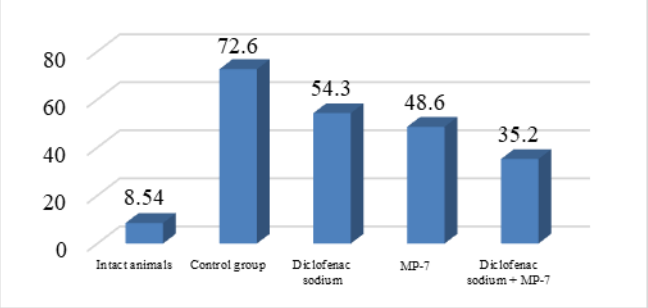

Fig.4 Effect of repeated administration of diclofenac sodium, MP-7, and their combination on serum IL-8 levels in rats with experimentally induced adjuvant arthritis.

The results of molecular docking demonstrated that the studied compounds are capable of effectively interacting with both orthosteric and potential allosteric sites of IL-1, IL-6, and IL-8 receptors, indicating the possibility of modulating key stages of cytokine-mediated inflammatory signaling. Within the framework of structure–activity relationship (SAR) analysis, a clear dependence of anti-inflammatory activity on specific chemical features of the molecules was revealed, including the nature of the carbamate fragment, the presence of a propargyl group, the degree of halogen substitution, and the distribution of electron density. Thus, the obtained data provide scientific justification for the significant influence of the chemical structure of propargyl carbamate esters and the synthesized 1,2,3-triazole derivatives based on them on their ability to interact with IL-1, IL-6, and IL-8 receptors and to exhibit pronounced anti-inflammatory activity.

In conclusion, the investigated compounds demonstrate high scientific and practical relevance as potential modulators of IL-1-, IL-6-, and IL-8-dependent inflammatory processes and may be considered a promising basis for the development of a new generation of anti-inflammatory drugs.

Author Contributions

Yakubkhodjaeva Malika Razakovna: Investigation, formal analysis, writing—original draft.

Data Availability

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

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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