Expression, Purification and Structural Analysis of Human IL-18 Binding Protein: A Potent Therapeutic Molecule for Allergy

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ABSTRACT

Background: While interleukin-18 (IL-18) plays an important role in the innate and adaptive immune responses, it can also cause severe allergic inflammatory reactions. Thus it is a molecule currently being targeted for therapy. The natural intrinsic inhibitor of IL-18 receptor activation, IL-18 binding protein (IL-18BP), shows a great potential for the treatment of allergy.

Methods: Expression and purification of recombinant human IL-18BP (rhIL-18BP) were performed using the baculovirus system to develop a therapeutic molecule for the treatment of IL-18-related diseases and to investigate the structural basis of its inhibitory mechanism.

Results: Purified rhIL-18BP potently inhibited the production of interferon-gamma by peripheral blood mononuclear cells in the presence of lipopolysaccharide and by human myelomonocytic KG-1 cells in the presence of IL-18 (IC₅₀ = 0.4 nM). Surface plasmon resonance showed a high affinity (Kd = 0.46 nM) for rhIL-18BP in binding hIL-18. Structural analysis indicated that the stoichiometry between IL-18 and IL-18BP is 1 : 1 in solution and the model structure of the complex suggests that the key residues on IL-18 (L5, K53, S55) and the estimated key residues on IL-18BP (F93, Y97, F104) could have interactions. The structural mechanism of IL-18BP inhibition might be a competition for Site 2 on rIL-18 so that IL-18BP can prevent IL-18 receptor alpha from binding to Site 2 and inhibit IL-18 receptor activation.

Conclusions: IL-18BP has unique features with respect to its structure, binding mode and inhibitory mechanism. It is a molecule that has a great potential for the therapy of allergy.

KEY WORDS

baculovirus, binding mode, IL-18 binding protein, purification, structure

INTRODUCTION

Interleukin-18 (IL-18) is a cytokine originally found to induce the production of interferon-gamma (IFN-γ) in T lymphocytes, and plays an important role in innate and adaptive immune systems.¹,² To initiate the IL-18 pathway, the IL-18 receptor needs to be activated, which requires IL-18 receptor alpha (IL-18Rα, formerly known as IL-1Rrp) and IL-18 receptor beta (IL-18Rβ, formerly known as IL-1RAcPL) to heterodimerize.² The structure of human IL-18 has been determined and it has shown that hIL-18 contains the β-trefoil fold that is similar to those found in interleukin-1 (IL-1) family members, making a new structural family in the interleukins.³,⁴ Also, using mutant recombinant human IL-18 (rhIL-18) proteins in receptor-binding and cellular response assays, three important binding sites have been identified.³ Two of these sites are important for binding to IL-18Rα, and the third is in-
volved in cellular responses but not in IL-18Rα binding.3

By comparing the structure and the receptor binding sites of IL-18 with those of the IL-1 family members, a two-step ternary complex formation that involves IL-18, IL-18Rα, and IL-18Rβ has been revealed.4 The formation of the ternary complex can then trigger a signal cascade that activates IL-1 receptor activating kinase (IRAK), tumor necrosis factor (TNF)-receptor associated factor 6 (TRAF6) and nuclear factor-kappa B (NF-κB).5,6

In medicine, the aberrant expression of IL-18 has been suggested to be responsible for several inflammatory conditions, such as allergies, autoimmune diseases, and neurological disorders.7,8 Serum concentrations of IL-18 and IgE have been correlated with disease severity in atopic dermatitis (AD) patients.7,9 In these patients, the serum concentration of IL-18 is significantly higher than that found in healthy individuals.9 Furthermore, in response to lipopolysaccharide (LPS), leukocytes prepared from the peripheral blood of AD patients produced more IL-18 than the cells of healthy individuals.7,8 In patients with bronchial asthma, the level of serum IL-18 is also elevated, and seems to correlate with disease severity.7,10-14 As well, polymorphic genes for IL-18 and IL-18R have been found in association with allergy.15-17 Collectively, these studies strongly suggest that IL-18 is responsible for causing and/or maintaining inflammatory conditions. Thus, IL-18 may be an ideal therapeutic target for the treatment of allergy.7

In clinical settings, humanized antibodies are widely used as therapeutic agents for the treatment of many diseases, including allergy.18 Although anti-IL-18 antibody has been proven to be effective for preventing liver damage in mice, to date, there are no antibody has been proven to be effective for pre-

METHODOLOGY

CONSTRUCTION OF EXPRESSION VECTOR FOR hIL-18BP

The IL-18BP isoform-a was selected from six iso-
forms of IL-18BP, because isoform-a most strongly inhibits IL-18 activity on human peripheral blood mono-nuclear cells (PBMCs) or human cell lines and has been widely used as the representative of the iso-
forms.31,32 Total mRNA was extracted from the blood of a healthy volunteer, and single-stranded cDNAs were synthesized at 72°C for 60 minutes using reverse transcriptase and oligo-dT primers. Only the coding region of the mature hIL-18BP isoform a (NM173042, residues 27-193 aa) was amplified by PCR. The amplified fragment was cloned into T-vector (Invitrogen, USA), because the amino acid residues 1 to 26 of the hIL-18BP are recognized as a signal peptide in mammalian cells (Fig. 1A). One of the primers used in the PCR contained an EcoRI site and a signal peptide sequence for Sf9 insect cells, and by design, these were placed immediately upstream of the first codon of the mature hIL-18BP. The second primer contained the His-6 tag sequence, a stop codon, and a NotI site. The purified PCR product was subcloned into the pFastBac1 vector (Invitrogen, USA). The DNA sequence of the clone was confirmed by bi-directional sequencing. The construct was called pFastBac1-hIL-18BP-His6 (Fig. 1B).

PRODUCTION OF RECOMBINANT BACULOVIRUS

Generation of recombinant baculovirus expressing hIL-18BP in Sf9 cells was carried out with a Bac-to-Bac baculovirus expression kit (Invitrogen, USA). In accord with the manufacturer's protocol, pFastBac1-hIL-18BP-His6 was introduced into E.coli DH10Bac (Invitrogen, USA) for the transposition of IL-18BP into baculovirus genomic DNA (bacmid). Colonies containing the recombinant bacmid were isolated using a miniprep plasmid isolation kit (Promega, USA). The recombinant bacmid DNA was then used to transfect Sf9 cells, which were plated at a density of 1 × 10⁶ cells per 35-mm well and then transfected with the bacmid DNA using Cellfectin reagent (Invitrogen, USA). The recombinant virus was harvested 72 hours post-transfection. Plaque assays were performed with the supernatants to determine the titer of recovered virion particles. Plaque assay and propagation of viruses were carried out according to the manual provided with the kit.

INSECT CELL CULTURE

Sf9 insect cells were cultured and routinely sub-cultured at 27°C. They were maintained either as a monolayer or as a suspension culture in SFM (Invitrogen, USA) without antibiotics. Insect
cells in a monolayer culture were plated at a density of 1.0 × 10^6 cells/well in six-well plates. These cells were used to transfect recombinant bacmid DNA or for the determination of the optimal multiplicity of infection (MOI). The cells in the suspension culture were grown in a 25 ml volume of SF-900 II SFM using TATEC (Japan) and were subjected to rotary shaking at a speed of 95 rpm (amplitude 35 mm). Stock flasks were maintained in a Bioshaker BR40LF.

**Fig. 1** Expression and purification of rhlL-18BP. A, Alignment for IL-18BPs. The signal peptide residues of the hIL-18BP in mammalian cells are boxed. Estimated key residues on hIL-18BP for hIL-18 binding are highlighted in yellow. B, A map of the construct used for expression of hIL-18BP in the baculovirus system. C, Purification of rhlL-18BP. Lane 1: medium, lane 2: DEAE, lane 3: NINTA, lane 4: Gel filtration. D, Deglycosylation by trifluoromethane sulfonic acid. Lane 1: before deglycosylation, lane 2: after deglycosylation.
Sf9 cells that were grown in suspension were seeded at 0.5 × 10^6 cells/ml and culture passage was performed every 3 days during the log phase (4–6 × 10^6 cells/ml).

**PURIFICATION OF hIL-18BP FROM BACULOVIRUS-INFECTED SF9 CELL CULTURE MEDIUM**

For the purification of rhIL-18BP, Sf9 cells were subcultured in 1 L Fernbach flasks. Each flask received 400 ml of the cell culture at a concentration of 0.5 × 10^6 cells/ml. Shaking speed was set to 71 rpm (Amplitude 50 mm). When the subcultured cells reached a concentration of 2.0 × 10^6 cells/ml, Sf9 cells were infected with the recombinant baculovirus at an MOI of 0.1 plaque-forming units (pfu) per ml and were incubated for up to 72 hours.

Secreted rhIL-18BP was processed by centrifugation and filtration. Then, the medium was applied to a DEAE-Sepharose (GE health care, Sweden) open column (Bio-Rad, USA) equilibrated with buffer A (50 mM sodium-phosphate, pH 6.0 containing 50 mM NaCl). The flow through was concentrated 10-fold using a tangential flow filtration system; Masterflex L/S (Barnant Company, USA) and viva flow 50 (Vivasciee AG, Germany). This was then diazylated against buffer B (50 mM Sodium Phosphate, 0.5 M NaCl, 10 mM Imidazol, pH 7.4) and insoluble material removed by centrifugation at 2 × 10^4 g; himac CR 20B2 (Hitachi, Japan). Diaziyozed sample was loaded onto a Ni-agarose (GE Healthcare, Sweden) column equili-brated with buffer B. The column was then washed with buffer B and bound proteins were eluted by an elution buffer B with varying imidazol concentrations (50, 100, 200, 300 and 500 mM). The eluted fraction of rhIL-18BP was concentrated and further purified using a superdex75 16/60 (GE Healthcare, Sweden) size exclusion column equilibrated with 50 mM potassium phosphate buffer pH 7.0, containing 150 mM KCl and 0.1 mM EDTA. The purity of the preparation was assessed by SDS-PAGE.

**DEGLYCOSYLATION OF rhIL-18BP BY TRIFLUOROMETHANE SULFONIC ACID**

Deglycosylation was performed in a draft chamber (DF-19RST, DALTON, Japan). Freeze dried IL-18BP (30 μg) in glass vial and trifluoromethane sulfonic acid (TFMS) (Sigma Aldrich, Japan) was chilled on ice prior to mixing. Fifty micro liters of TFMS was added in a glass vial and gently mixed. The vial was then incubated for 50 hours on ice and neutralized with a solution of 500 μl of 1M-Tris. Neutralized rhIL-18BP was concentrated with a MicroconYM-10 (Millipore, USA) and diazylated against 20 mM sodium phosphate buffer of pH 7.0 containing 150 mM NaCl before SDS-PAGE.

**N-TERMINAL SEQUENCING BY EDMAN DEGRADATION**

Purified proteins were electro-botted onto a polyvinylidene difluoride membrane (Amersham Biosciences, UK) after SDS-PAGE for 1 hour at 200 mA in solution that contained 25 mM Tris, 192 mM glycine, and 10% methanol. The membrane was briefly stained with CBB R250 (Wako, Japan) and de-stained extensively in 45% and 90% methanol containing 7% acetic acid. The amino acid sequence analysis of the recombinant protein was carried out by an Edman degradation technique using a pulse liquid automatic sequencer (Model 491HT, Applied Biosystems, USA).

**FUNCTIONAL ANALYSIS OF rhIL-18BP**

Biological activity of rhIL-18BP was assayed by measuring its ability to inhibit the production of IFN-γ. This was performed as previously described with minor modifications. Briefly, PBMCs were isolated from three volunteers and suspended at 1 × 10^6 /ml in the culture medium. PBMCs were cultured in the presence or absence of 1 ng/ml of the LPS with or without rhIL-18BP (400 ng/ml) for 24 hours at 37°C in a humidified atmosphere containing 5% CO2. The cell culture supernatants were collected in test tubes, and the samples were spun to get rid of cells and then stored at −80°C until assay. The concentration of IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA), as previously described.

**MEASUREMENT OF rhIL-18BP-MEDIATED INHIBITION OF IFN-γ PRODUCTION IN KG-1 CELLS**

In hIL-18 inhibition assay, the level of inhibition is determined by the amount of IFN-γ produced by the target cells. A detailed description has been reported. Briefly, human myelomonocytic KG-1 cells (ATCC CCL246) were grown in RPMI1640 (Invitrogen, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma Aldrich, USA), L-glutamine (2 mmol/L) (Wako, Japan), penicillin (100 U/ml) (Meiji, Japan), and streptomycin (100 μg/ml) (Meiji, Japan). The rhIL-18 and rhIL-18BP prepared in our laboratory were mixed in the RPMI1640 medium noted above. The concentration of rhIL-18 was 4 ng/ml and rhIL-18BP was from 0 to 250 ng/ml. The mixed samples were incubated at 37°C for 1 hour. Then, 100 μl of the mixture was added to the wells of a 96-well plate (Nunc, Denmark), which contained 100 μl of KG-1 cells (3 × 10^6 cells/ml) (The final concentration of rhIL-18 was 2 ng/ml and rhIL-18BP was from 0 to 125 ng/ml). The plate was further incubated at 37°C for 24 hours in 5% CO2. The culture supernatants were collected, and the amount of IFN-γ produced by KG-1 cells was determined by ELISA, IC50, the concentration of antagonists required to inhibit 50% of IFN-γ production by KG-1 cells stimulated with hIL-18, was then calculated.
MEASUREMENT OF rhIL-18BP’S BINDING ACTIVITY BY SURFACE PLASMON RESONANCE EXPERIMENT

In vitro affinity of the hIL-18 for rhIL-18BP was measured at 25°C by surface plasmon resonance (SPR) using a BIAcore3000 (BIAcore, Sweden). A specific binding surface was prepared by coupling the anti-penta-His antibody (QIAGEN K.K, Japan) to a CM5 sensor chip by the standard amine coupling method, as recommended by BIAcore. Then, 6-his tagged rhIL-18BP was injected over the sensor chip and immobilized. The coupling density was limited to 120 resonance units (RU). Samples of hIL-18 were diluted to varying concentrations ranging from 2 to 32 nM in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.0 mM EDTA, 0.005% (v/v) surfactant P-20). For dissociation constant analysis, diluted hIL-18 samples were injected over the sensor chip at a flow rate of 20 μl/min. The sensor surface was regenerated by two 60-second pulses of 0.2 M glycine-HCl, pH 2.2. The sensorgrams obtained from various concentrations of rhIL-18 were fitted with BIAEVALUATION software (BIAcore, Sweden) in a 1-to-1 binding model.

ANALYTICAL GEL FILTRATION OF rhIL-18BP

The molecular mass of purified rhIL-18BP and the complex of rhIL-18 and rhIL-18BP were determined by size exclusion chromatography. A superdex200 10/30 GL column (GE Healthcare, Sweden) attached to a FPLC system (GE Healthcare, Sweden) was utilized and the study conducted at 4°C. The column was equilibrated with 50 mM potassium phosphate buffer of pH 7.0, containing 150 mM KCl and 0.1 mM EDTA. The column was calibrated with the following gel filtration standards (Bio-Rad Laboratories, USA): thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B12 (1.35 kDa) at a flow rate of 0.25 ml/min. Samples with only hIL-18BP, only rhIL-18 or a mixture of rhIL-18BP and rhIL-18 at a 1:2 ratio were used for the analysis. Protein elution was monitored and detected by UV absorption at 280 nm. We plotted the logarithm of the molecular weight versus the elution volume, and calculated the correlation line. The elution volume of rhIL-18BP and the complex of rhIL-18/rhIL-18BP were plotted and the deduced molecular weight determined.
characterization of the purified rhIL-18BP

High expressions of rhIL-18BP were obtained using the baculovirus expression system. We were able to obtain a yield of 2.5 mg/L of rhIL-18BP after Ni-NTA column purification (Fig. 1C). A further purification by gel filtration showed excellent purity and yielded 1.8 mg/L (Fig. 1C). To check the purity of the protein, electrophoresis was performed. Although the band appeared smearable, when we deglycosylated it with TFMS, a single sharp band of 18 kDa in size was detected (Fig. 1D). Edman degradation analysis of rhIL-18BP revealed the expected five N-terminal amino acid residues: (LVRAT). The sequence verified the identity of the expressed protein and indicated that the signal peptide from the vector was correctly cleaved at the N-terminal residue of mature rhIL-18BP.

**FUNCTIONAL ACTIVITY OF THE rhIL-18BP**

The biological activity of rhIL-18BP was tested by measuring its ability to inhibit the production of IFN-γ by LPS-stimulated PBMCs from volunteers (Fig. 2A). The rhIL-18BP showed distinct inhibitory actions against IFN-γ production by PBMCs in response to LPS. rhIL-18BP only partly inhibited IFN-γ production by PBMCs from volunteer #1, while it almost completely inhibited those from volunteers #2 and #3 (Fig. 2A). This diversity might be partly explained by the different amounts of IL-18 released from PBMCs of individual volunteers and/or in part by the different productions of other IFN-γ-related cytokines, such as IL-12 and IL-15. As well, rhIL-18BP inhibited the production of IFN-γ by human KG-1 cells in the presence of IL-18 (IC50 = 0.4 nM, Fig. 2B).

His-tagged rhIL-18BP was immobilized on a BIAcore sensor chip coated with anti-His-tag antibodies and its binding activity monitored in real time with a BIAcore 3000 sensor. A sensorgram of rhIL-18 showed a rapid increase in signal during the association phase and a slow decline in the dissociation phase (Fig. 2C), demonstrating a fast on-rate and a slow off-rate. The estimated Kd of 0.46 nM was similar to results previously reported, indicating that the produced rhIL-18BP had proper structural folding.

**STRUCTURAL ANALYSIS OF rhIL-18BP**

Gel filtration analysis revealed that rhIL-18BP is a monomer in solution (Fig. 3A) and that a complex of rhIL-18 and rhIL-18BP could be made in the same molar ratio (Fig. 3B). Together with the molecular weight of the complex, it is suggested that the complex could be made by one rhIL-18 and one hIL-18BP (Fig. 3B).

Based on the results of analytical gel filtration, a model structure of the rhIL-18/rhIL-18BP complex was constructed using the NMR structure of hIL-18 and the crystal structure of the domain 3 of IL-1Rα as templates (Fig. 4). The analysis of the amino acid sequence alignment for IL-18BP revealed that the residues that are involved in the binding of hIL-18 are

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**HOMOLOGY MODELING OF THE STRUCTURES**

The sequence alignment of IL-18BP from different species was performed using ClustalW (http://www2.ebi.ac.uk/clustalw/) with a BLOSUM matrix. Homology modeling was performed using MOE software (CCG, Inc., Canada) with a combination of segment-matching and modeling of indels. The templates used for the modeling were as follows: the NMR structure of hIL-18 (PDB: 1J0S) and the crystal structure of the domain 3 of hIL-1Rα (PDB: 1ITB) for the hIL-18: hIL-18BP complex; the NMR structure of hIL-18 (PDB: 1J0S) and the crystal structure of the hIL-10/ anti-IL-10 Fab complex (PDB: 1LK3) for the hIL-18/human anti-hIL-18 Fab interaction. The modeled structures were refined by further energy minimization.

**RESULTS**

**CHARACTERIZATION OF THE PURIFIED rhIL-18BP**

High expressions of rhIL-18BP were obtained using ClustalW (http://www2.ebi.ac.uk/clustalw/) with a BLOSUM matrix. Homology modeling was performed using MOE software (CCG, Inc., Canada) with a combination of segment-matching and modeling of indels. The templates used for the modeling were as follows: the NMR structure of hIL-18 (PDB: 1J0S) and the crystal structure of the domain 3 of hIL-1Rα (PDB: 1ITB) for the hIL-18: hIL-18BP complex; the NMR structure of hIL-18 (PDB: 1J0S) and the crystal structure of the domain 3 of IL-1Rα as templates.

**STORAGE ANALYSIS OF rhIL-18**

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**Fig. 3 Stoichiometric analysis of the rhIL-18/rhIL-18BP complex. A, Gel filtration of rhIL-18BP. B, Gel filtration of the rhIL-18BP/rhIL-18 complex. A solid line indicates the sample that contained a mixture of rhIL-18BP and rhIL-18 at 1:2 ratio, a dashed line, indicates the sample with only rhIL-18.**
**Fig. 4** A structural model of the hIL-18/hIL-18BP complex. **A**, Surface of hIL-18. Functional binding residues on hIL-18 for hIL-18BP are colored in orange. **B**, A structural model of the rhIL-18BP monomer. Estimated functional key residues of hIL-18BP for hIL-18 are shown as atoms in red. **C**, A model of the hIL-18/hIL-18BP complex (hIL-18 as a ribbon model, hIL-18BP as a surface representation, colored as in A or B).

**Fig. 5** Human IL-18 interacts differently with different proteins through its three binding sites. **A**, Receptor binding sites on hIL-18. Sites 1, 2 and 3 are indicated in red, orange, and blue, respectively. **B, C, D**, Models of complexes between hIL-18 and hIL-18Rα (in grey), hIL-18BP (in yellow) and human Anti-hIL-18 Fab (in yellow).
highly conserved between species. Our model showed that the three key residues (F93, Y97, and F104) that are involved in binding IL-18 make hydrophobic patches that align with the binding interfaces of hIL-18 (Fig. 4C). The three key residues of hIL-18 for binding (L5, K53, and S55) have been shown to be involved in the interaction with hIL-18BP.

**DISCUSSION**

Our previous structural analysis of the IL-18/IL-18 receptor interaction showed that there are 3 distinct and important binding sites on the surface of IL-18 (Fig. 5A). Two of the sites (Sites 1 and 2) are responsible for binding to IL-18Rα while the third site is involved in binding to IL-18Rβ (Fig. 5A, B). Site 2 residue is also important for IL-18BP binding (Fig. 5A, C). A recent study showed that a single-chain Fv (scFv) of an anti-human IL-18 antibody binds to Site 3 of IL-18 (Fig. 5A, D). scFv (h18–108) showed a moderate binding affinity (Kd = 50 nM), whereas the Fab fragment of h18–108 showed a higher affinity (Kd = 3.7nM), suggesting that the stability of the binding surface structure could improve the affinity.

Further, hIL-18BP, which has a smaller molecular mass than scFv, showed a greater than 100-fold higher affinity (Kd = 0.46 nM) than scFv. This suggested that the evolutionally improved structure of IL-18BP provided an optimal binding surface complementarity. Based on this, perhaps one could optimize the binding affinity of h18–108 by modifying the surface structure through *in vitro* mutagenesis or through a computational design, as it has been demonstrated for the anti-epidermal growth factor receptor drug, Cetuximab.

IL-18 activates IL-18 receptor through a two-step binding mechanism as shown in Figure 6. According to this model, the inhibitory mechanisms of IL-18BP and the Fab fragment of h18–108 antibody are different (Fig. 6). IL-18BP inhibits the receptor activation by competing for Site 2 on IL-18, which is also a binding site for IL-18Rα. On the other hand, the Fab fragment of h18–108 antibody binds to Site 3 on IL-18 to block IL-18 from binding to IL-18Rβ (Fig. 6). The IC50 of IL-18BP has been shown to fall in the range of 0.04–0.46 nM, while it is 100 nM for the Fab frag-
ment. This huge difference in IC50 may reflect the type of inhibitory mechanism that is employed (Fig. 6). Interestingly, an intact form of h18-108 antibody, an IgG molecule, has a much higher affinity with a Kd of 0.64 nM and an IC50 of 5 nM. This improvement is most likely the result of its overall stability and the bivalency of the intact antibody molecule. Regardless of the affinity, these different molecules will be useful for determining the precise mechanism by which IL-18 receptor is activated or inhibited. Information gained from such studies will undoubtedly help us design potent therapeutic reagents.

A number of drugs targeting and neutralizing the deleterious effects of cytokines have been developed. Etanercept is a soluble fusion protein that is composed of two tumor necrosis factor alpha (TNF-α) receptors (p75) and a Fc fragment of a human IgG1 molecule; it has been shown to be effective and safe in patients with rheumatoid arthritis. It has been shown to be effective and safe in patients with rheumatoid arthritis. Regardless of the affinity, these different molecules will be useful for determining the precise mechanism by which IL-18 receptor is activated or inhibited. Information gained from such studies will undoubtedly help us design potent therapeutic reagents.

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