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An MD2 Hot-Spot Mimicking Peptide that Suppresses TLR4-Mediated Inflammatory Response In Vitro and In Vivo

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Abstract

A truncated peptide was shown to retain the structure of the TLR4-binding hot-spot region of MD2, disrupting with the TLR4/MD2 interactions. The peptide not only demonstrated strong binding affinity in the fluorescence polarization assay, but also showed high specificity in macrophage cells. Furthermore, MD2-I was able to suppress neuropathic pain in animal models.

Keywords

toll-like receptor 4; myeloid differentiation factor 2; peptide inhibitor; peptidomimetic design; analgesic

Pain is perceived first by sensory nerves in the periphery, that then propagate this message to the pain processing centers in the central nervous system (CNS). Immune cells that reside in the CNS, called glial cells, have been implicated in proinflammatory processes.^[1] These inflammatory signals are involved in the development of diseases such as neuropathic pain,^[2] and adverse effects of current pain management therapies (e.g. opioids), including opposition to both acute and chronic pain relief, increasing tolerance to opioid effects, and opioid dependence.^[3] The identification of novel inflammatory triggers that lead to CNS diseases provides an exciting new strategy to reduce inflammatory responses within the CNS thereby suppress pathological pain and enhance opioid efficacy for pain control.

Specifically, the innate immune receptor toll-like receptor 4 (TLR4) has been shown to mediate neuropathic pain,^[2] via a chain reaction of intracellular signals resulting in an increase in proinflammatory cytokines and other inflammatory mediators that importantly contribute to pain enhancement. Several previous reports have focused on inhibiting TLR4-mediated glial activation to reverse neuropathic pain.^[3] However, a major challenge for targeting TLR4 is achieving high selectivity, as TLR4 is one of more than 10 homologous toll-like receptors (TLRs) expressed by glial cells.^[4] Among all TLRs, TLR4 activation requires an accessory protein, myeloid differentiation factor 2 (MD2).^[5] Our goal is to

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validate that the TLR4/MD2 complex is a target for therapeutic regulation of the TLR4-mediated inflammation in the CNS.

A 17-residue peptide (MD2-I) was previously reported to reproduce the TLR4-binding region of the MD2 protein (Figure 1A).^[6] The truncated sequence contains six charged residues critical for the TLR4 recognition (Table 1, highlighted using underline).^[7] In this design, a disulfide bridge between Cys95 and Cys105 along with the secondary structures of the truncated peptide help to retain the geometry of the critical residues in 3D space, mimicking the TLR4-binding surface of the full-length MD2 protein. A peptide, MD2-III, with the critical residues mutated, was used as a negative control. In this report, we report further investigations of the MD2-I peptide as a disruptor of the TLR4/MD2 complex both *in vitro* and *in vivo*. First, we characterized the binding of MD2-I with purified TLR4 protein using biophysical methods. A dissociation constant (K_D) of $0.40 \pm 0.08 \mu\text{M}$ was determined for MD2-I binding to TLR4 using a one-site saturation model. Next, the selectivity of MD2-I was tested in macrophage cells that express a variety of TLRs, showing MD2-I selectively inhibits TLR4 signaling. A general kinase profiling was also carried out against twelve representative kinases to demonstrate that MD2-I is specific for the TLR4 signalling. The peptide design was further validated using an all-atom molecular dynamic (MD) simulation in an explicit solvation model. Finally, *in vivo* studies showed that MD2-I, but not the control peptide MD2-III, could suppress neuropathic pain *in vivo*, presumably by inhibiting the TLR4-induced inflammation.

Previously, we reported that MD2-I abolishes TLR4 signalling by antagonizing its native ligand, lipopolysaccharide (LPS), binding to the TLR4/MD2 complex.^[6] To further confirm the binding mode of MD2-I, we developed an *in vitro* biophysical binding assay to determine the direct binding of MD2-I with TLR4. We successfully expressed and purified the extracellular construct (a.a. 1–632) of human TLR4 in HEK293 cells.^[8] The activity of the TLR4 protein is validated by testing its binding to the full-length MD2 protein expressed in insect cells (Supplementary Figure 3A). Further, the K_D of LPS binding to the MD2 protein used in these experiments was determined to be $0.06 \mu\text{M}$, which is in agreement with the previous literature report (Supplementary Figure 3B).^[9] The purified TLR4 protein was then used to titrate the nitrobenzoxadiazole (NBD) labelled MD2-I (NBD-MD2-I) or MD2-III (NBD-MD2-III) peptide with an ϵ -aminohexanoic acid linker. Fluorescence polarization, which corresponds to the tumbling rate of the MD2 peptides in solution, was monitored to determine their binding to TLR4 (Figure 1B). Upon addition of TLR4, the polarization value of MD2-I showed a robust increase, leveling off at a maximum of approximately 0.25. This elevation of polarization values is consistent with ligand/receptor association with comparable sizes.^[10] The binding data were then fitted into a one-site saturation model using standard regression analysis. A fitting value of $R > 0.99$ was obtained, suggesting that MD2-I binds to TLR4 in a specific one-site binding manner. Further, the K_D value of NBD-MD2-I binding to TLR4 was determined to be $0.40 \pm 0.08 \mu\text{M}$, indicating that MD2-I, retains most of the binding affinity of the full-length MD2 binding to TLR4 ($K_D = 0.012 \mu\text{M}$).^[11] By contrast, the control peptide, NBD-MD2-III showed negligible binding activity toward TLR4 protein, implying that the MD2-I employs the same critical residues found in the full-length MD2 to recognize TLR4.

A major challenge of developing inhibitors to target a particular TLR is to achieve high selectivity. There are more than 10 TLRs reported in human and at least 13 in rodents.^[12,13] All these TLRs share a homologous structural scaffold, posing challenges for the development of selective inhibitors. Our design strategy was based on the disruption of the TLR4/MD2 complex formation as TLR4 is the only TLR that has been confirmed to require MD2 for signalling.^[5] Nitric oxide (NO) production in macrophages was monitored as the probe to TLR activation (Figure 2).^[14] Macrophage cells express a variety of TLR receptors,

providing a valid system to test the selectivity of inhibitors targeting a particular TLR. The augmentation of NO production shows that a particular TLR pathway was activated with treatments of TLR-specific ligands. With 50 μ M MD2-I peptide present, RAW 264.7 macrophage cells showed significant reduced signals of LPS-induced TLR4 activation, with negligible inhibitory effects observed in TLR1/2, TLR2/TLR6, and TLR7 signalling. Further, the control peptide (MD2-III) shows no inhibition on the TLR4 signaling.

The specificity of MD2-I was further examined by screening against twelve representative kinases (AKT1, CAMK1, DDR2, GSK-3 α , MAPK1, MET, PAK1, PDGFRB, PIM1, PKC- γ , PLK4, and SRC) using the previously reported KinaseSeekerTM method, a luciferase fragment complementation assay that provides a direct measurement of their interactions with each respective kinase.^[16] In the presence of 50 μ M MD2-I, eight of the twelve kinases retained >99% of their activity, and the other four retained 85%–95% of the kinase activity (Supplementary Figure 4). The results demonstrated that the active dose of MD2-I required for TLR4 inhibition does not substantially perturb the activity of these biologically essential enzymes. Taken together, these results supported that MD2-I targets the TLR4 pathway with high specificity.

In the absence of an x-ray crystal structure of the TLR4/MD2-I complex, we carried out an all-atom molecular dynamics (MD) simulation with an explicit solvation model to understand the molecular recognition of MD2-I binding to TLR4 (Figure 3). With such an x-ray structure inspired model building, we specifically wanted to understand: (1) whether the structural integrity of the TLR4/MD2-I complex is maintained in the absence of full-length MD2; (2) how different is the binding between MD2-I, compared to full-length MD2, and TLR4; and (3) do we observe dissociation of the TLR4/MD2-I complex during the 10 nanosecond time course of the simulation? The 3.1 Å x-ray structure (PDB ID: 3FXI) of the TLR4/MD2 complex was used as the seed structure for the simulation.^[7] The TLR4-binding region of MD2 was kept intact while the rest of the protein was removed. MD simulations were performed using the NAMD software suite^[17] with the CHARMM22 protein force field^[18] and TIP3P water model.^[19] The system was solvated with 55825 TIP3P water molecules in a cubic box of 122 Å³. Counter-ions were placed randomly in a box around the solute at a concentration of 150 mM KCl. The temperature was maintained in all the simulations at 298 K using Langevin dynamics. During the NVT-equilibration run, a 50000-step energy minimisation using a conjugate gradient algorithm was followed in the following order: (1) gradual heating from 0 to 298 K in 500 ps, (2) solvent equilibration for 500 ps with the protein backbone atoms constrained, and (3) then an equilibration of 1 ns without any constraints. Next production runs of 10 nanoseconds in the NPT ensemble was carried out with a time step of 1 fs. The hydrogen atoms were constrained using the SHAKE^[20] and SETTLE^[21] algorithms. Periodic boundary conditions were applied in three dimensions. Long-range electrostatics was taken into account via the particle mesh Ewald scheme.^[22,23] All simulations were run in parallel on a 64-processor Linux cluster.

As shown in Figure 3B the consistent root mean square deviation (RMSD) value (~2.0 Å) of backbone atoms of the TLR4/MD2-I complex during the MD simulations shows that overall structural integrity is maintained and no large scale structural fluctuations are observed. Importantly, during the 10-nanosecond time course of MD simulations, dissociation of MD2-I from the target (TLR4) was not observed. Further, as shown in Figure 3C and 3D, the critical residues for the full-length MD2 binding to TLR4 retain the same three dimensional arrangement within MD2-I, supporting the notion that MD2-I is a structural mimic of the TLR4-binding hot-spot region of the full-length MD2. In addition, we did not observe major rearrangements in the peptide backbone in a standalone simulation of MD2-I peptide in the water environment. The backbone structure in the bound (TLR4) and unbound (water) are comparable. Presumably, the Cys95-Cys105 disulfide linkage contributes in

maintaining this overall backbone structural integrity. Although thorough understanding of atomic level interactions can only come from x-ray structures or extensive site-directed mutagenesis, the current MD simulation is encouraging since the results imply that the truncated peptide may indeed retain the structure of the TLR4-binding region of MD2, providing us with a prototype for future peptidomimetic inhibitor designs.

Existing small molecule TLR4 antagonists have been shown to reverse allodynia in experimental pain models, including chronic constriction injury (CCI).^[24] We further studied the anti-allodynic effects of the MD2-I peptide using in vivo animal models. MD2-I was tested in rats subjected to CCI of the sciatic nerve of the left hind leg.^[25] This is a classic model of nerve injury induced pain enhancement. This pain model induces bilateral allodynia. That is, in response to injury of one sciatic nerve, there is a decrease in response threshold to calibrated pressure stimuli applied to either of the hind paws (von Frey test). The response thresholds (grams force) were assessed prior to CCI surgery (baseline; BL), 4 and 11 days after surgery (D4, D11; to confirm development of allodynia), and then 1, 3, and 24 hours after MD2-I or MD2-III was delivered intrathecally into the peri-spinal cerebrospinal fluid via the lumbar approach, for a final dose of approximately 250 μ M in the intrathecal space (Figure 4). Consistent with its biochemical indices as a TLR4/MD2 inhibitor, MD2-I produced a partial suppression of allodynia evident at 3 hours, compared to saline vehicle. By contrast, the control peptide, MD2-III, in which all critical residues were mutated, showed negligible inhibitory effects. We also studied the stability of MD2-I peptide in a cell culture medium (DMEM) in the presence of abundant serum (10% FBS) at 37 °C. The amount of intact MD2-I peptide was determined at various points (0, 3, and 24 hours) during the time course of a day by calibrated gel filtration assay. Little time-dependent variation of the absorption corresponding to the MD2-I abundance demonstrates that this peptide is stable in the serum-containing medium (Supplementary Figure 5).

Last, we have shown that MD2-I demonstrates minimal toxicity by: (1) MD2-I showed little cytotoxicity using trypan blue staining assays in various cell lines, including HEK 293, BV2, and RAW 264.7 cells; (2) MD2-I does not induce haemolysis in whole human blood at concentrations up to 500 μ M; and (3) in the previously described animal test, no abnormal behaviours were observed after injections of the peptide stock solutions. These indications supported the notion that MD2-I specifically reduces the TLR4 activation, ruling out the possibility that the in vitro and in vivo inhibitory activities by MD2-I are due to its toxicity.

In summary, the MD2-I peptide binds to TLR4 in a similar manner to the full-length MD2, and selectively suppresses TLR4 signalling without non-specifically affecting activities of other homologous TLRs and representative kinases. The binding mode of MD2-I was further confirmed by direct biophysical binding assays with purified TLR4 and an all-atom MD simulation using an explicit solvation model. Further, MD2-I has demonstrated moderate potency in an animal model, which is particularly promising given protein-protein interfaces have been regarded as daunting targets for drug discovery.^[27] Supported by these results, MD2-I validates a new strategy to suppress inflammation within CNS by disrupting the association between TLR4 and MD2. Even much optimization is needed, this truncated peptide may potentially provide a prototype for the future anti-analgesia peptidomimetic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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26. The convergence of these two MD2 peptides 24 hours after the administration perhaps involves in vivo metabolism, which is currently under investigations.
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Figure 1.

(A) Global view of TLR4 in complex with the truncated region of MD2 (PDB ID: 3FXI).^[7] (B) Fluorescence polarization assay results of MD2-I and MD2-III binding to the extracellular domain of TLR4. To a solution of 200 nM nitrobenzoxadiazole (NBD) labelled MD2-I peptide, TLR4 protein is titrated. The binding plot is then fitted into a one-site saturation model using standard regression analysis. Good fit ($R > 0.99$) implies that MD2-I binds to TLR4 in the predicted manner. Negligible change was observed with the control peptide, NBD-MD2-III.

Figure 2.

Inhibitory effects of 50 μ M MD2-I/MD2-III on various TLR pathways measured by nitric oxide (NO) production in RAW 264.7 macrophage cells. TLRs are activated by TLR-specific ligands in the presence or absence of 50 μ M MD2-I/MD2-III peptide. TLR1/TLR2 is activated by N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R,S)-propyl]-[R]-cysteiny]-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine.3HCl (Pam3CSK4) (250 ng/ml);^[15] TLR4 is activated by lipopolysaccharide (LPS) at 50 ng/ml; TLR2/6 is activated by peptidoglycan (PGN) (50 μ g/ml); TLR7 is activated by 4-amino-2-(ethoxymethyl)- α,α -dimethyl-1H-imidazo[4,5-c]quinoline-1-ethanol (R848) (100 nM). MD2-I selectively inhibits the NO production induced by LPS without affecting other homologous TLRs, while MD2-III demonstrates no inhibitory effect on the TLR4 signaling.

Figure 3.

All-atom molecular dynamics (MD) simulations of the MD2-I peptide binding to TLR4. (A) Superimposition of the x-ray crystallographic structure of the TLR4-binding region of MD2 in complex with TLR4 with the MD predicted structure, showing minimal structural deviation. The x-ray structure is shown in red while the final MD-predicted structure from a 10 ns run is shown in green. (B) RMSD fluctuations of the backbone atoms of the TLR4/MD2-I complex during the MD simulations, showing overall structural integrity is maintained (i.e. no large scale structural fluctuations are observed). Close-up views of the MD2-I peptide binding to TLR4: (C) MD2-I (stick) projects the side chains of the critical amino acid residues in a similar way to the TLR4-binding region of MD2 (blue ribbon). (D) MD2-I (red) superimposes with the TLR4-binding region of full-length MD2 (green) from the 3.1 Å x-ray crystal structure.

Figure 4.

Effect of MD2-I (filled squares) and MD2-III (filled circles) on enhanced pain (allodynia) induced by unilateral injury of the sciatic nerve (chronic constriction injury; CCI). After pre-surgery (baseline; BL) assessment of response thresholds to calibrated pressure stimuli applied to the ipsilateral and contralateral hindpaws (von Frey test), CCI surgery was performed. This produced allodynia, as verified 4 (D4) and 11 (D11) days later. After pre-drug assessment (Pre-Inj), rats were injected intrathecally as indicated and reassessed 1, 3 and 24 hours later.^[26]

Table 1

Sequence alignment of human and murine MD2. Underlined residues are critical binding residues. The disulfide-forming cysteine residues are highlighted in bold. Italic residues are mutated in the control peptide MD2-III.

MD2	SEQUENCE
Human	R ⁹⁰ KEVICRGSDDDYSFCRAL <u>K</u> GETVNTTISFS ¹²⁰
Murine	R ⁹⁰ KEVLCHGHDDDY <u>SFCRAL</u> <u>K</u> GETVNTSIPFS ¹²⁰
MD2-I	CRGSDDDY <u>SFCRAL</u> <u>K</u> GE
MD2-III	CRGSAAAYSFCALAG <i>A</i>