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Development of a Novel Noncompetitive Antagonist of IL-1 Receptor

Christiane Quiniou,* Przemyslaw Sapienha,* Isabelle Lahaie,* Xin Hou,* Sonia Brault,* Martin Beauchamp,* Martin Leduc,* Lenka Rihakova,* Jean-Sébastien Joyal,* Sylvain Nadeau,* Nikolaus Heveker,* William Lubell,† Florian Sennlaub,¶ Fernand Gobeil, Jr.,§ Greg Miller,‡ Alexey V. Pshezhetsky,* and Sylvain Chemtob²*

IL-1 is a major proinflammatory cytokine which interacts with the IL-1 receptor I (IL-1RI) complex, composed of IL-1RI and IL-1R accessory protein subunits. Currently available strategies to counter pathological IL-1 signaling rely on a recombinant IL-1 receptor antagonist, which directly competes with IL-1 for its binding site. Presently, there are no small antagonists of the IL-1RI complex. Given this void, we derived 15 peptides from loops of IL-1R accessory protein, which are putative interactive sites with the IL-1RI subunit. In this study, we substantiate the merits of one of these peptides, rytvela (we termed “101.10”), as an inhibitor of IL-1R and describe its properties consistent with those of an allosteric negative modulator. 101.10 (IC₅₀ ≈ 1 nM) blocked human thymocyte proliferation in vitro, and demonstrated robust in vivo effects in models of hyperthermia and inflammatory bowel disease as well as topically in contact dermatitis, superior to corticosteroids and IL-1ra; 101.10 did not bind to IL-1RI deficient cells and was ineffective in vivo in IL-1RI knockout mice. Importantly, characterization of 101.10, revealed noncompetitive antagonist actions and functional selectivity by blocking certain IL-1R pathways while not affecting others. Findings describe the discovery of a potent and specific small (peptide) antagonist of IL-1RI, with properties in line with an allosteric negative modulator. The Journal of Immunology, 2008, 180: 6977–6987.

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Abbreviations used in this paper: IL-1RI, IL-1 receptor type I; IBID, inflammatory bowel disease; BP, blood pressure; TNBS, 2,4,6-trinitrobenzene sulfonic acid; MBP, mean BP.

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these peptides would conceivably often exhibit allosteric modulatory properties (18, 19). Allostersim can alter signaling modalities (18) and down-stream responses without completely inhibiting receptor function (7), and thus confer greater selectivity. This has, for instance, been shown for thiorechrome, a selective allosteric enhancer of the M4 muscarinic receptor (19), for TNF-αRI (20), and for adhesion molecules such as the LFA-1 integrin of lymphocytes where a peptide binds to an allosteric site and interferes with some (but not all) of its function (21).

Along these lines, in relevance to IL-1R, it has recently been demonstrated that a recombinant of the major extracellular portion of the IL-1RαP (~75 kDa) exhibits in vivo efficacy in a model of autoimmune arthritis without interfering with all actions exerted by IL-1RI (22). On the basis of the overall rationale presented above, we generated peptides derived from extracellular loops and interdomain regions of IL-1RαP and screened them against IL-1-induced effects. We hereby describe for the first time 101.10 (rytvely), a small selective peptide antagonist of IL-1RI, which exhibits properties consistent with those of an allosteric modulator, and is effective in models of acute inflammation.

Materials and Methods

Animals, cells, and peptides

Animals were used according to a protocol of the Animal Care committee of Hôpital Ste-Justine along with the principles of the Canadian Council on Animal Care. Sprague-Dawley rats and CD-1 mice were obtained from Charles River Laboratories. IL1R gene knockout (-/-) mice generated on the B6129S1-Il1r-/- background and corresponding wild-type controls B6129S were purchased from Jackson Laboratories (23). Human thymocyte TF-1 cells were obtained from American Type Culture Collection. All D-peptides were custom synthesized by Sigma-Aldrich (>95% purity).

Peptide design

Regions of the IL-1R accessory protein were identified based on crystallography and modeling data (16, 24), which were supported by hydrophobic and flexibility profiles, as well as homology domains using computational analysis (ProDom (25), PROSITE (26), Predict Protein (26), and ProtScale (27)). Fifteen corresponding homologous peptides (all D-octadeca, sense (NH₂-COOH) and anti-sense (COOH-NH₂)) were derived from primary sequences of extracellular regions (loops and interdomain regions) of the IL-1RαP regions.

IL-1-induced hyperthermia

Sprague-Dawley rats (300–330 g) were anesthetized with 2% isoflurane and placed under a radiant warmer to maintain core (rectal) temperature at ~37.5°C. A polypropylene tube (PE-90) was inserted in the jugular vein for injections. The femoral artery was catheterized (PE-90) to collect blood samples. The probe of an electronic thermometer was inserted ~4 cm into the rectum to measure temperature. 101.10 (1 mg/kg; estimated concentration in maximum efficacy range (~200 mM); see Fig. 2), Ibuprofen (40 mg/kg) or vehicle (normal saline) was injected 10 min before IL-1β (5 μg/kg). Rectal temperature was measured at different time points thereafter.

For arteriole-venule injections, a burr hole was drilled through the skull 1.5 mm lateral to the midline and 1.2 mm posterior to the bregma on the right side. A 10-mm 20-gauge stainless steel hypodermic blunt needle was inserted 4–4.5 mm below the surface of the skull into the right lateral ventricle and secured to the skull with acrylic cement (28); EP3 agonist M&B28767 was injected. The jugular vein was exposed as above. Animals were pretreated with 101.10 (1 mg/kg i.v.) and administered intracerebroventricularly with an EP3 agonist M&B28767 (2 μL of 1 μM solution; estimated concentration of 50 nM for cerebroventricle volume ~40 μL (29). Rectal temperature was monitored as described above.

Trinitrobenzene sulfonic acid-induced model of inflammatory bowel disease

The efficacy of 101.10 was tested on a model of colon inflammation induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). TNBS was administered intrarectally to Sprague-Dawley rats anesthetized with 2% isoflurane (30, 31). In brief, 120 mg/ml TNBS dissolved in 50% ethanol (vol/vol) was injected into the rectum/sigmoid colon (8 cm from anus) (total volume of 0.25 ml per rat; 30 mg total) via a polyethylene tubing (PE50). The control rat received 0.25 ml of vehicle (normal saline). Dexamethasone (0.75 mg/kg/day) (32), IL-1Ra (8 mg/kg/day) (33), and infliximab (10 mg/kg/day) (34) were injected i.p. (within 15 min of TNBS) once a day, and 101.10 (1 mg/kg/day) twice a day. Some animals were also treated with intrarectal 101.10 (1 mg/kg/day) to assess its efficacy upon local application to epithelium (which could also be of clinical interest).

Forty eight hours after administration of TNBS, rats were euthanized by CO₂ inhalation and colon removed and assessed for macroscopic observations (abdominal adhesions, faecal consistency, ulcerations, and discoloration). Tissue was then rolled over ~10 cm length along its transverse axis, fixed, and cut for histology; this enabled histological evaluation in the same tissue over a reasonable length (damage to surface epithelium, crypt distortion, and ulcerations, neutrophil infiltration). Tissues were stained with hematoxylin and phloxin. Tissue injury was evaluated macroscopically and microscopically by three investigators blinded to treatment assignments, based on an adapted version of Peterson’s scale (30); mean of scores was calculated for each animal.

Tissue neutrophil invasion was assessed by myeloperoxidase assay. Myeloperoxidase activity was determined as described (35). In brief, tissue specimens (200–400 mg) were homogenized three times (30 s, 4°C) in 50 mM phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethylammonium bromide, sonicated for 10 s, and exposed to three freeze-thaw cycles. Samples were centrifuged at 20,000 × g for 20 min. A total of 100 μL supernatant was diluted in 2.9 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 526 μM of O-dianisidine hydrochloride. Enzymatic reaction was started with the addition of 0.0005% of peroxide. Myeloperoxidase activity was calculated by dividing the absorbancy (460 nm at 25°C) change per min (total 5 min) with the extinction coefficient for O-dianiside (ε = 1.13 × 10⁴ M⁻¹ cm⁻¹), and was normalized to protein concentration; 1 unit myeloperoxidase activity was defined as that degrading 1 μmol of peroxide per min at 25°C. Values were presented as percent of those in TNBS-injected vehicle-treated (control) rats.

Wright stain was used for histological confirmation of inflammatory cell infiltration into tissues. Intestinal sections were mounted on slides covered with 750 μL of Wright staining solution (Fluka) for 1 min, covered with 1.5 ml of distilled water for 2 min and finally washed with distilled water and mounted with Gel/Moun (Biomedica). Digital images of intestines were obtained (Nikon Dxm 1200). Intestine total length and intact villi were measured with the Image-Pro Plus software (5.1 version). The percentage of intact villi was evaluated by dividing the length of intact villi by the total intestinal length (36). T lymphocytes were counted on intestinal sections incubated for 2 h at room temperature with anti-CD4 Ab (mouse anti-CD4; US Biological) diluted in PBS containing 0.4% Triton X-100 (Sigma-Aldrich), 1% BSA (fraction V; MP Biomedicals), and counterstained with a tagged secondary Ab (goat anti-mouse IgG; Molecular Probes). Intestinal sections were mounted and positive T cells counted (per mm²) on digitized images as reported (37).

Phorbol myristate acetate-induced dermatitis

The efficacy of 101.10 (applied topically) was assessed in a model of cutaneous inflammation induced with 0.05% PMA in acetone applied to ears of CD-1 mice, II/r knockout mice, and wild-type congeners B6129S (38). PMA was applied to both ears, while one ear was treated 45 min after PMA with 20 μL of different concentrations of 101.10 in PEG-400. Ear thickness was measured with a caliper. Forty two hours after the start of treatment, Evans blue dye was injected intracardiac to determine capillary extravasation (see below), and 4 mm ear punches made and weighed. Ear punches were then minced and incubates in dimethyl formamide at 80°C for 3 h. Supernatant was centrifuged and absorbance measured at 620 nm with a background correction of 740 nm (39, 40), and normalized for tissue weight; concentration was determined from Evan’s blue standard curve. PGE₂ was also measured in the tissue (see below).

Western blots

Western blots of p38, phospho-p38, JNK, phospho-JNK, Erk1/2, phospho-Erk1/2, IκB, phospho-IκB, and IL-1R were performed as previously described (41). Essentially rat thymocytes or microvascular endothelial cells

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were preincubated (45 min) at 37°C with the peptide 101.10 (10⁻⁷ M), followed by 30 min incubation with IL-1β (50 pM), IL-6 (0.5 ng/ml), or IL-18 (100 ng/ml). Cells were then lysed, run on 12% SDS-PAGE, and immunoblots were revealed with specific Abs (Calbiochem, Santa Cruz, Cell Signaling) of total and phosphorylated proteins.

[^3]H]thymidine incorporation

Cell proliferation was assessed by incorporation of[^3]H]thymidine as we described (42). Essentially human TF-1 cells were cultured in complete RPMI supplemented with GM-CSF (2 ng/ml; BD Biosource). Cells were deprived of growth factors for 18 h before preincubation with 101.10 followed by treatment with IL-1β (1 ng/ml). For IL-1β dose-response curves and Schild Plot conversion, cells were preincubated with a constant concentration of 101.10 and different concentrations of IL-1β (10⁻⁷ M) followed by incubation with[^3]H]thymidine for 2 h at room temperature. Similarly, thymocytes were preincubated (20 min) with 101.10 (10⁻³ M) and subsequently treated with different concentrations of IL-1β (10⁻⁷ to 10⁻⁶ M), after which[^3]H]thymidine (1 Ci/well; Amersham) was added for another 24 h. Cells were harvested, washed four times with PBS (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and lysed with 0.1 N NaOH/0.1% Triton-X100. Radioactivity was measured (Beckman Multi-Purpose Scintillation Coulter Counter LS6500).

PGE₂ concentrations

PGE₂ was measured in cell cultures, plasma, and tissues as we described in detail (43). TF-1 cells were preincubated 45 min at 37°C with different concentrations of peptides or 9 nM of IL-Rα, after which IL-1β (50 pM) was added to the medium. After 24-h incubation, PGE₂ was measured on growth medium. Measurement of PGE₂ in tissues and plasma was performed as follows. Ears were homogenized in cold indomethacin (10 μM)-containing buffer, whereas plasma was collected in EDTA- and indomethacin (10 μM)-containing tubes; both were passed through C18 columns after which PGE₂ was measured by radioimmunoassay (Amersham).

Radiolabelled ligand binding

Radiolabelled binding of cytokines was performed as comparably described (44, 45). Freshly isolated rat thymocytes (10⁶) were suspended in binding buffer (PBS, HEPES 10 mM, 0.02% NaN₃, 0.05% gelatin, and preincubated (20 min) with increasing concentrations of nonradiolabelled IL-1β or 101.10 followed by incubation with[^125]I-IL-1β for 2 h at room temperature. Similarly, thymocytes were preincubated (20 min) with 101.10 (10⁻³ M) and subsequently treated with different concentrations of[^125]I-IL-1β (10⁻³ M) for 2 h at room temperature. PGE₂ concentrations were determined using the GraphPad Prism 4 software. To ascertain binding profiles performed in thymocytes (low number of IL-1-binding sites (46, 47), experiments were also conducted on NIH3T3 cells (∼5800 IL-1-binding sites/cell).

![FIGURE 1. A, Ribbon-like model of IL-1RI (1ITB), IL-1, and IL-1RacP and identification of regions of derived effective peptides. B, Primary sequence of the IL-1RacP. Colored sequences refer to corresponding loops indicated on A; blue, 101.10; turquoise: 108, green, 106, red, 103.](http://www.jimmunol.org/)

### Table I. Sequence of peptides and targeted regions of the extracellular portion of the accessory protein

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Assigned No.</th>
<th>Peptide Sequences (Sense)</th>
<th>IL-1RαC Target Loops Sequences</th>
<th>IL-1RαC Regions Targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>101.10</td>
<td>NH₂-rytvela-COOH</td>
<td>NH₂-VAAAKVKQKVAPPRTVEVLAC-COOH</td>
<td>D3-juxtamembranous</td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>NH₂-nklpvhlk-CCOH</td>
<td>NH₂-PMLPVHLKLYIEY-COOH</td>
<td>Loop D1-D2</td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>NH₂-vgspknayppp-COOH</td>
<td>NH₂-VKVGSPKNAVPVTHSPN-COOH</td>
<td>Loop D2-D3</td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>NH₂-wtldgkkpddl-COOH</td>
<td>NH₂-WTIDGBKPD-D1-COOH</td>
<td>Loop in D3</td>
<td></td>
</tr>
</tbody>
</table>
Binding of $^{[125]}	ext{I}101.10$ to IL-1R-expressing cells was confirmed by cross-linking. IL-1R-expressing and -deficient thymocytes ($10^6$) were prepared as described above with $[125\text{I}]101.10$ (100 nM) in absence or presence of 1 and 10 μM unlabelled 101.10 for 45 min at 37°C (ascertaining equilibrium for peptide binding). The nonpermeable cross-linker (Bis(sulfosuccinimidyl)suberate [BS3]; 11 Å) was then added to a final concentration of 2.5 mM and samples were incubated at 4°C for 30 min to minimize active internalization of BS3 (48) (Fig. 2A). The reaction was quenched with 20 mM Tris pH 7.5 for 15 min at room temperature. Cells were centrifuged at 4000 rpm for 10 min, lysed for 30 min on ice (150 μl of lysis buffer), and electrophoresed on SDS-PAGE under nonreducing conditions. Autoradiography and Western Blot analysis with anti-IL-1R Ab were performed using the Tukey-Kramer method. Statistical significance was set at $p < 0.05$. Data are presented as mean ± SEM of three experiments each performed in duplicate; $*, p < 0.01$ compared with IL-1-induced.

Data analysis

Results were analyzed by one- or two-way ANOVA factoring for concentration or treatments. Postanova comparisons among means were performed using the Tukey-Kramer method. Statistical significance was set at $p < 0.05$. Data are presented as mean ± SEM.

Results

Peptides and screening of efficacy

Using the rationale presented above regarding intramolecular peptides that interfere with actions of the protein of interest (8–14) we identified extracellular regions of the IL-1RαcP reported to interact with IL-1R1 subunit (11,18). Fifteen corresponding homologous peptides (all D-octa-deca, sense (NH$_3$-COOH) and anti-sense (COOH-NH$_3$)) were designed based on crystallography and modeling data (16, 24), which were supported by hydrophobic and flexibility profiles as well as homology domains using computational analysis (ProDom (25), PROSITE (26), Predict Protein (26), ProtScale (27)); BLAST analysis was coherent with selectivity of peptides for IL-1RαcP. IL-1RαcP regions chose exhibited interspecies homology for human, rat, and mouse. Screening of peptide efficacy was performed on IL-1-induced PGE$_2$ formation. Four of the peptides (termed 103, 106, 108, and 101.10 (all D-); 0.1 μM) derived from regions of the IL-1RαcP depicted in Fig. 1 and presented in Table I effectively inhibited PGE$_2$ formation to varying degrees (Fig. 2A). Interestingly, IL-1-induced p38 phosphorylation was only inhibited by 101.10 (rytvela) and as expected by human recombinant IL-1ra (Fig. 2A); whereas 103 and 108 increased p38 phosphorylation, and 106 was ineffective, consistent with possible allosteric modulation of receptor signaling by 103, 106, and 108 (16, 49). Because 101.10 was reproducibly effective even in inhibiting IL-1-induced IkB phosphorylation (data not shown) and exhibited high potency (see below), we decided to focus and further characterize this small heptapeptide (0.8 kDa).

Efficacy and potency of 101.10

Compilation of data reveals that 101.10 inhibited dose-dependently IL-1-induced PGE$_2$ formation ($IC_{50} = 0.5$ nM; $Emax = 50\%$, consistent with screening) and IL-1-induced human thymocyte (TF-1 cell) proliferation ($IC_{50} = 2$ nM; $Emax ≥ 90\%$) (Fig. 2B).

A

A

B

C

B

C

FIGURE 2. In vitro efficacy of IL-1RαcP-derived peptides. A, Effects of peptides (0.1 μM) and IL-1ra (9 nM) on IL-1 (50 pM)-induced PGE$_2$ formation and p38 phosphorylation on human TF-1 cells. Top gels represent phosphorylated p38 and bottom gels represent total p38. Values on top of p38 gels refer to time (min). Values are mean ± SEM of three experiments each performed in duplicate; *, $p < 0.01$ compared with IL-1-induced. B, Dose response of 101.10 and scrambled peptide (verytla) on IL-1 (1 ng/ml)-induced PGE$_2$ synthesis and cell proliferation in human TF-1. Values are mean ± SEM of 8 experiments each performed in duplicate; *, $p < 0.01$ compared with scrambled peptide or $10^{-8}$ M 101.10 concentration (ANOVAs).

FIGURE 3. Binding of $^{[125]}	ext{I}101.10$. A, Saturation isotherm of $^{[125]}	ext{I}101.10$ in thymocytes from wild-type and IL-1R1$^{-/-}$ mice. Values are mean ± SEM of percent of maximum binding in three experiments each performed in duplicate. B, Western blot of IL-1R1 receptor (+/+) and IL-1R1$^{-/-}$ with anti-sense (COOH-NH$_3$).
effects were observed in a different IL-1-responsive cell type, specifically microvascular endothelial cells; scrambled peptide (very-

|125I|101.10 bound specifically in a saturable manner with an affinity constant (KD) of 5 nM only to thymocytes containing IL-

|101.10 at two different initial concentrations of |125I|101.10; Kᵢ = 4 nM. D. Displacement of bound |125I|IL-1 (25 pM) by IL-1 in the presence of increasing concentrations of 101.10 in rodent thymocytes. E. Displace-

ment of bound |125I|IL-1 (25 pM) by IL-1 in the presence of increasing concentrations of 101.10 in NIH3T3 cells; insert reveals 101.10 binding sites/cell in NIH3T3 cells. D and E. Note the right shift of displacement curves by 101.10. For C–E, values are mean ± SEM of three experiments each performed in duplicate.

Selectivity of 101.10

Selectivity of 101.10 was further tested on effects of homologous cytokine of the IL-1 family, namely IL-18, as well as on other proinflammatory cytokines such as IL-6. IL-18-induced respectively TNF-α- and LPS-dependent JNK and p38 phosphorylation were unaffected by 101.10 (Fig. 5A). Likewise, IL-6-induced Erk1/2 phosphorylation was unaltered by 101.10 (Fig. 5A).

IL-1 causes hyperthermia via formation of PGE2 that in turn activates its EP3 receptor (50, 51). We tested whether this effect can be attenuated by 101.10. IL-1 caused a 1°C increase in core temperature associated with a rise in PGE2 plasma levels (Fig. 5B). 101.10 (1 mg/kg; estimated concentration in maximum efficacy range (≈200 nM)) markedly diminished IL-1-induced peak hyperthermia and attenuated the associated rise in plasma PGE2 in rat (Fig. 5B). As expected the prostaglandin synthase inhibitor ibuprofen (40 mg/kg, as we have shown to inhibit PGE2 formation
(43) also diminished IL-1-induced hyperthermia (Fig. 5C). To ascertain that observed effects of 101.10 in vivo were not mediated via another relevant receptor, namely EP3, animals were treated with EP3-selective agonist PGE2 analog M&B28767 (52) in absence and presence of 101.10. M&B28767-induced hyperthermia, which was unaffected by 101.10, consistent with in vitro selectivity of 101.10 (Figs. 3 and 5).

IL-1 is also known to contribute to systemic hypotension secondary to bacterial endotoxins such as LPS (53). We tested the effects of 101.10 in wild-type and IL-1RI−/− mice. LPS caused a (maximum) 35% decrease in mean BP (MBP) of wild-type mice; 101.10 pretreatment attenuated the drop in MBP to 15% (Fig. 5D), without affecting baseline (~100 mm Hg). IL-1RI−/− mice have a nearly identical baseline MBP to wild-type animals. In IL-1RI−/− mice LPS caused a decrease in MBP similar to that seen in 101.10-treated wild-type mice; 101.10 did not further affect MBP in IL-1RI-deficient animals treated with LPS (Fig. 5D). Data further substantiate in vivo specificity of 101.10.

**In vivo efficacy of 101.10 in animal models of inflammatory conditions**

IL-1 contributes significantly to numerous inflammatory conditions such as inflammatory bowel disease (54, 55) and contact dermatitis (56) including respectively in the TNBS-induced model of inflammatory bowel disease (57) and phorbol ester-induced dermatitis (58). Intragastric TNBS caused pronounced inflammation of intestinal mucosa and submucosa at 48 h, as revealed by destruction of epithelium, crypts and submucosal region, associated with invasion of inflammatory cells (Fig. 6B). 101.10 dose-dependently improved preservation of intestinal integrity during this hyperacute phase of inflammatory destruction by TNBS, and its effects were significantly superior to dexamethasone (and equivalent to infliximab; Fig. 6G and Table II); the scrambled peptide was ineffective. Interestingly, focus on microscopic evaluation of villus integrity (the most important overall criteria) (Fig. 6G), T cell abundance (Fig. 6, H–K), and
myeloperoxidase activity (which reflects neutrophil infiltration) (Table II), suggested superior efficacy of 101.10 (noncompetitive IL-1RI inhibitor; Fig. 4) over the competitive inhibitor IL-1ra (6); Fig. 6G). In addition, 12 h after administration of the proinflammatory irritant TNBS, systemic treatment with 101.10 was also found to diminish mucosal and submucosal destruction, albeit without affecting myeloperoxidase activity (Table II), likely because of insufficient time to clear invading neutrophils, which are however less cytotoxic during inhibition of IL-1RI. Interestingly, pretreatment with intrarectal 101.10 (1 mg/kg/day) also attenuated the index of neutrophil invasion (myeloperoxidase activity) to 56 ± 9% of control values ($p < 0.05$).

Because of transepithelial (rectal) efficacy of 101.10 in TNBS-induced model of gut inflammation, we proceeded to verify the efficacy of topical 101.10 in a model of cutaneous inflammation induced by PMA; topical application of phorbol esters such as PMA induces an acute inflammatory reaction contributed by IL-1...
101.10 (in PEG-400) applied twice a day to PMA-treated ears of CD-1 mice diminished dose- and time-dependently redness and edema formation measured by ear thickness and ear weight (estimated EC$_{50}$ = 10 nM; Fig. 7). These observations were consistent with a dose-dependent reduction in capillary extravasation (measured by Evan’s Blue tissue concentration) and a decrease in tissue PGE$_2$. 

Table II. Effect of 101.10 and other drug treatments on histologic evaluation of TNBS-induced colon inflammation in rat$
^{a}$

<table>
<thead>
<tr>
<th>Preventive Treatment</th>
<th>Macroscopy (Score of 2)</th>
<th>Microscopy (Score of 5)</th>
<th>Myeloperoxidase (% of Control (TNBS))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNBS (n = 8)</td>
<td>1.57 ± 0.04</td>
<td>3.7 ± 0.2</td>
<td>100</td>
</tr>
<tr>
<td>+ 101.10 (n = 8)</td>
<td>0.7 ± 0.1*</td>
<td>2.4 ± 0.3*</td>
<td>47 ± 7*</td>
</tr>
<tr>
<td>+ IL-1ra (n = 3)</td>
<td>0.84 ± 0.1*</td>
<td>2.0 ± 0.5*</td>
<td>63 ± 5*</td>
</tr>
<tr>
<td>+ Infliximab (n = 3)</td>
<td>0.87 ± 0.1*</td>
<td>2.4 ± 1.4*</td>
<td>60 ± 7*</td>
</tr>
<tr>
<td>+ Dexamethasone (n = 8)</td>
<td>ND</td>
<td>3.2 ± 0.3†</td>
<td>66 ± 18†</td>
</tr>
<tr>
<td>Treatment 101.10 (n = 7)</td>
<td>1.1 ± 0.1*</td>
<td>2.7 ± 0.1*</td>
<td>123 ± 18</td>
</tr>
</tbody>
</table>

$^{a}$Macroscopy and microscopy scores are adapted from Peterson’s scale (24). ND, not determined. Values are mean ± SEM. *, p < 0.05 compared to TNBS alone; †, p < 0.05 compared to 101.10 (pretreatment).

FIGURE 7. Efficacy of topical 101.10 in PMA-induced dermatitis. CD-1 mice ears were treated either with PMA (0.05%, applied once daily) or PMA followed immediately with 101.10 mixed in PEG-400 (applied topically twice daily to contralateral ear). Ear thickness was measured throughout the experiment. At 42 h, animals were euthanized and 4-mm ear punches made and weighed; some animals were injected with Evan’s blue to detect extravasation. A, Photographic representations of dose-dependent response to 101.10 applied to right ear; note marked reduction in redness of 101.10-treated right ears compared with untreated left ones. B, Time- and dose-dependent effect of 101.10 on ear weight and thickness of ear punches. C, Concentration-dependent effect of 101.10 on degree of capillary extravasation (measured by Evans blue in tissue). D, Efficacy of 101.10 (100 nM) on tissue PGE$_2$ concentrations. For B–D, values are mean ± SEM of three to five experiments; *, p < 0.01 compared with values without asterisks. E, Effects of 101.10 (100 nM) on PMA-induced inflammatory edema in wild-type and IL-1RI$^{-/-}$ B6129S1 mice. 101.10 attenuated edema formation in wild-type but not in IL-1RI$^{-/-}$ mice, wherein edema is less pronounced than in wild-type congeners. Values are mean ± SEM percent of control (without PMA) of three to four experiments; *, p < 0.01 compared with PMA in corresponding animal group.
concentrations (Fig. 7C); topical IL-1ra was ineffective (data not shown), as usually expected with proteins. Finally, parameters of PMA-induced edema (ear weight and thickness; latter not shown) were (as expected) diminished in \( \text{IL-1R}^{--} \) animals to levels approaching those after 101.10 in wild-type mice; 101.10 was ineffective in \( \text{IL-1R}^{--} \) animals (Fig. 7E).

**Discussion**

Based on evidence that IL-1RaCp interacts with the IL-1R subunit of the IL-1RI receptor complex (16) and that recombinant extracellular portion of IL-1RaCp can interfere with IL-1RI actions (22), we designed peptides that reproduce various relevant IL-1RaCp regions. Of these one small heptapeptide, termed “101.10,” was found to be particularly effective in inhibiting IL-1-induced effects in vitro, although not necessarily fully (as seen with PGE\(_2\)). 101.10 was a potent, selective, and reversible noncompetitive inhibitor of IL-1RI, and also exhibited modulatory properties, notably, by not interacting with the ligand binding (orthosteric) site, albeit by affecting IL-1-binding affinity and by interfering variably with different in vitro responses to IL-1. These features distinguish it from the large molecule competitive inhibitor IL-1ra (59). Consistent with IL-1-induced in vitro and in vivo (specifically hyperthermia) effects, 101.10 displayed effective anti-inflammatory capacity in acute inflammatory conditions involving IL-1. Findings describe a new small unprecedented noncompetitive antagonist of IL-1RI with valuable and increasingly desirable modulatory pharmacologic properties, consistent with those of an allosteric negative modulator that exhibits functional selectivity (7, 60–65).

The peptides we designed arose from loops and interdomain regions of the IL-1RaCp (Fig. 1) and possess high flexibility profiles, enabling interaction with the IL-1RI subunit (16), which requires appropriate conformational changes. Primary sequence peptides reproducing specific protein regions have successfully been used to interfere with the effects of various receptors (8, 10–13, 66), and the effects of such peptides coincide with those of specific corresponding mutations (67, 68). Because these regions of interest are often remote from the natural ligand-binding site (orthosteric site), derived molecules are noncompetitive and can modulate ligand-binding affinity; these features are in line with characteristics of allosteric modulators (61, 69–71), and based on data presented, apply to 101.10. 101.10 binds specifically to IL-1R including after cross-linking (11 Å) but not to the IL-1-binding site, and accordingly is a noncompetitive antagonist (18, 19) of IL-1-induced effects (Figs. 3 and 4); because 101.10 appears to modulate (rather than totally interfere with) IL-1 binding to the IL-1RI (Fig. 4, D and E) which, in turn, facilitates complex formation with IL-1RaCp (4), 101.10 can partly but not necessarily fully dissociate the complex, as suggested by cross-linking of [\(^{125}\)I]101.10 to the IL-1RI/IL-1RaCp complex (Fig. 3C). The noncompetitive property of 101.10 on IL-1-induced actions is further substantiated by the inability of increasing concentrations of IL-1 to overcome antagonist effects of 101.10 on IL-1-induced TF-1 proliferation, which is correspondingly reflected in plateauing of the Schild plot (Fig. 4A); in contrast, orthosteric antagonism by definition can be overcome by increasing concentrations of the natural ligand (88).

Another feature observed with allosteric modulators relates to their ability to modulate ligand-binding affinity; this was also seen with 101.10 which diminished binding affinity of IL-1 as revealed by the right shift of the curve of [\(^{125}\)I]IL-1 displacement by IL-1 (Fig. 4D). The magnitude of change in both affinity and efficacy induced by such a receptor modulator is indicative of conformational modifications represented by the cooperativity constant \( \alpha \) a measure of how the orthosteric and allosteric ligand perceive each other (65, 67, 72). Crystallographic analyses of protein complexes often fail to detect small conformational changes (\( \leq 2 \) Å), which may have profound effects on receptor function and are more readily appreciated by pharmacological binding and efficacy profiles (73). The \( \alpha \) constant for 101.10 to induce a two-fold shift of the IL-1-induced proliferation dose-response was positive but below a value of 1, which again suggests noncompetitive negative modulation on IL-1 potency toward its receptor (74). Altogether, one notes that 101.10 moderately decreases binding affinity of IL-1 but markedly depresses (some of) its function (Fig. 4); these observations contrast with those of orthosteric (competitive) inhibitors where changes in ligand binding somewhat correspond to those in function (60, 61, 75).

As alluded to above specific mutations (67, 76) or small molecules can affect some but not all functions evoked by a receptor (77–79). This property is referred to as pharmacological permissivity (7, 61, 65). This concept also appears to apply to 101.10, which partially inhibited IL-1-induced PGE\(_2\) production but fully antagonized p38 (Fig. 2); this paradigm is further illustrated with peptides arising from other IL-1RaCp regions (Fig. 1), namely 103, 106 and 108, which affected differently IL-1-induced PGE\(_2\) and p38 phosphorylation (Fig. 2). This functional selectivity is made possible by ligands which bind in ways that affect the dynamic conformation of the receptor to interact with its natural ligand and associated proteins needed to activate normal signaling pathways (65, 74, 75); hence, such ligands can alter signaling modalities (18), which may confer greater selectivity (19) and reduce side effects (61), compared with orthosteric antagonists which disable all functions triggered by the receptor. These features seem to apply to 101.10 in line with what has lately been reported by other (noncytokine) receptors (77–80).

In agreement with its specific anti-IL-1RI actions in vitro, 101.10 exerted corresponding effects in vitro by curtailing IL-1-induced hyperthermia and hypotension (Fig. 5). Given the dominant role of IL-1 in acute bouts of inflammation a contribution for IL-1 in models of inflammatory conditions is also anticipated (1, 57, 58). Indeed, colon inflammation is characterized by a Th1 response, with high levels of IL-12, IL-6, IL-18, TNF-\( \alpha \), and IL-1, mostly produced by monocytes and macrophages (81, 82). In skin, IL-1 is mainly found in keratinocytes, fibroblasts, and endothelial cells, and is a sequestered pool ready to be released upon injury (83). 101.10 was effective in in vivo models of inflammatory conditions, as demonstrated in two distinct models of inflammation induced in gut by TNBS and on cutaneous tissue by PMA (Table II; Figs. 6 and 7), consistent with reported efficacy of IL-1ra in analogous models (84–87). In contrast to genetically intact animals, 101.10 was ineffective on IL-1-dependent physiologic and inflammatory parameters induced in IL-1RI \(^{--} \) mice (Figs. 5 and 7), consistent with its specificity (Figs. 3 and 5).

In summary, we hereby document the discovery and pharmacological properties of a small stable (D-) peptide antagonist of IL-1RI, namely 101.10, which is rationally derived from an extracellular loop region of IL-1RaCp (see Fig. 1) and exhibits properties consistent with those of an allosteric negative modulator. As a competitive inhibitor of IL-1, IL-1ra interferes with all actions of IL-1RI, including desirable ones related to innate immunity, and hence increases the risk of cancer and seemingly of infections (85). In this report, we have described the first small molecule (peptide) antagonist of a cytokine receptor, notably of IL-1RI, which seems to integrate allosteric modulatory properties; 101.10 is specific, potent and effective in vitro and in vivo (IL-1-implicated) models of inflammation after systemic and topical applications. Because 101.10 appears to some extent more effective than the competitive antagonist IL-1ra in vivo inflammatory conditions (Table II and Fig. 6), 101.10 (and small like-compounds) could.
The authors have no financial conflict of interest.

Disclosures
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