

RX-207, Small Molecule Inhibitor Of Protein Interaction With Glycosaminoglycans (SMIGs), Reduces Experimentally Induced Inflammation And Increases Survival Rate In Cecal Ligation And Puncture (CLP) Induced Sepsis

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Running Title Small molecule inhibitors of deleterious inflammation and sepsis

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Abstract

The fused quinazolinone derivative, RX-207, is chemically and functionally related to small molecule inhibitors of protein binding to glycosaminoglycans (SMIGs). Composed of a planar aromatic amine scaffold, it inhibits protein binding to glycosaminoglycans (GAGs). RX-207 reduced neutrophil migration in thioglycollate induced peritonitis (37%), inhibited carrageenan induced paw edema (32%) and cerulein induced pancreatitis (28%) and increased animal survival in the mouse model of cecal ligation and puncture (CLP) induced sepsis (60%). The mechanism of RX-207 action, analyzed by UV spectroscopy, confirmed that which was elucidated for chemically related anti-inflammatory SMIGs. RX-207 binding to cell surface GAGs can account for the inhibition of neutrophil recruitment via the microvasculature and as a consequence the reduction of neutrophil mediated tissue damage in the animal models of inflammation and improved survival of mice in CLP induced sepsis.

Keywords: heparin binding protein; glycosaminoglycan; neutrophil; inflammation; sepsis

Introduction

Contemporary medicine treats inflammatory disorders with a variety of medications. Corticosteroids are the most effective drugs for acute inflammation and are efficacious, at a low dose, in treating chronic inflammation [1]. Inflammatory disorders that are refractory to treatment, such as acute pancreatitis and sepsis, can be fatal. The absence of effective treatment of sepsis is not for want of effort, or resource. There are more than four hundred reports of preclinical studies of the pro-inflammatory cytokine, tumor necrosis factor alfa (TNF- α), alone and more than one hundred clinical trials have been carried out on anti-inflammatory drug candidates [2]. Many and various explanations have been given for the clinical failure of drugs in treating sepsis [3]. One, for example, is the difficulty in defining the disease [4], another is the temporal heterogeneity in sepsis development and a third explanation is the biological heterogeneity resulting from patient age, comorbidities and pathogens [5]. What is true for sepsis and inflammatory disorders in general, is that inflammation is the net result of the activities of multiple biochemical pathways. Modulation of any one pathway, therefore, is unlikely to influence the course of the illness in a significant way. Profound changes in the endothelium of venules, as a result of sepsis, cause vasodilation, loss of barrier function and widespread tissue edema [6]. Neutrophils, the principal effectors of endothelial damage in sepsis [7], are potential targets for anti-inflammatory drugs. Therapeutic modulation of inflammatory neutrophils potentially could reduce tissue edema and prevent the damage caused by the reactive oxygen species, proteases and pro-inflammatory cytokines that they secrete [8]. Anti-inflammatory small molecule inhibitors of protein binding to glycosaminoglycans (SMIGs) have been described [9] and their mechanism of action in experimental models of inflammation elucidated [10]. The current manuscript describes the anti-inflammatory activity of a novel SMIG,

demonstrates its efficacy in experimental sepsis and delineates and confirms the
mechanism of action that can account for the inhibition of inflammatory neutrophil
adhesion and activity in the disease.

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Materials and Methods

Materials

Quinazolinone derivatives were purchased from, or custom-synthesized by Life Chemicals Ltd. (Kiev, Ukraine) and ChemDiv (San Diego, CA); heparin sodium salt (porcine intestinal mucosa, Cat# 51551), chondroitin 6-sulfate sodium salt (shark cartilage, Cat# C4384), carrageenan (Cat# C-3889), cerulein (Cat# C-9026), Tween 80, DMSO, thioglycollate (Cat# T-0632) were purchased from Sigma-Aldrich Corporation, (St Louis, MO); Türk's reagent was purchased from (Merck, Germany, Cat# 109277); Balb/c mice were provided by Velaz (Prague, Czech Republic); the solubility of RX-207 is 20 mg/mL in 50 mM citric acid.

Methods

Thioglycollate-induced peritonitis¹

BALB/c mice (6 weeks old, ~19 g in weight, 12 mice/group) received p.o. inhibitor compound in 0.1 mL methylcellulose 1 hour before administration of thioglycollate. Control groups received vehicle only. Mice were injected intraperitoneally with 1 mL of 3% thioglycollate broth [11]. Mice were sacrificed after 3 hours, and the peritoneal cavities were lavaged with 5 mL of ice-cold saline containing 2 mM EDTA to prevent clotting. After red blood cell lysis, leukocytes were counted in a hemocytometer. Neutrophils were counted after staining with Türk's reagent. Data was expressed as mean \pm SEM, and statistical analysis was performed by Student *t* test.

Quinazolinone (RX-207) formulation

RX 207 was dissolved in a mixture of propylenglycol (41%, Fluka, Biochemika), PEG400 (9%, Fluka, Biochemika) and water (pharmaceutical grade).

Carrageenan-induced paw edema¹

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2 Acute edema was induced in the right hind paw of BALB/c mice (8 weeks old, ~21 g in
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4 weight, 12 mice/group) by injecting 0.05 mL of freshly prepared solution of 1% carrageenan
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6 after 60 min of test compound administration [12]. The left paw received 0.05 mL of saline,
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8 which served as a control. Carrageenan was injected under the plantar region of right hind
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10 paw and the paw thickness was measured at 4 hours after carrageenan challenge using a
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12 Mitutoyo engineer's micrometer expressed as the difference between right and left pad as
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14 mean \pm SEM. Results were evaluated by Student's *t* test.
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Acute pancreatitis¹

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20 Acute pancreatitis was induced in Balb/c mice (8 weeks old, ~22 g in weight, 10-11 mice per
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22 group) by injecting cerulein (50 μ g/kg, i.p., X7; [13]). The cerulein model of acute
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24 pancreatitis is a commonly used pharmaceutical industry model. Test compounds were
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26 administered i.m or i.v. 1h prior to first cerulein injection. One hour after the last cerulein
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28 injection blood samples were taken. Serum amylase was analysed by standard methods
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30 [14]. Amylase data were calculated as units per volume and expressed as mean \pm SEM.
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32 Results were evaluated by Student's *t* test.
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Cecal ligation and puncture (CLP, a model of sepsis)¹

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41 CLP was performed in the control and experimental Balb/c mice (8 weeks old, ~22 g in
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43 weight, 9-10 mice per group) as described by Fink [15]. The experimental mice were given
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45 RX-207 or RX-205 (20 mg/kg, 0.1ml, i.m.), 24 hours before surgery and once per day for the
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47 duration of the study. Survival data were evaluated by Log-rank (Mantel-Cox) test.
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UV absorption spectroscopy

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53 UV absorption spectra were recorded on a JASCO J-715 spectropolarimeter at 25 ± 0.2 °C.
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55 Temperature control was provided by a Peltier thermostat equipped with magnetic stirring.
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57 Each spectrum represents the average of two scans obtained by collecting data at a scan
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1 speed of 200 nm/min. 2.3 mM stock solution of RX-207 was prepared using ultra-pure water
2 and a small volume of ethanol. 25 or 30 μ L stock solution of the ligand was added to 1.8 mL
3 ultra-pure water in a rectangular quartz cell of 1 cm optical path length (Hellma, USA). Drug
4 samples obtained by this way were subsequently titrated by small aliquots of concentrated
5 aqueous solution of heparin and chondroitin 6-sulfate. Due to their poly-disperse nature,
6 glycosaminoglycan (GAG) concentrations were expressed using the molecular weight of the
7 average repeating disaccharide units: 665 and 503 g/mol for heparin and chondroitin 6-
8 sulfate, respectively. Absorption curves of drug-GAG mixtures were corrected by subtracting
9 the spectra of blank water solution.
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25 ¹ The animal experiments were reviewed and approved by the Ethical Committee for animal
26 experimentation of the Institute of Animal Physiology, approved by the State Veterinary and
27 Food Administration of the Slovak Republic and were performed in accordance with Slovak
28 legislation based on EC Directive 86/609/EEC on the protection of animals used for
29 experimental and other scientific purposes.
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Results

RX-207 and RX-205, inhibitors of neutrophil migration

An *in vitro* screen of SMIGs identified the anti-inflammatory quinazolinone, RX-211, with IC₅₀ values in the low micro-molar range and broad activity against inflammatory chemokines and cytokines [9]. Derivatives of related compounds, the fused quinazolinones, RX-207 (azepino[2,1-b]quinazoline-6,7,8,9,10,12-hexahydro-12-oxo-*N*-(1-methylethyl-piperidinyl-4)-3-carboxamide, Figure 1A and RX-205 (pyrrolo[2,1-b]quinazoline-1,2,3,9-tetrahydro-9-oxo-*N*-(1-methyl-piperidinyl-4)-6-carboxamide), Figure 1B, were predicted to have anti-inflammatory activity in animal models of inflammation.

Thioglycollate induced peritonitis that results in a massive neutrophil infiltrate into the peritoneum [16], is a frequently used animal model to study acute inflammation [17]. Oral administration of RX-205 and RX-207 (50 mg/kg) inhibited neutrophil migration into the peritoneal cavity by 38% and 37%, respectively (Figure 2).

RX-207 anti-inflammatory activity

Carrageenan-induced inflammation is characterized by the migration of leukocytes, mainly neutrophils, to the site of inflammation [18]. Carrageenan-induced edema of the mouse paw is a model of acute local inflammation to evaluate the anti-inflammatory activity of investigational compounds [12]. Intra-muscular (i.m.) injection of RX-207 (1 mg/kg, 0.1ml) inhibited carrageenan-induced paw edema in mice (32%), as measured by a reduction in paw thickness (Figure 3). RX-205 (25 mg/kg), administered i.v., reduced paw thickness by 24% ($p < 0.001$, data not shown).

Supra-physiologic doses of cerulein cause acute pancreatitis in mice reproducing morphological and biochemical features of the human disease [13]. Models of cerulein

1 induced pancreatitis, characterized by increased serum amylase activity, have been used to
2 study acute pancreatitis and to test potential drugs [19]. RX-207 (25 mg/kg, 0.1ml),
3 administered i.m., reduced serum levels of amylase by 28% (Figure 4). RX-205 (20 mg/kg),
4 administered i.v., reduced amylase activity by 34% ($p < 0.01$, data not shown).
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10 11 12 **RX-207 and RX-205 therapeutic activity**

13 Cecal Ligation and Puncture (CLP) causes poly-microbial sepsis, a procedure used for
14 modeling sepsis *in vivo* [15]. CLP in the mouse model of sepsis resulted in 70% mortality
15 (Figure 5). Treatment with RX-207 and RX-205 (20 mg/kg each, 0.1ml, i.m), 24 hours before
16 surgery and once per day for the duration of the study, increased survival rate by 60% and
17 50%, respectively. Body weight decreased to the same extent (22-23% of starting values) in
18 both experimental and control groups (Figure 6).
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32 **Mechanism of RX-207 action**

33 The related quinazolinone derivatives, RX-207 and RX-205 (30 μ M) inhibited binding of the
34 pro-inflammatory chemokine, MCP-3, to heparin, a highly sulfated form of the cell surface
35 GAG, heparan sulfate (data not shown). The inhibition of MCP-3, the only one of ten heparin
36 binding chemokines, cytokines and selectins tested, was an indication of target specificity.
37 RX-207 and RX-205 (30 μ M) inhibited binding of the pro-inflammatory chemokine, IP-10, to
38 the extracellular matrix GAG, chondroitin sulfate (CS, data not shown), extending the range
39 of GAGs targeted by SMIGs. Spectroscopic analysis was carried out to elucidate the
40 interaction of RX-207 with the GAGs. UV absorption spectra of RX-2007 were determined in
41 the absence and presence of CS and heparin. The spectrum, recorded in water, exhibited
42 multiple peaks at a wavelength greater than 200 nm, the most intense one ($\epsilon_{max} \sim 35,000 \text{ M}^{-1}$
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1 $^1 \text{ cm}^{-1}$) centered around 235 nm (Figure 7). Heparin induced a dose dependent reduction in
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3 the molar absorption coefficient (ϵ_{max}), the experimental proof of quinazolinone binding to
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5 the GAG.
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8 At the initial phase of the titration, heparin produced a moderate (12%) but abrupt
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10 reduction in the intensity of the main UV peak (Figure 8). Further addition of heparin
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12 induced opposite, but slower changes, restoring the molar absorption coefficient close to its
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14 original value (Figure 8). Compared with heparin, interaction of RX-207 with CS resulted in a
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16 very small decrease (~3%) of the ϵ_{max} value (Figure 8). A small concentration of NaCl
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18 promptly restored the original absorption intensities of RX-207 in the presence of heparin
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20 and CS (Figure 9). No circular dichroism signals were induced either by heparin or CS (data
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22 not shown).
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31 **Discussion**

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33 Activated neutrophils have an extended half-life and an altered biochemical profile [20].
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35 They remain in the blood stream, express inflammatory signals that alter blood vessel
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37 permeability [21] and release reactive oxygen species, proteolytic enzymes and pro-
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39 inflammatory cytokines [8]. Neutrophil apoptosis and phagocytosis of the apoptotic cells
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41 resolve the inflammatory response [22]. Inordinate neutrophil activity impairs innate anti-
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43 oxidant and anti-protease activity and stimulates indiscriminate proteolysis, the hallmark of
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45 inflammatory disorders [23]. Enteral administration of the quinazolinone related derivatives,
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47 RX-207 and RX-205, inhibited thioglycollate induced neutrophil migration (Figure 2),
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49 parenteral administration of RX-207 inhibited carrageenan induced paw edema (Figure 3)
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51 and intramuscular injection of RX-207 inhibited a neutrophil mediated increase in serum
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1 amylase in cerulein induced acute pancreatitis (Figure 4). RX-207 appeared to be
2 responsible for inhibiting neutrophil recruitment and consequent inflammation in the three
3 animal models of disease.
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7 Intramuscular injection of RX-207 and RX-205 significantly reduced mouse mortality, 60%
8 and 50% respectively, in CLP-induced sepsis (Figure 5). The weights of the experimental and
9 the control animals leveled off at 80% of starting values (Figure 6) and a dose of 1,500
10 mg/kg (RX-205, toxicity study, not shown) did not produce any adverse clinical symptoms.
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13 The efficacy in CLP-induced sepsis, a poly-microbial sepsis that reflects clinical reality more
14 than any other animal model of systemic inflammation [24], is predictive of therapeutic
15 potential.
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26 RX-207 inhibited the selective binding of MCP-3 to heparin and IP-10 binding to CS.
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28 Spectroscopic analysis of RX-207 was carried out to study its interaction with the respective
29 GAGs (Figure 7). Heparin induced dose-dependent hypochromism [25], a reduction in ϵ_{\max}
30 that is experimental proof of RX-207 binding to the GAG. UV hypochromism is due to
31 electronic interactions between the planar quinazolinone moieties of RX-207 molecules
32 (dimers or oligomers) arranged proximally along the heparin chain. The largest reduction in
33 the ϵ_{\max} of RX-207 was observed around RX-207:disaccharide molar ratio of 2:1 (Figures 7
34 and 8), suggesting the association of two RX-207 molecules per disaccharide unit of the
35 heparin chain. Restoration of the molar absorption coefficient to its original value by the
36 further addition of heparin (Figure 8) might be indicative of a shift in the stoichiometry, back
37 from 2:1 to 1:1. Due to the molar excess of heparin binding sites, the monomeric association
38 of RX-207 became more prevalent, canceling the hypochromism. It seems that the structure
39 of the disaccharide unit of CS (lower charge density vs. heparin, Figure 9) did not favor the
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1 dimeric binding of RX-207. A low concentration of NaCl was observed to promptly restore
2 the original absorption intensities of RX-207 in the presence of either heparin or CS,
3 suggesting that Na⁺ ions effectively disrupt the Coulombic interactions between the acidic
4 groups of GAGs and the protonated piperidine nitrogen of RX-207 (Figure 1). The
5 dissociation of the RX-207-GAG complexes provides a rationale for the inactivity of RX-207
6 in inhibiting protein binding to heparin in the presence of salt.
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15 RX207 inhibits pro-inflammatory MCP-3 binding to heparin (current manuscript) and anti-
16 inflammatory SMIGs inhibit binding of the pro-inflammatory cytokines, IL-6 and IL-8 [9].
17 Exactly how the anti-inflammatory SMIGs influence cytokine expression has not yet been
18 investigated. This is a question that should be addressed given that the anti-inflammatory
19 cytokine, IL-10, protects mice from bacterial sepsis [26] and the anti-inflammatory IL-13
20 prevents lethality in a mouse model of neonatal sepsis in humans, neonatal endotoxin shock
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34 The endothelial lumen of the micro-vasculature is decorated with glycocalyx, an
35 extracellular matrix that mediates vascular barrier function, leukocyte adhesion and anti-
36 oxidant defense [28]. Leukocyte adhesion molecules, embedded in the glycocalyx, are
37 shielded from circulating leukocytes. Sepsis causes glycocalyx shedding, exposes lymphocyte
38 adhesion molecules and liberates endogenous damage associated molecular patterns
39 (DAMPs, [29]), including heparan sulfates, themselves inducers of sepsis [30]. Neutrophils,
40 adhering to the glycocalyx and extravasating into proximal tissue, release heparin binding
41 protein (HBP, [31]) that binds to cell surface proteoglycans and induces vascular leakage
42 [32]. Spectral analysis showed that RX-207 bound directly to the GAG, heparin (Figure 7),
43 confirmation of the mechanism of action elucidated for chemically distinct, but functionally
44 related anti-inflammatory SMIGs [10]. Glycocalyx of the inflamed micro-vasculature
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1 provides the pathologic context for anti-inflammatory SMIG activity. SMIGs have the
2 potential to prevent inflammatory neutrophils adhering to microvasculature and
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5 extravasating into proximal tissue, inhibit heparin binding proteins that cause vascular
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7 leakage and neutralize sepsis inducing heparan sulfates.
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10 At the time of submitting this manuscript no small molecule therapy for sepsis had been
11 approved [33]. The efficacy of SMIGs in a severe experimental model of sepsis (current
12 report), colitis and experimental autoimmune encephalomyelitis [10], underscores the
13 potential of the glycocalyx in general and GAGs in particular, as biological targets for the
14 treatment of deleterious inflammation. RX-207 and RX-205 are chemically related to
15 therapeutic quinazolinones, a number of which have anti-inflammatory activity [34], [35],
16 [36]. Anti-inflammatory SMIGs, with defined biological targets and an elucidated mechanism
17 of action, are potential therapeutics for diverse, but pathogenically related inflammatory
18 disorders.
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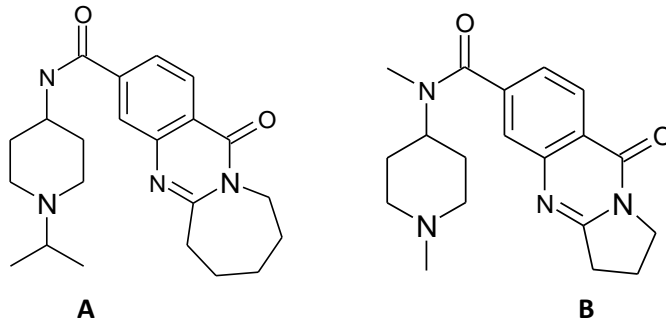


Fig. 1 (A) RX-207, (B) RX-205

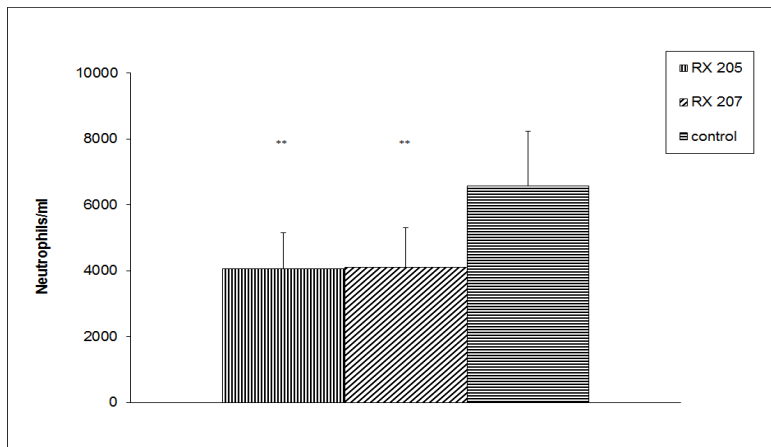


Fig. 2 Inhibition of neutrophil migration by RX-205 and RX-207 (50 mg/kg, *per os*) in a mouse model of thioglycollate induced peritonitis (** $p < 0.01$)

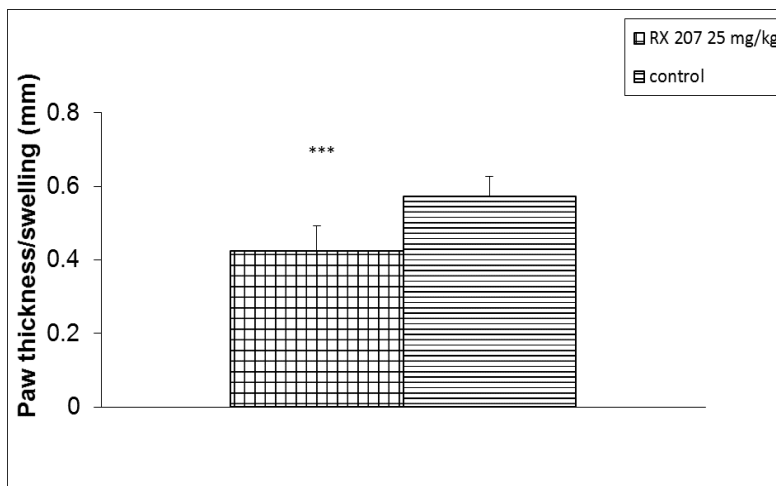


Fig. 3 Anti-inflammatory activity of RX-207 in a mouse model of carrageenan-induced edema. RX-207 (1 mg/kg) was administered i.m. one hour before injecting carrageenan. Paw edema was determined 4 hours after administering carrageenan. The Y axis represents difference in paw thickness between carrageenan treated and non-treated paws (** $p < 0.001$)

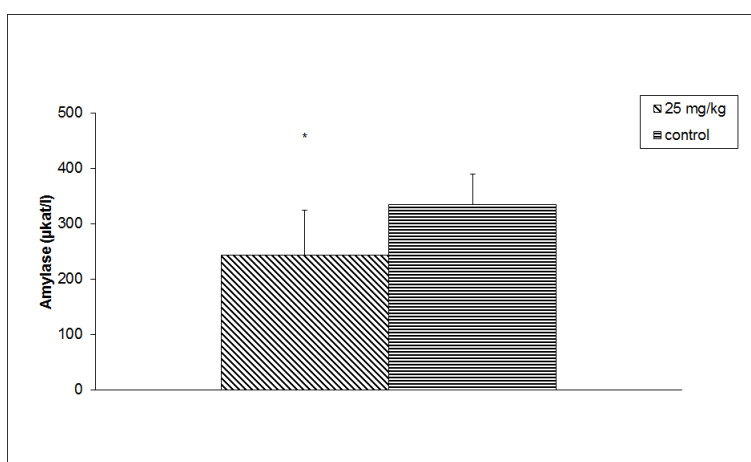


Fig. 4 Therapeutic activity of RX-207 in a mouse model of acute pancreatitis. RX-207 (25 mg/kg) was administered i.m. one hour before injecting cerulein. Serum amylase was analyzed one hour after the last injection of cerulein (* $p < 0.05$)

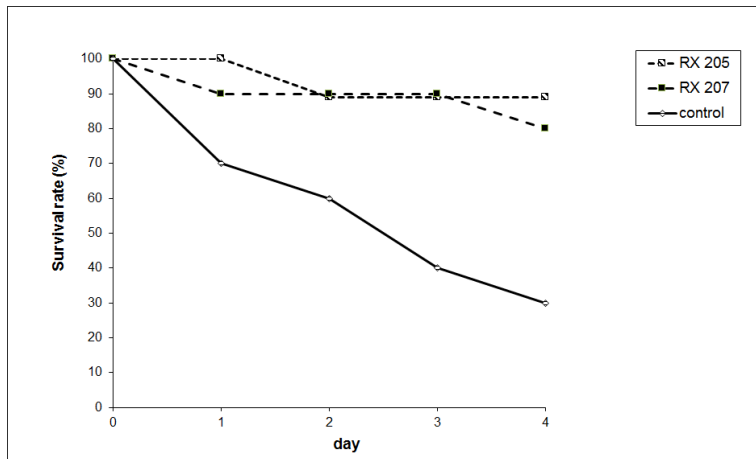


Fig. 5 Therapeutic activity (survival rate) of RX-205 (20 mg/kg, i.m., *p=0.0123) and RX-207 (20 mg/kg, i.m., *p=0.0233) in the mouse model of CLP-induced sepsis. *Log-rank (Mantel-Cox) test for CLP survival

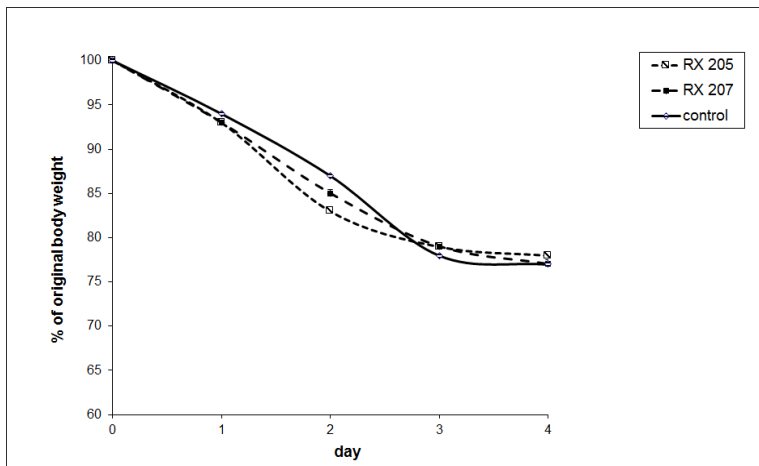


Fig. 6 Change in body weight of mice treated with RX-205 (20 mg/kg, i.m.) and RX-207 (20 mg/kg, i.m.) in the mouse model of CLP-induced sepsis

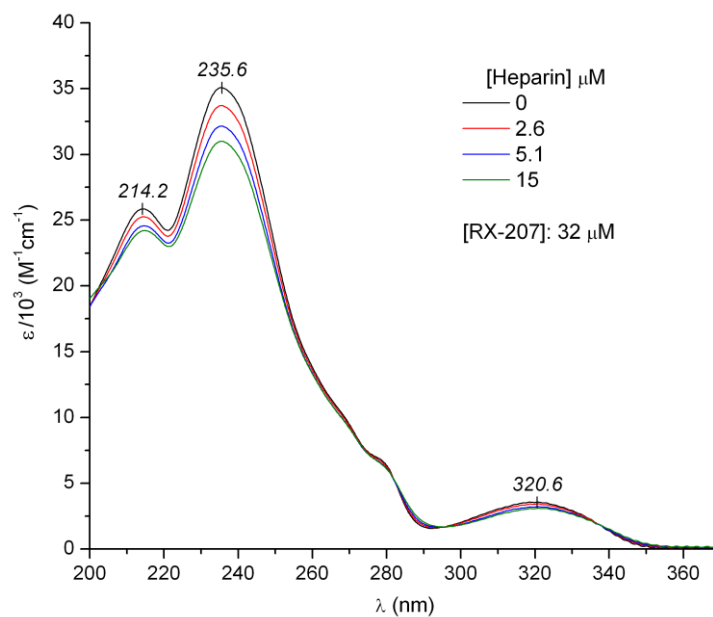


Fig. 7 Effect of heparin on the UV absorption spectrum of RX-207 measured in aqueous solution

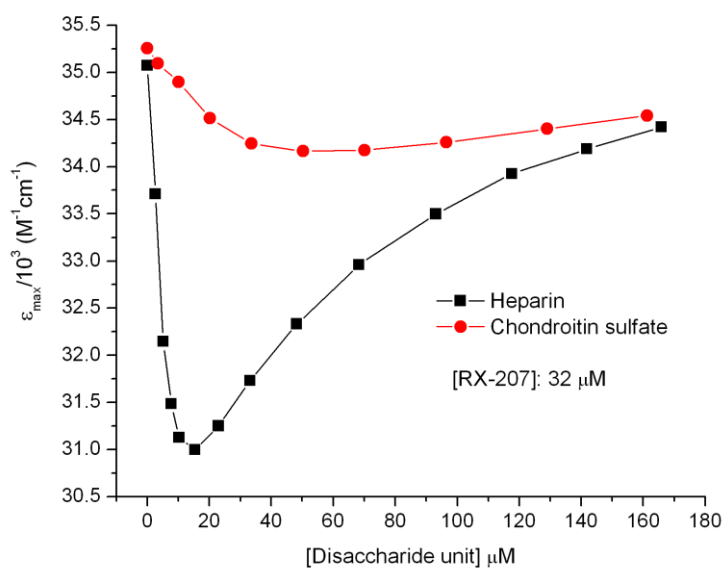


Fig. 8 Effect of addition of heparin and CS on the ϵ_{max} value of RX-207

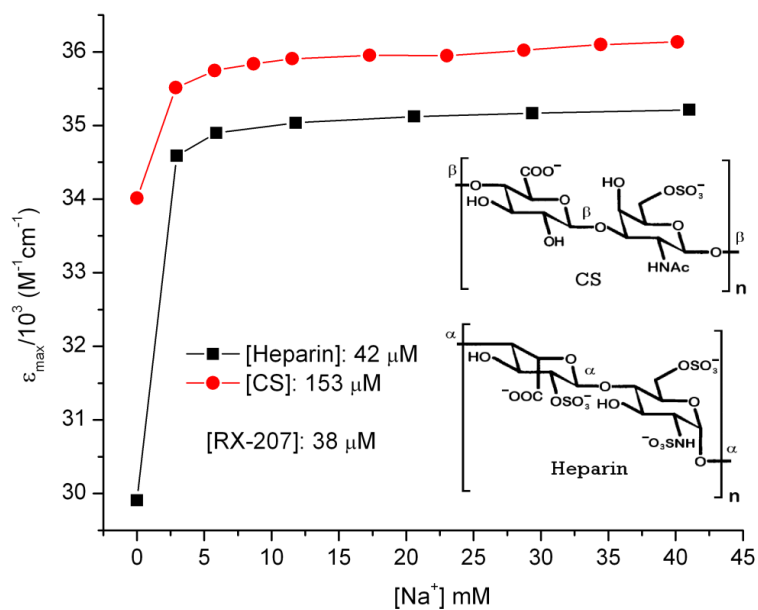


Fig. 9 Effect of sodium chloride on the ϵ_{\max} of RX-207 bound to heparin and CS. Inset shows the average repeating disaccharide unit of CS and heparin