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Intravesical Toll-like receptor 7 agonist R-837: Optimization of its formulation in an orthotopic mouse model of bladder cancer

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Abstract

Objective—To study the immune response caused by the intravesical administration of the immunomodulator R-837 in various formulations and to estimate its therapeutic potential for bladder cancer.

Methods—Female C57BL/6 mice were intravesically treated with different formulations of R-837, a Toll-like receptor 7 agonist used for treating genital warts and skin malignancy. The tested formulation mixtures contained different ratios of lactic acid, a thermosensitive poloxamer polymer (Lutrol F127) and 2-(hydroxypropyl)- β -cyclodextrin (HP β CD). Induction of tumor necrosis factor α (TNF α) and keratinocyte-derived chemokine (KC) was analyzed by Luminex microbeads assay. The therapeutic potential of intravesical administration of R-837 was assessed in an orthotopic, syngeneic mouse model of bladder cancer using MB49 cells.

Results—Intravesical administration of R-837 in lactic acid alone induced systemic and bladder TNF α and KC in a dose-dependent manner. Formulations including poloxamer decreased systemic absorption of R-837 and significantly reduced systemic and local induction of KC. Addition of HP β CD in the poloxamer formulation particularly reversed levels of systemic and local levels of TNF α and KC. Histological examination showed that poloxamer-HP β CD formulation allowed infiltration of mononuclear cells into urothelium and lamina propria. In studies using orthotopic mouse bladder cancer, the tumor loads in R-837-treated mice were significantly lower than those in vehicle-treated or non-treated mice.

Conclusion—The optimized poloxamer-HP β CD formulation of R-837 shows therapeutic potential for bladder cancer while avoiding adverse side-effects.

Keywords

bladder cancer; cyclodextrin; formulation; poloxamer; Toll-like receptor 7 agonist

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Conflict of interest: Roberto Maj and Alcide Barberis are employees of Telormedix. Lorenzo Leoni is a former employee of Telormedix.

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Introduction

Urinary bladder cancer is the fifth most common malignancy among men in Western society. The majority of bladder cancer cases are diagnosed as non-invasive, superficial tumors that are potentially curable by surgical and immune therapy.¹ Superficially growing tumors can be removed by transurethral resection but the recurrence rate is high. To prolong the tumor-free intervals after surgical resection, intravesical treatment with *Mycobacterium bovis* Bacillus-Calmette-Guerin (BCG), the vaccine strain against tuberculosis infection, is currently used as the initial treatment for high-grade superficial tumors (i.e. T1, carcinoma *in situ*, bulky Ta, or repeated recurrences).²

M. bovis BCG treatment induces non-specific local inflammation in the bladder accompanied by various proinflammatory cytokines (interleukin (IL)-2, IL-6, IL-8 and tumor necrosis factor α (TNF α))³ and chemokines, that in turn initiate infiltration of immune cells in the bladder urothelium.^{4,5} BCG instillation causes non-specific stimulation of the immune system, which induces local infiltration of the bladder wall by activated T cells derived by cell mediated immunity.⁶

R-837, a Toll-like receptor 7 (TLR7) agonist, is a potent immune modulator currently used as a first-line topical therapy for genital warts and superficial basal cell carcinomas.⁷⁻⁹ In addition, R-837 has been used for the treatment of malignant skin lesions including melanoma and basal cell carcinoma.⁹ R-837 induces proinflammatory cytokines and chemokines *in vitro* and *in vivo*¹⁰ that attract immune cells to the local site of administration.¹¹ Imidazoquinolines have been shown to direct cytotoxic effects to bladder cancer cells and induce them to secrete proinflammatory cytokines.¹² It is further described that imidazoquinolines have anti-tumor effects in orthotopic bladder cancer mouse models.¹² The objective of the present study was to optimize the formulation of R-837 to improve therapeutic application. The systemic and local inflammation induced by various formulations of R-837 was compared. The anticancer efficacy of R-837 in thermosensitive poloxamer polymer was evaluated in murine orthotopic bladder cancer models.

Methods

Mice

Six- to 8-week old female C57BL/6 mice were purchased from Charles River Laboratory (Wilmington, MA, USA). TLR7-deficient mice were a gift from S. Akira (Osaka University, Osaka, Japan) and backcrossed for 10 generations onto the C57BL/6 background mice. All mice were housed under standard conditions in the University of California, San Diego (UCSD) Animal Facility. All procedures and protocols received prior approval by the Institutional Review Board of UCSD.

Reagents

R-837 and Lutrol F127 (Fig. S1) were provided by Telormedix SA (Bioggio, Switzerland). Lactic acid was purchased from Fisher Scientific (Pittsburgh, PA, USA). 2-(hydroxypropyl)- β -cyclodextrin (HP β CD) was purchased from Sigma Aldrich (St Louis, MO, USA) (Fig. S1). R-837 was dissolved at the final concentration of 1% (w/v, 41.7 mM) in 0.1% lactic acid (lactic acid formulation). Lutrol F127 was added to 0.1 M lactic acid to make 20% (poloxamer formulation). 5% HP β CD was incorporated into 15% Lutrol F127, in 0.1 M lactic acid (poloxamer-HP β CD formulation). All solutions were filtered by 0.22 μ m filter before administration.

***In vivo* pharmacological study**

Mice were anesthetized with Ketamin-HCl (100 mg/kg) and xylazine hydrochloride (10 mg/mL) and catheterized using a 20 G Teflon intravenous catheter (Terumo Co. Somerset, NJ, USA). One hundred and fifty, 500 or 1500 nmoles R-837 in 50 or 100 μ L vehicles were intravesically administered, respectively. One hundred and twenty μ L volume was used for installation of 5000 nmoles. The R-837 solution was kept in the bladder for 20 min. In preliminary experiments, we compared serum cytokine levels at 2 h post-administration of 20-min and 60-min instillation periods using R-837 in lactic acid formulation. There was no significant difference in serum levels of TNF α and KC between the 20-min and 60-min instillation period. Therefore, a 20-min instillation time was used in the present study. At the various time points, serum and bladder lavage were collected. The bladder lavage was collected following administration of 100 μ L saline. The levels of cytokines were measured by Luminex microbead assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The minimum detection levels of TNF α , and KC were 5 pg/mL and 25 pg/mL, respectively. Total cytokines per bladder was calculated by instillation volume (0.1 mL) \times (concentration of TNF α or KC/mL lavage). The level of R-837 in serum was analyzed by Chimán SRL (Rottofreno, Italy), a contract laboratory. Briefly, R-837 and metabolites were extracted from serum and quantified using high performance liquid chromatography on a reversed phase C8 column with ultraviolet detection at 242 nm.

Histological examination

Mice were administered 100 μ L 0.1% R-837 in poloxamer-HP β CD formulation once, or 50 μ L three times at four-day intervals. At 24 h after the last administration, the bladders were collected. The fixed bladders were paraffin-embedded and stained with hematoxylin–eosin (HE) by UCSD Cancer Center Histology Core.

Implantation, treatment and assessment of tumor

C57BL/6 mice were anaesthetized and treated with 100 μ L of 0.1 mg/mL poly-L-lysine for 20 min to enhance tumor cell adhesion.^{13,14} After treatment with poly-L-lysine, 1×10^6 MB49 cells were implanted, as described previously.^{13–15} The treatment was performed on days 3, 6 and 9. MB49-implanted mice with no treatment or vehicle treatment served as controls. Mice were killed on day 11 and the weight of bladder was measured.¹⁴

Statistical analysis

A software package (Prism 4.0, GraphPad, San Diego CA, USA) was used for statistical analyses as indicated in the figure legends. The statistical differences were analyzed by unpaired Student-*t*-test to compare two groups and one-way ANOVA to compare multiple groups. In the one-way ANOVA, Dunnett's post hoc testing was used to compare the value to the control group and Bonferroni's post hoc testing was used to compare multiple groups to each other. A value of $P < 0.05$ was considered statistically significant.

Results

Intravesical administration of R-837 induced systemic inflammation

R-837 is known to be insoluble in water and sparingly soluble in common pharmaceutical solvents. Because acid solution increases the solubility of R-837,¹⁶ 0.1 M lactic acid was used to prepare 1% R-837 solution (~ 41.7 mM). Mice intravesically received 150, 500, 1500 and 5000 nmoles. Serum TNF α and KC were induced in a dose-dependent manner (0.1 M lactic acid in Fig. 1a,b). The levels of these factors in the bladder lavages were 10–100 times lower than the levels in their serum ($P < 0.001$ at 1500 and 5000 nmoles, Fig. 1c,d).

Addition of poloxamer polymer prevented absorption of R-837 and systemic induction of cytokines

Systemic induction of proinflammatory cytokines causes anorexia and fatigue, so called 'sickness syndrome' in hosts.¹⁷ To avoid systemic absorption as well as increase the local contact of R-837 to the urothelium, thermosensitive poloxamer polymer, Lutrol F127, was added to the formulation. The levels of TNF α (Fig. 2a,b) and KC (Fig. 2c,d) in both serum and bladder were significantly reduced in mice that received 5000 nmoles of R-837 in this formulation compared with the formulation with lactic acid.

To evaluate the effects of the poloxamer polymer on the systemic absorption of R-837 via bladder urothelium, the sera from mice that received 1500 nmoles R-837 were collected at 2, 4, 6, 24 and 48 h. In mice receiving R-837 either in lactic acid alone or in poloxamer formulation, maximum serum concentration of R-837 was observed 2 h after administration (Fig. 2e) ($P < 0.01$). The levels of serum R-837 increased in a dose-dependent manner (Fig. 2f). Addition of poloxamer polymer in the formulation reduced the serum levels of R-837 at 2 h time points ($P < 0.01$, Fig. 2e) and at 5000 nmoles ($P < 0.01$, Fig. 2f). These data indicate that inclusion of poloxamer in the formulation significantly reduces systemic absorption of R-837.

Incorporation of HP β CD restores systemic and local inflammation

To improve physical stability and achieve complete release from the poloxamer polymer, R-837 was incorporated with HP β CD to poloxamer in lactic acid (poloxamer-HP β CD formulation). The systemic levels of TNF α and KC were evaluated and compared with the other formulations (Fig. 3). Adding the poloxamer polymer reduced the serum KC levels to almost baseline levels, compared with lactic acid formulation ($P < 0.001$, Fig. 3a,b). Addition of HP β CD to the poloxamer formulation significantly restored the serum KC level (Fig. 3b), but not TNF α (Fig. 3a). A similar trend was seen in local levels of KC (Fig. 3c).

Evaluation of local inflammation in the bladder by intravesical administration of R-837 in poloxamer-HP β CD formulation

To evaluate local inflammation in the bladder, mice received intravesically 0.1% R-837 in poloxamer-HP β CD formulation as vehicle. Influx of inflammatory cells in the bladder was evaluated by histological examination (Fig. 4). Because patients received repeated intravesical treatments in the clinical application, we tested the effect of repeated administration of R-837 in poloxamer-HP β CD formulation on days 0, 4 and 8. After the first instillation of R-837, the cell infiltration was initiated (Fig. 4c) compared with the vehicle alone (Fig. 4a). After the third treatment, substantial infiltration of mononuclear cells in the lamina propria in the bladder treated with R-837 was observed (Fig. 4d), while few cells infiltrated in the vehicle- or saline-treated bladders (Fig. 4b,e). Reduced cell infiltration was observed in the bladder of TLR7 deficient mice treated with R-837 in poloxamer-HP β CD formulation similar to saline-treated mice, indicating that inflammation induced by R-837 in the bladder was TLR7 dependent (Fig. 4f).

Evaluation of inhibition of local tumor growth of R-837 in poloxamer-HP β CD formulation in mouse orthotopic bladder cancer models

Orthotopic bladder tumor models were generated using MB49, a cell line derived from transitional cell carcinoma of the murine urinary tracts. Mice bearing the MB49 bladder tumor were treated three times (days 3, 6 and 9) with 50 μ L of 0.1% R-837 in poloxamer-HP β CD formulation as vehicle. The treatment group showed significantly lower average tumor loads compared with the non-treated or vehicle-treated group ($P < 0.01$, Fig. 5). Thus,

treatment with R-837 in poloxamer-HP β CD formulation maintained the bladder weight to nearly that of non-tumor bearing (naïve) mice.

Discussion

R-837, a specific TLR7 agonist, is currently approved for treatment of skin disorders such as viral warts and superficial basal cell carcinomas.^{7,8} R-837 directly inhibits tumor growth and has potent therapeutic activity in superficial bladder cancer.¹⁸ In the present study, thermosensitive poloxamer polymer was used to ensure prolonged local contact and minimize systemic absorption of R-837. Polymer formulation reduced systemic absorption of R-837 from bladder urothelium with enhanced local infiltration of immune cells. Incorporating HP β CD in the formulation improved the physical stability, achieving a clear homogeneous solution. This formulation improved the induction of local chemokine and showed anti-tumor effects in an orthotopic mouse model of bladder cancer.

Intravesical BCG administration is a well established immune therapy of superficial bladder cancer.¹⁹ Although live BCG bacilli provides a significant advantage to evoke the immune response, use of live BCG requires careful biohazard precaution for health care staff and patients.²⁰ BCG remains only partially effective and serious side-effects, including high fever, pneumonia, hepatitis and sepsis, might occur. Efforts to develop safer and more effective therapy for bladder cancer continue. Among those, attempts to use the individual TLR agonist to treat bladder cancer have been reported.^{12,14,21} Repeated application of the TLR9 agonist improved survival and reduced tumor loads.²¹ TLR7 agonist R-837 directly affects human and mouse bladder cancer cells to induce chemokine secretion, and to induce apoptosis and reduce tumor growth.¹² We showed in the present study that R-837 in poloxamer-HP β CD formulation could initiate a substantial local innate immune reaction.

Because acid formulation increased solubility of R-837, we initially tested R-837 dissolved in 0.1 M lactic acid that is commonly used in the pharmaceutical industry. R-837 in lactic acid formulation was absorbed systemically and caused significant induction of systemic inflammation. Live BCG bacilli adhere to the surface of the bladder wall,^{22,23} and provide sustained immune stimulation to urothelium and local immune cells for prolonged periods. Thermosensitive poloxamer polymer reduces the drug release and holds the drug concentration on the surface of the cells, and prevents systemic absorption of the drug.²⁴ To recapitulate the advantageous pro-inflammatory properties of live BCG bacilli infection and to reduce the systemic absorption, as well as increase the surface contact of the drug, thermosensitive poloxamer polymer was added to the lactic acid formulation. The poloxamer formulation significantly reduced the systemic absorption of R-837 through the bladder surface and reduced the systemic cytokine induction to baseline levels.

The component of the present study that was of particular interest was that the formulation including HP β CD restored the induction of KC by R-837 compared with that of poloxamer alone. Complexation using HP β CD is a common approach for increasing drug solubility and stability in aqueous media.^{25,26} Moreover, inclusion of HP β CD in polymers is used in vaginal delivery systems.²⁷ Because 20% thermosensitive poloxamer was observed to solidify in the bladder within a short time, causing obstruction of the urethra, the concentration of poloxamer was reduced to 15%. Specifically, incorporating HP β CD in the polymer in an orthotopic mouse model of bladder cancer.

Effective anti-tumor immune therapy requires appropriate recruitment of immune cells.^{5,28} Histological examination shows substantial immune cell infiltration into the submucosal space (i.e. the lamina propria) by R-837, similar to that found following intravesical administration of TLR9 agonists.¹⁴ Cell infiltration in the bladder induced by R-837

diminished in TLR7-deficient mice, indicating the inflammation was TLR7 dependent, not caused by vehicle or mechanical injury. The integrity of the urothelium was well maintained after repeated administration of vehicle alone (poloxamer-HP β CD formulation).^{10,29}

In summary, our results suggest the formulation of poloxamer and HP β CD of drugs with low solubility might exert favorable properties, such as a slow release profile and longer surface contact, while avoiding possible systemic adverse effects. Optimized poloxamer-HP β CD formulations might increase the maximum tolerated dose of R-837 and improve patient compliance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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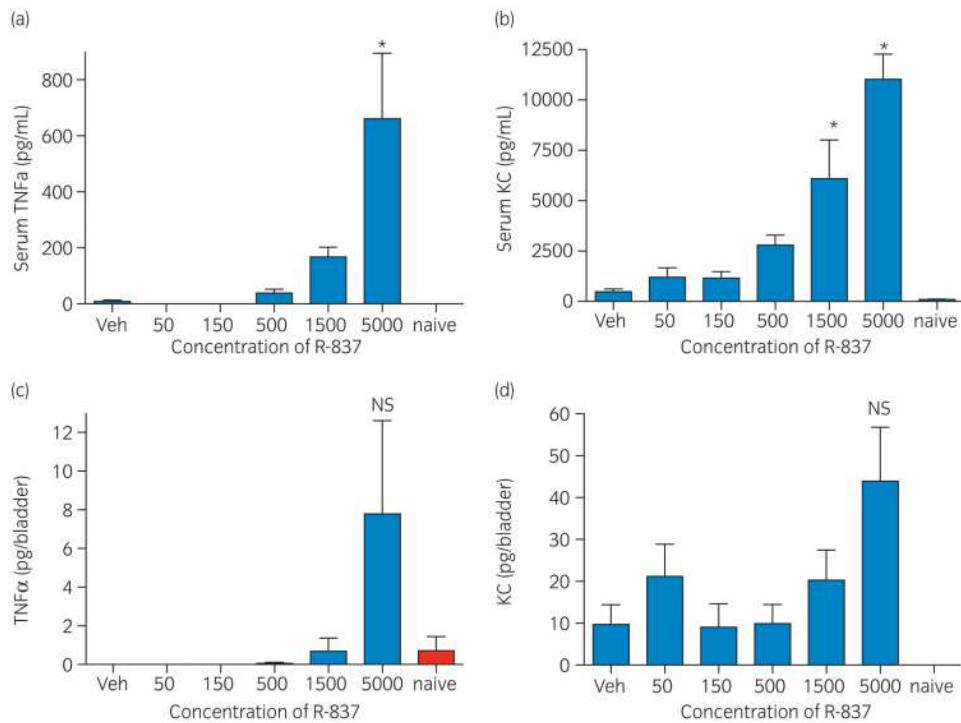
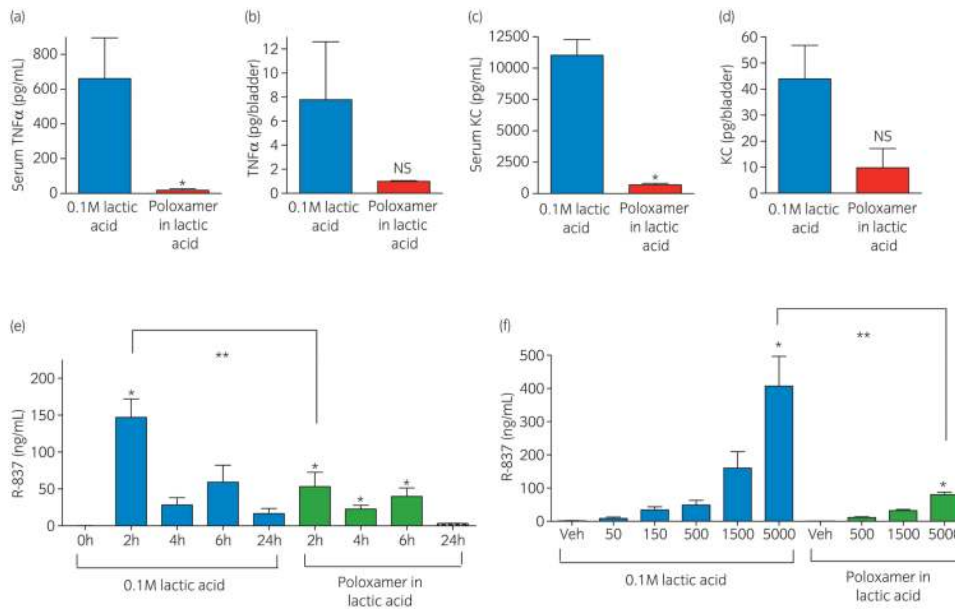


Fig. 1. Cytokine and chemokine induction in serum and bladder lavage by intravesical administration of R-837 in 0.1% lactic acid or poloxamer in 0.1M lactic acid. Mice ($n = 8$) intravesically received various doses of R-837 in lactic acid formulation. Two hours after administration, sera and bladder lavage were collected. The levels of tumor necrosis factor α (TNF α) (a) and keratinocyte-derived chemokine (KC) (b) in serum, and TNF α (c) and KC (d) in the bladder lavage were measured. *Statistically significant. NS, not significant; Veh, vehicle.

**Fig. 2.**

Pharmacokinetics of R-837 in mice after intravesical administration. (a–d) Mice ($n = 8$) were administered 5000 nmoles R-837 in lactic acid or poloxamer formulation. The levels of serum tumor necrosis factor α (TNF α) (a), TNF α in bladder lavage (b), serum keratinocyte-derived chemokine (KC) (c), and KC in bladder lavage (d) were measured. *Statistically significant. NS, not significant. (e) Mice ($n = 6-8$) were administered 1500 nmoles R-837 in lactic acid or poloxamer formulation. Sera were collected 2, 4, 6 and 24 h after administration. (f) Mice ($n = 6$) were administered various doses of R-837 in 100 μ L in lactic acid or poloxamer formulations. Sera were collected 2-4 h after administration. The levels of R-837 were determined by Chimian SRL. Data shown are pooled values from two independent experiments (mean \pm SEM.). *, **Statistically significant. NS, not significant compared with treated mice at 0 h time point or mice treated with vehicle (Veh) alone.

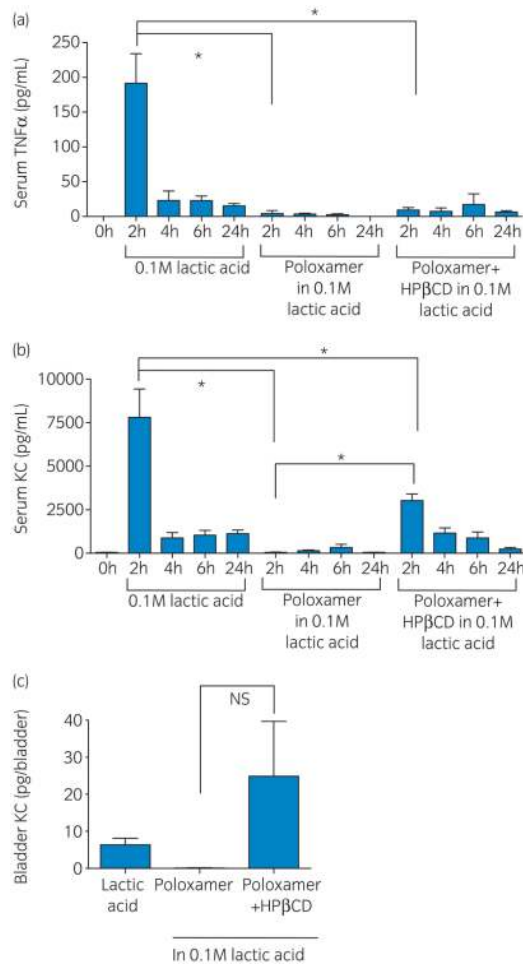


Fig. 3. Incorporation of 2-(hydroxypropyl)- β -cyclodextrin (HP β CD) partially restores systemic tumor necrosis factor α (TNF α) and keratinocyte-derived chemokine (KC) levels. Mice ($n = 8$) were administered 1500 nmoles R-837 in lactic acid, poloxamer or poloxamer-HP β CD formulation. The levels of TNF α (a) and KC (b) are shown. Data presented are pooled values from two independent experiments (mean \pm SEM). *Statistically significant. (c) C57BL/6 mice were injected with 5000 nmoles R-837 in lactic acid, poloxamer or poloxamer-HP β CD formulations. The levels of KC in the lavage were assessed by Luminex beads assay. NS, not significant ($P > 0.05$).

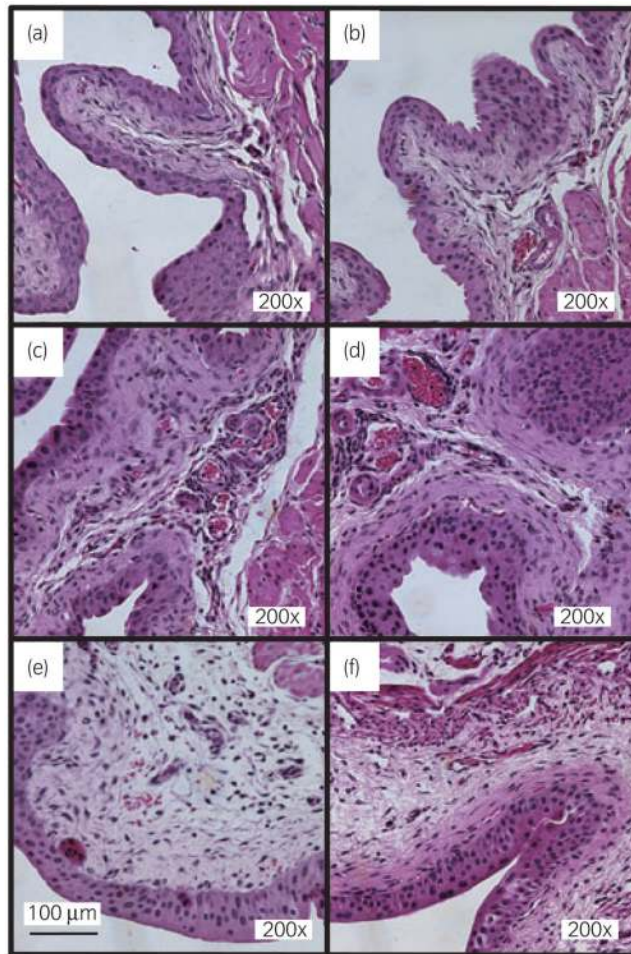


Fig. 4. Representative histology of murine bladder treated with 0.1% R-837 in poloxamer-2-(hydroxypropyl)- β -cyclodextrin (HP β CD) formulation. C57BL/6 (a–e) or Toll-like receptor 7 (TLR7) deficient mice (ko) (f) were intravesically treated with 0.1% R-837 in poloxamer-HP β CD formulation on day 0 (single treatment), 50 μ L on days 0, 4 and 8 (three times treatment). The bladders were collected on day 1 for single treatment (a and c) or on day 9 for three times treatment (b, d, e and f) and stained with HE. Mice treated with vehicle (Veh) alone and naïve mice served as controls (b and e). (a) Single treatment Veh (wild type C57BL/6). (b) Three times treatment Veh (wild type C57BL/6). (c) Single treatment R-837 (wild type C57BL/6). (d) Three times treatment R-837 (wild type C57BL/6). (e) Three times treatment saline (wild type C57BL/6). (f) Three times treatment R-837 (TLR7 ko). Scale bar, 100 μ m. Original magnification, \times 200.

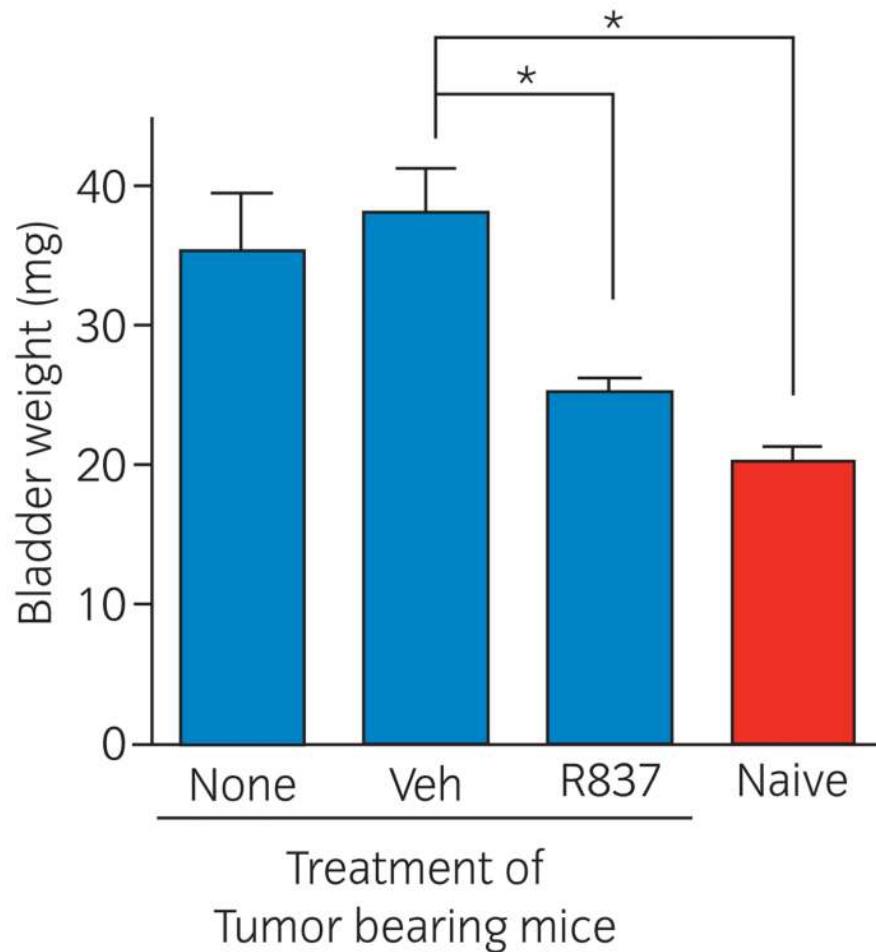


Fig. 5. Reduced bladder weight of mice bearing MB49 treated 0.1% R-837 in poloxamer-2-(hydroxypropyl)- β -cyclodextrin (HP β CD) formulation. Mice ($n = 11$) bearing MB49 bladder tumor intravesically received 50 μ L 0.1% R-837 in poloxamer-HP β CD formulation on days 3, 6 and 9. Mice were killed on day 11 and the bladder weight was measured. Naive mice without tumor implantation (naïve), MB49 implanted-mice with no treatment (None) or vehicle (Veh) treated mice served as controls. *Statistically significant.