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EFFECTS OF THE COMPOUNDS MV8608 AND MV8612 OBTAINED FROM *Mandevilla velutina* IN THE MODEL OF HEMORRHAGIC CYSTITIS INDUCED BY CYCLOPHOSPHAMIDE IN RATS

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Abstract

Hemorrhagic cystitis (HC) is a common side effect observed in patients under chemotherapy with cyclophosphamide (CYP). The urotoxic side effects of CYP are attributed to the metabolic compound acrolein, and can be partially prevented by the uroproctector agent 2-mercaptoethene sulfate (Mesna). The present study analyzed the anti-inflammatory and the antinociceptive effects of compounds MV8608 and MV8612 obtained from *Mandevilla velutina* in the rat model of CYP-induced HC. Male Wistar rats were used (6 to 8 per group, 220-250g). Hemorrhagic cystitis was induced by a single administration of CYP (100 mg/kg, ip). Three behavioral parameters, breathing rate, closing of the eyes, and specific posture were used as nociception indexes, and scored at different time intervals (15-180 min) after cystitis induction. As inflammatory parameters, hemorrhage presence, edema formation, and bladder weight were determined at 24 h after CYP administration. The neutrophil migration was assessed by means of myeloperoxidase (MPO activity), 4 h after cystitis induction. As expected, Mesna treatment was able to reduce in a significant manner the all the inflammatory and the nociceptive parameters induced by CYP. Of note, the administration of MV8608 significantly inhibited the hemorrhage formation and the neutrophil recruitment, while the MV8612 treatment markedly reduced the bladder weight, without interfering with neutrophil influx. Interestingly, the treatment with either MV8608 or MV8612 markedly reduced the nociceptive responses. The present results clearly indicate that MV8608 and MV8612 might represent important alternatives to prevent side effects, especially the nociception, following chemotherapy with CYP.

Keywords: cystitis, cyclophosphamide, *Mandevilla velutina*, compounds MV8608 and MV8612.

Introduction

Cyclophosphamide (CYP) is an oxazaphosphorine alkylating agent, widely used alone or in combination with other chemotherapeutic drugs for the treatment of various solid tumors and B cell malignant diseases. It is also employed in certain non-neoplasic conditions, such as nephrotic syndrome, rheumatoid arthritis and systemic lupus erythemathosus and for conditioning before bone marrow transplantation (Chow et al. 2006; Levine and Jarrard 1993; Matsuoka et al. 2007; Ozcan et al. 2005; Safron et al. 1997). Hemorrhagic cystitis is a well known complication that limits the clinical use of CYP (Assreuy et al. 1999; Chow et al. 2006; Chow et al. 2007; Kranc et al. 1992; Linares-Fernández and Alfieri 2007; Sakura et al. 2008). The incidence of this side effect ranges from 2 to 40 %, and might reach 68 % in patients undergoing bone marrow transplantation (Chow et al. 2006; Levine and Jarrard 1993), with a mortality rate of 4 % (Chow et al. 2007). The main features of hemorrhagic cystitis are urothelial damage, edema, necrosis, ulceration, hemorrhage, neovascularization, and leukocyte infiltration (Assreuy et al. 1999), and rarely transitional cell carcinoma of the bladder (Assreuy et al. 1999; Chow et al. 2006; Wong et al. 2000). Noteworthy, the damage caused by CYP can also affect other organs, in addition to the bladder (Wong et al. 2000).

The urotoxicity of CYP is likely associated to the renal excretion of 4-hidroxy metabolites, especially acrolein, which is formed from hepatic microsomal enzymatic hidroxylation (Chow et al. 2006; Korkmaz et al. 2001; Korkmaz et al. 2005; Kranc et al. 1992). Since acrolein was recognized as the toxic agent of hemorrhagic cystitis, it became clear that prevention could be possible. Currently, vigorous diuresis and continuous bladder irrigation are used to decrease the incidence of hemorrhagic cystitis (Crocitto et al. 1996). In addition, some agents, which detoxify cyclophosphamide and its metabolites, such as 2-mercaptoethene sulfate (Mesna), can be used to prevent the incidence of hemorrhagic cystitis (Crocitto et al. 1996). Nevertheless, in some cases, these therapeutic measures are only

partially effective. Due to that, other methods have been used for the prevention and/or treatment of CYP-induced hemorrhagic cystitis (HC), including the oral administration of disulfiram and N-acetylcysteine, hyperbaric oxygen, superhydratation, or intravesical instillation of saline, formalin, phenol, silver nitrate, prostaglandin E_1, E_2, and F_{2alpha} (Chow et al. 2006). More aggressive procedures involve hypogastric artery embolization, cystectomy with ileal conduit, and bladder augmentation (Chow et al. 2006).

Mandevilla velutina (Apocynaceae) is a native plant to the Brazilian Savanna, and is frequently prescribed by folk medicine in the form of infusion or alcoholic extract of its rhizome as an anti-inflammatory preparation and for the treatment of venomous snake bites (Mattos et al. 2006a; Mattos et al. 2006b; Santos et al. 2003). The non-peptidic compounds MV8608 and MV8612 have been isolated from the rhizome of *M. velutina*. The compound MV8612 has been characterized as an steroidal glycoside (Figure 1A), whilst the compound MV8608 (also known as *velutinol A*) is an aglycone steroid (Figure 1B) (Mattos et al. 2006a). Previous studies have demonstrated that these compounds are able to reduce inflammatory and nociceptive responses in several animal models (Calixto et al. 1991; Santos et al. 2003).

In this context, the present study aimed to evaluate the effects of MV8608 and MV8612 pregnane compounds on the nociceptive and inflammatory events in the rat model of hemorrhagic cystitis induced by CYP, in comparison to the reference compound Mesna.

Materials and methods

Animals

Non-fasted male Wistar rats (6 to 8 per group, 220-250g) were used in this study. The animals were housed in groups of five and maintained in controlled temperature (22 ± 2 ºC) and humidity (60 - 70 %), under a 12 h light-dark cycle. Food and water were available *ad libitum*. Animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. All the tests were performed between 8:00 AM and 6:00 PM. The studies reported in this manuscript followed the "Principles of Laboratory Animal Care" from NIH publication No. 85-23 and ethical guidelines for investigation of experimental pain in conscious animals. The Institutional Ethics Committee approved all the experimental procedures. The number of animals and intensity of noxious stimuli were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Drugs and reagents

The following drugs and reagents were used: Cyclophosphamide, Mesna (Mitexan, Baxter Oncology Gmbh, Frankfurt, Germany); hexadecyltrimethyl ammonium bromide, tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO); NaPO4, hydrogen peroxide, NaCl, Tween 80, and Tween 20 (all from Merck, Haar, Germany). The compounds MV8612 and MV8608 were isolated from the rhizomes from *M. velutina*, as described previously (Calixto et al. 1985). The grade of purity of the compounds was > 95 %. All the dilutions were made in NaCl 0.9 % solution (saline), except the compounds MV8608 and MV8612, which were prepared in a 1 % Tween 80 plus 1% absolute ethanol diluted in saline. The doses of compounds MV8608 and MV8612 or Mesna were chosen on the basis of the literature (Korkmaz et al. 2001; Mattos et al. 2006a; Santos et al. 2003). Separate groups of animals received the same volume of vehicle and were used as control.

Treatments

Hemorrhagic cystitis was induced by a single administration of CYP (100 mg/kg, ip). The treatment with the Mesna (21.5 mg/kg), or with the compounds MV8608 (10 mg/kg) and MV8612 (11.8 mg/kg) was performed in three doses; the first one was given 30 min prior CYP, and the following two doses were given 4 and 8 h after the administration of CYP, except in the experiments for assessing nociception or MPO activity, in which the compounds were administered as a single ip dose, 30 min prior to CYP.

Nociceptive response

The design used in the present study was similar to that described by Boucher (2000), with minor modifications. The rats were placed individually in observation boxes and were acclimatized for a 30 min period prior to behavioral testing. Experiments were always performed between 8:00 AM and 12:00 AM to minimize the potential 24 h variations in the behavioral responses. The single ip administration of CYP (100 mg/kg) produced changes in three behavioral parameters: breathing rate, opening of the eyes, and posture, reflecting the nociceptive alterations associated to hemorrhagic cystitis. Therefore, these parameters were adopted as nociceptive indexes and scored every 15 min, for 180 min after administration of CYP. All the experiments were performed blind, each experimenter scoring two rats in parallel. For the breathing rate, every 10 cycles per min diminution was scored as 1, with control values of about 140 cycles per min. For the opening or closing of the eyes, the following scores were assumed: 0 for complete opening, corresponding to normal eyes; 10 for complete closing; 5 for half-closed eyes; and 2 and 7 for the two intermediate positions (between open and half-closed and between half-closed and closed, respectively). Finally, regarding the posture changes, when either the rounded-back with the whole body aligned or

complete limpness were observed, the score was 10. When no specific posture was seen over the 15-min observation period, the score was 0.

Gross evaluation and wet weight determination

The gross evaluation was based on criteria established by Gray (1986). All bladders were dissected free from connecting tissues and transected at the bladder neck. Wet weight of each bladder was recorded, and expressed as mg per 100 g of animal. Each bladder was additionally examined macroscopically for edema formation, which was categorized as: 3 = severe, 2 = moderate, 1 = mild or 0 = none . Edema was considered severe when fluid was seen externally in the walls of the bladder, as well as internally. When the edema was confined to the internal mucosa it was reported as moderate; when it was between normal and moderate, the edema was defined as mild. Upon examination, the bladders were also surveyed for bleeding in the walls and categorized into four designations: 3 = intravesical clots, 2 = mucosal hematomas, 1 = telangiectasia or dilatation of the bladder vessels and 0 = normal.

Histological evaluation

Following the gross evaluation, the bladders were fixed in buffered formalin solution (10 %) for 24 h. After this period, the samples were embedded in paraffin, and stained with hematoxylin and eosin. A pathologist who was blinded to the treatment reviewed each specimen, considering the following parameters, as proposed by Gray et al. (1986), with some modifications: normal (normal epithelium, no inflammatory cell infiltrate or ulcers); mild (diminished epithelial cells, flattening with submucosal edema, mild hemorrhage, few ulcerations); moderate (mucosal erosion, inflammatory cell infiltrate, fibrin deposition, hemorrhage, and multiple ulcerations); severe (mucosal erosion, inflammatory cell infiltrate, fibrin deposition, multiple ulcerations, and transmural hemorrhage with severe edema).

Neutrophil myeloperoxidase assay

Neutrophil recruitment to the rat bladder was measured by means of tissue MPO activity, according to the method described before (Passos et al. 2004).The bladder tissues were removed at different time-points (2, 4, 6, 8, 12 and 24 h after) CYP injection. Then, samples were homogenized at 5 % (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 5000 g for 20 min at 4°C. The pellet was resuspended in 5 % (w/v) hexadecyltrimethyl ammonium bromide buffer (pH 5.4) and the samples were frozen. Upon thawing, the samples were re-centrifuged and 25 µl of the supernatant were used for MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM NaPO4, and 0.3 mM hydrogen peroxide. The absorbance was measured at 595 nm, and the results are expressed as OD per milligram (mg) of tissue. As the increase in MPO activity peaked 4 h following CYP administration, this time interval was adopted for most experiments.

Statistical Analysis

All the results are presented as the mean ± standard error mean of 6 to 8 animals per group. For the nociception evaluation, the inhibition indexes were determined based on the area under the curve. The statistical comparison of the data was performed by one-way Analysis of Variance (ANOVA), followed by Bonferroni's post-hoc test. *P*-values smaller than 0.05 ($P < 0.05$) were considered significant.

Results

CYP-induced nociceptive behavior

As described previously (Boucher et al. 2000), the ip injection of CYP (100 mg/kg) was able to induce marked modifications in the behavior of freely moving conscious rats (Figure 2), according to assessment of breathing rate, closing of the eyes and occurrence of specific postures. Data on Figure 2A show that the reference compound Mesna (21.5 mg/kg, ip, 30 min prior to CYP administration) produced a partial, but significant reduction of CYP-induced nociceptive behavior, with an inhibition percentage of 24 ± 7 %. Interestingly, the pretreatment of animals with either compound MV8608 (10 mg/kg ip, 30 min prior to CYP) or MV8612 (11.8 mg/kg, ip. 30 min beforehand) resulted in a marked inhibition of nociceptive responses evoked by CYP (Figures 2A and 2C). The percentages of inhibition obtained for MV8608 and MV8612 were 67 ± 1 % and 58 ± 2 %, respectively.

Gross Evaluation

Confirming the literature data (Gray et al. 1986), the damage score of edema and hemorrhage in the CYP group was significantly higher than the score of 0 in the saline group (Figure 3). Treatment of rats with Mesna (21.5 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection) caused a significant inhibition of hemorrhage (50 ± 9 %) and edema (50 ± 16 %) induced by this chemotherapeutic agent (Figure 3 A and B). Interestingly, the administration of MV8608 (10 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection) was able to inhibit only the hemorrhage formation (58 ± 8 %) (Figure 3 A), while the MV8612 treatment (11.82 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection) did not display any significant effect on edema and hemorrhage scores (Figure 3).

Wet Bladder Weight

The edema induced by the ip injection of CYP was also assessed by determining the mean empty bladder wet weight of the control group per 100 g of body weight, 24 h after CYP administration. Data depicted in Figure 4 A show that treatment with Mesna (21.5 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection) produced a significant lessening of the wet bladder weight, with a reduction percentage of 68 ± 7 %. Likewise, a significant reduction of CYP-induced increase in wet bladder weight was also observed following the treatment of animals with MV8612 (11.8 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), with an inhibition percentage of 38 ± 15% (Figure 4 A). On the other hand, MV8608 (10 mg/kg), given at the same schedule of treatment, failed to significantly affect this parameter.

Neutrophil myeloperoxidase assay

The cystitis caused by CYP was also characterized by an increase in MPO activity in the bladder compared with that in the saline group ($P < 0.01$). Treatment with Mesna (21.5 mg/kg, ip, 30 min prior to CYP administration) resulted in a significant inhibition of MPO activity in the bladder of CYP-treated rats (68 ± 8%). Surprisingly, the treatment with MV8608 (10 mg/kg ip, 30 min prior to CYP) significantly reduced the MPO activity with reduction percentage of 45 ± 11%, while the MV8612 treatment (11.8 mg/kg, ip, 30 min beforehand) did not display any significant effect on MPO activity (Figure 4 B).

Histological Evaluation

Histological analysis demonstrated that treatment with Mesna, MV8608 and MV8612 produced a diminishment of HC characteristics. A brief description of the main histological findings is presented bellow.

In the CYP group, there were marked changes in all layers of the bladder. In this group, epithelium was thin and sometimes denuded. The submucosa was enlarged, presented severe hemorrhagic cystitis with edema, transmural hemorrhage and inflammation. In the Mesna Group, the animals displayed characteristics of normality. The epithelium was found to be thick with normal size nuclei and with mild abnormal attenuation. The submucosa presented mild edema and no inflammation. The muscularis had a normal appearance. The animals treated with MV8608 and MV8612 presented moderate mucosal ulceration, submucosal mild edema and hemorrhage, and the muscularis had normal appearance. The representative images of histological analysis are shown in Figure 5 (A-E).

Discussion

Hemorrhagic cystitis is a well known adverse effect observed in some patients under treatment with the oxazaphosphorine alkylant agent CYP. This undesirable side effect is likely the major reason that limits the clinical use of CYP. It has been demonstrated that acrolein formed from hepatic microsomal enzymatic hidroxylation of CYP is the metabolite responsible for the urotoxicity caused by CYP (for review see: Korkmaz et al., 2007). In this regard, one of the most important features of acrolein is the ability to activate intracellular reactive oxygen species (ROS) and nitric oxide production. It leads to depletion of cellular thiols and gene activation, either directly or subsequently to the activation of transcription factors, such as nuclear factor-κB (NF-κB) an activator protein-1 (AP-1). The stimulation of NF-κB and AP-1 drives the expression of cytokines (such as TNF-α and IL-1β) and inducible nitric oxide synthase (iNOS), resulting in additional ROS production. As a final consequence, DNA damage and cell necrosis take place and HC is manifested by urothelial damage, pain, edema, necrosis, ulceration, hemorrhage, neovascularization, and leukocyte infiltration (Assreuy et al. 1999; Korkmaz et al. 2007). Various methods for the prevention and treatment of CYP-induced HC have been employed, with variable results. The prophylactic administration of Mesna has been demonstrated to be a useful measure for preventing HC, although it is not efficient for treating established lesions in the bladder.

Both extract from *M. velutina* and its active compounds have been previously reported to exhibit systemically anti-inflammatory and antinociceptive actions, mainly due their ability to interfere with kinin actions (Calixto et al. 1985). Therefore, the present study evaluated the effects of two plant-derived compounds MV8608 and MV8612 derived from *M. velutina*, on nociceptive and inflammatory parameters in the model of HC induced by CYP in rats, in comparison the reference drug Mesna.

Data provided in the present study show that pretreatment with Mesna consistently reduced the edema, the hemorrhage and the increased bladder weight, observed in the HC model induced by CYP. Our results confirm previous literature data supplied on the protective anti-inflammatory effects of Mesna (Freedman et al. 1984; Morais et al. 1999). Mesna represents a thiol compound, which entered clinical trials as a systemic uroprotective agent in the late 70s (Freedman et al. 1984; Morais et al. 1999). The systemic administration of Mesna results in regional detoxification of the urinary system, as the interaction between acrolein and Mesna results in the formation of an inactive compound, which possibly contributes to its anti-inflammatory effects (Crocitto et al. 1996; Morais et al. 1999). In previous experiments, in which we used only one dose of compounds (data not shown), either MV8608 or MV8612 failed to significantly prevent the inflammatory studied parameters. This lack of effect was probably due to pharmacokinetic factors. Therefore, we decided to investigate the effects of compounds when given at the same schedules of treatment used for Mesna. In that condition, we observed that MV8608 significantly inhibited the formation of hemorrhage, whereas the MV8612 markedly reduced the increase in wet weight of bladder, denoting that the lack of anti-inflammatory effects in early experiments were due to the different schedules of treatment. Afterward, we have also assessed the bladder MPO activity as an indirect indicative of neutrophil migration (Masuda et al. 2006). The enhancement of MPO activity following CYP administration in rats was demonstrated beforehand (Linares-Fernández and Alfieri 2007). Our data showed that this parameter was significantly reduced by treating animals with the reference compound Mesna. Furthermore, increased MPO activity induced by CYP injection was markedly prevented by treatment with compound MV8608, whereas MV8612 failed to significantly alter this response.

The expression of the immediate early gene-encoded proteins c-Fos and Krox-24 observed in different spinal and hindbrain structures (Lantéri-Minet et al. 1995) reflects the

nociceptive process resulting from the CYP-induced HC. In fact, painful symptoms associated to HC represent serious limitation factors for the quality of life of patients under treatment with CYP (Morais et al. 1999). In this context, previous studies have demonstrated that MV8608 and MV8612 displayed prominent antinociceptive effects in different animal models of spontaneous nociception (Correa and Calixto, 1993). Therefore, we decided to examine the ability of the compounds MV8608 and MV8612 obtained from *M. velutina* on the pain-like behavior elicited by CYP. As described previously (Boucher et al. 2000), the ip injection of CYP (100 mg/kg) was found able to induce marked behavioral modifications of freely moving conscious rats, which are mainly manifested by decreased breathing rate, closing of the eyes and occurrence of specific postures. The results of the present study showed that both compounds MV8608 and MV8612 produced a striking reduction of the nociception induced by CYP, as demonstrated by a general reduction of the three evaluated nociceptive parameters. Noteworthy, the antinociceptive actions reported herein for compounds MV8608 and MV8612 do not appear to involve unspecific central effects, as both compounds failed to significantly alter the locomotor activity or the motor coordination of animals (data not shown). Conversely, the administration of the reference uroprotective agent Mesna produced only a partial reduction of the nociceptive responses evoked by CYP in rats. Although, the anti-inflammatory effects of Mesna were generally greater that those seen for *M. velutina*-derived compounds, the compelling anti-nociceptive effects of MV8608 and MV8612 might justify their potential therapeutic application for controlling CYP-induced visceral pain.

Previous literature data have demonstrated that MV8612 produces a pronounced systemic, spinal and supraspinal antinociception in bradykinin, formalin, and capsaicin nociception models in mice, by mechanisms likely related to the activation of small and large conductance calcium-activated K^+ channels and an interaction with G_i/G_o pertussis toxin-sensitive G proteins (Santos et al. 2003). Furthermore, it has been demonstrated that

compound MV8608 (*velutinol A*) is able to block the inflammatory and nociceptive responses mediated by the activation of the inducible kinin B_1 receptors in rodents (Mattos et al. 2006a). Finally, some additional pieces of evidence have suggested that MV8612 and MV8608 hold selective effects on kinin-mediated responses (Henriques et al. 1991; Calixto et al. 1998; Mattos et al. 2006a), and they are able to inhibit phospholipase A2-mediated inflammation (Neves et al. 1993). Therefore, it is possible to suggest that one or more of the aforementioned mechanisms might be responsible for the antinociceptive and anti-inflammatory effects demonstrated for MV8608 and MV8612 in the HC model induced by CYP.

The present study clearly shows that pregnane compounds isolated from the rhizome of *M. velutina* MV8608 and MV8612, display marked antinociceptive actions in the rat model of HC induced by CYP, accompanied by partial anti-inflammatory effects. Considering the literature evidence indicating some collateral effects in patients under treatment with Mesna, such as hypersensivity-like reactions, fever, nausea and vomiting (Khaw et al. 2007), new promising alternatives to control HC induced by CYP are of high interest. In addition, taking into account the expressive antinociceptive effects displayed by MV8608 and MV8612, it is tempting to suggest that a combination of these compounds and Mesna might furnish favorable results in the bladder damage and visceral pain in CYP-induced HC.

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Legends to the Figures

Figure 1. Chemical structures of MV8608 (A) and MV8612 (B).

Figure 2. (A) Effect of treatment with Mesna (21.5 mg/kg, ip, 30 min before CYP injection), MV8608 (10 mg/kg, ip, 30 min prior to CYP), or MV8612 (11.8 mg/kg, ip, 30 min prior to CYP) on the nociceptive responses in CYP-induced HC. (B) Area under curve of the nociceptive reponses in CYP-induced HC. Each point on the curve represents the mean of 6-8 animals and the vertical lines show the S.E.M. **P<0.01; ***P<0.001 denote the significance levels in comparison to control values; ###P<0.001 denote the significance levels in comparison to saline values.

Figure 3. Effect of treatment with Mesna (21.5 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), MV8608 (10 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), or MV8612 (11.8 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection) on macroscopic hemorrhage (A) and edema (B) evaluation in CYP-induced HC (24 h). Each column represents the mean of 6-8 animals and the vertical lines show the S.E.M. *P<0.05; **P<0.01 denote the significance levels in comparison to control values; ###P<0.001 denote the significance levels in comparison to saline values.

Figure 4. (A) Effect of treatment with Mesna (21.5 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), MV8608 (10 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), or MV8612 (11.8 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection) on weight bladder in CYP-induced HC (24 h). (B) Effect of treatment with

Mesna (21.5 mg/kg, ip, 0.5 h before CYP injection), MV8608 (10 mg/kg, ip, 30 min prior to CYP), or MV8612 (11.8 mg/kg, ip, 30 min prior to CYP) on myeloperoxidase activity in CYP-induced HC (4 h). Each column represents the mean of 6-8 animals and the vertical lines show the S.E.M. *P<0.05; **P<0.01; ***P<0.001 denote the significance levels in comparison to control values; ###P<0.001 denote the significance levels in comparison to saline values.

Figure 5. Histological analysis of representative bladder walls in cross-sections. (A) **Saline:** bladder with normal appearance, with absence of edema, hemorrhage, or inflammation. (B) **CYP:** severe hemorrhagic cystitis with edema, transmural hemorrhage, mucosal ulceration, and inflammation. (C) **Mesna:** near normal appearance. (D) **MV8608:** mild submucosal edema, minimal hemorrhage, few ulcerations. (E) **MV8612:** mild submucosal edema, hemorrhage and erosions. All panels, hematoxylin-eosin stain. Original magnification x 10 in all panels.

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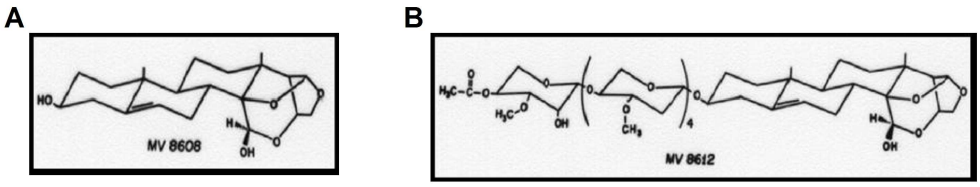

Chemical structures of MV8608 (A) and MV8612 (B).
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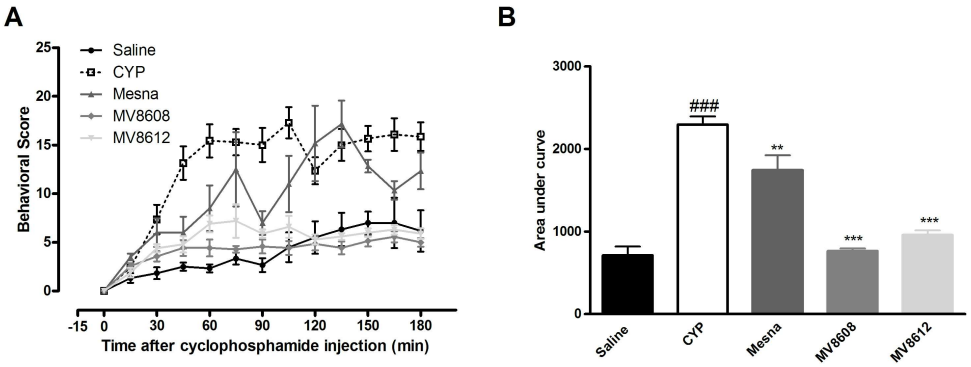

(A) Effect of treatment with Mesna (21.5 mg/kg, ip, 30 min before CYP injection), MV8608 (10 mg/kg, ip, 30 min prior to CYP), or MV8612 (11.8 mg/kg, ip, 30 min prior to CYP) on the nociceptive responses in CYP-induced HC. (B) Area under curve of the nociceptive reponses in CYP-induced HC. Each point on the curve represents the mean of 6-8 animals and the vertical lines show the S.E.M. **P<0.01; ***P<0.001 denote the significance levels in comparison to control values; ###P<0.001 denote the significance levels in comparison to saline values.
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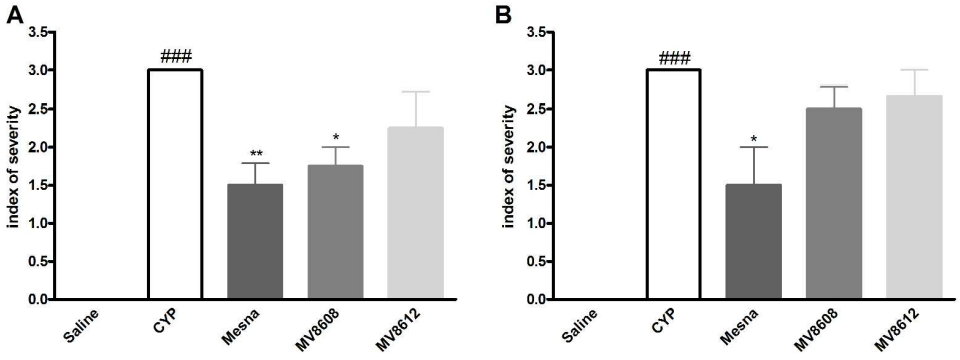

Effect of treatment with Mesna (21.5 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), MV8608 (10 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), or MV8612 (11.8 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection) on macroscopic hemorrhage (A) and edema (B) evaluation in CYP-induced HC (24 h). Each column represents the mean of 6-8 animals and the vertical lines show the S.E.M. *P<0.05; **P<0.01 denote the significance levels in comparison to control values; ###P<0.001 denote the significance levels in comparison to saline values.
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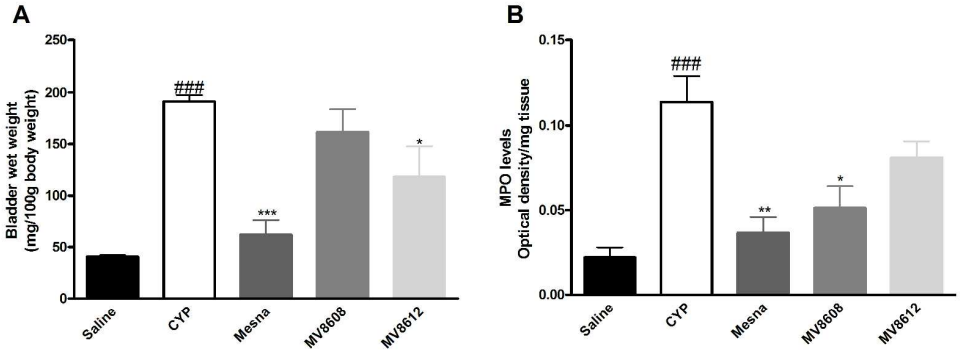

(A) Effect of treatment with Mesna (21.5 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), MV8608 (10 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection), or MV8612 (11.8 mg/kg, ip, 3 doses, 0.5 h before, and 4 and 8 h after CYP injection) on weight bladder in CYP-induced HC (24 h). (B) Effect of treatment with Mesna (21.5 mg/kg, ip, 0.5 h before CYP injection), MV8608 (10 mg/kg, ip, 30 min prior to CYP), or MV8612 (11.8 mg/kg, ip, 30 min prior to CYP) on myeloperoxidase activity in CYP-induced HC (4 h). Each column represents the mean of 6-8 animals and the vertical lines show the S.E.M. *P<0.05; **P<0.01; ***P<0.001 denote the significance levels in comparison to control values; ###P<0.001 denote the significance levels in comparison to saline values.
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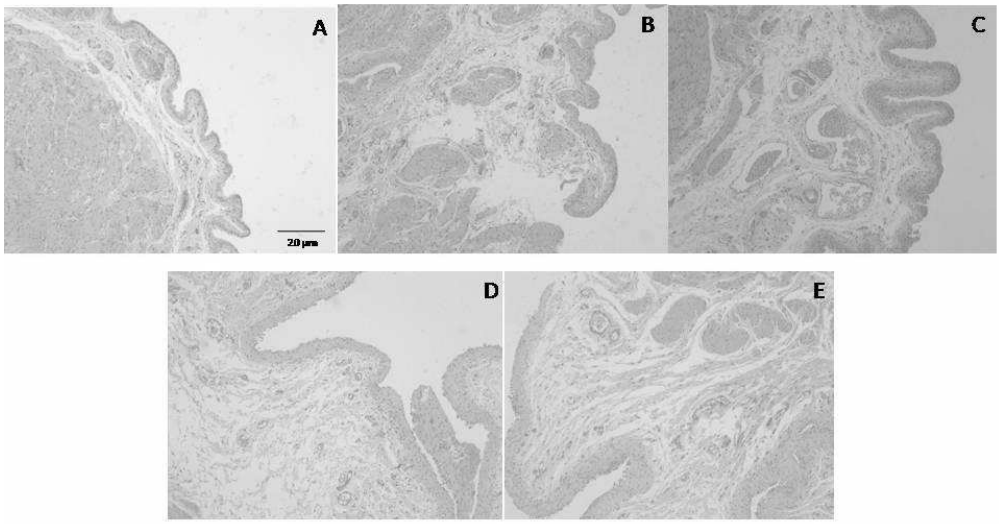

Histological analysis of representative bladder walls in cross-sections. (A) Saline: bladder with normal appearance, with absence of edema, hemorrhage, or inflammation. (B) CYP: severe hemorrhagic cystitis with edema, transmural hemorrhage, mucosal ulceration, and inflammation. (C) Mesna: near normal appearance. (D) MV8608: mild submucosal edema, minimal hemorrhage, few ulcerations. (E) MV8612: mild submucosal edema, hemorrhage and erosions. All panels, hematoxylin-eosin stain. Original magnification x 10 in all panels.
254x190mm (96 x 96 DPI)