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Research Paper

Carvacryl acetate, a novel semisynthetic monoterpene ester, binds to the TRPA1 receptor and is effective in attenuating irinotecan-induced intestinal mucositis in mice

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Abstract

Objectives We aimed to determine whether carvacryl acetate acts as a TRPA1 receptor agonist and its effects against irinotecan (CPT-11) induced intestinal mucositis in mice.

Methods TRPA1 structure was obtained from a protein databank, and the 3D structure of carvacryl acetate was determined. Appropriate binding conformations were discovered via automatic docking simulations. To determine the effect of carvacryl acetate *in vivo*, mice were treated with either DMSO 2%, CPT-11, carvacryl acetate followed by CPT-11, or HC-030031, a TRPA1 antagonist, followed by carvacryl acetate. Jejunum samples were taken and structural, inflammatory and antioxidant parameters were studied.

Key findings Eight amino acids residues in TRPA1 established stable interactions with carvacryl acetate, which led to pharmacological efficacy against CPT-11-induced intestinal mucositis via reduction of both neutropenia and bacteremia, increase in villi height and crypt depth, decrease in pro-inflammatory cytokines (interleukin-1β, keratinocyte chemoattractant and tumour necrosis factor-α) and decrease in malondialdehyde and nitric oxide metabolite levels in the jejunum.

Conclusions Carvacryl acetate is a promising anti-inflammatory and antioxidant agent, a fact confirmed through observations of its interactions with TRPA1 in CPT-11-induced intestinal mucositis in mice.

Introduction

Carvacryl acetate (5-isopropyl-2-methylphenyl acetate, $C_{12}H_{16}O_2$), a semisynthetic monoterpene ester, has shown anti-inflammatory and antinociceptive effects in experimental models of peritonitis and paw oedema.[1] The main properties of carvacryl acetate include: molecular weight – 192.26; refractive index – 1.497; boiling point – 94.56 °C at 760 mmHg; enthalpy of vapourization – 48.414 kJ/mol; density – 0.994 g/cm^3.[2] Due to the relatively high toxicity of its precursor carvacrol compared to that of other phenols and esters, carvacryl acetate was thought to be a less toxic semisynthetic derivative of carvacrol with the same or improved pharmacological properties. The difference between carvacrol and carvacryl acetate is the replacement of the hydroxyl group by an ester group in the latter, which is attributable to carvacryl's lower toxicity and higher stability. Carvacryl acetate inhibits inflammatory mediators and neutrophil migration.[1] However, information about the effect of carvacryl acetate on specific inflammatory diseases is scarce, particularly on disorders of the gastrointestinal tract.

Widely distributed in the gastrointestinal tract, the ankyrin-repeat transient receptor potential (TRPA1) is a cation channel and sensor for cell damage signals, including reactive oxygen species and inflammatory mediators.[3] Some compounds, such as carvacrol and HC-030031,[4] act as a TRPA1 agonist and antagonist, respectively.

Intestinal mucositis, an inflammatory process that results in morphological and physiological changes in the small intestine mucosa, is generally caused by the use of antineoplastic drugs, such as irinotecan hydrochloride (CPT-11). CPT-11 is a semisynthetic analogue of camptothecin and a selective inhibitor of the enzyme topoisomerase I.[5,6] It is one of the main neoplastic drugs in use today. As intestinal mucositis is severe, studies on how to minimize its negative effects, or that clarify how intestinal mucositis develops are essential, because they can help discover potential therapeutic targets. Due to its wide distribution in the intestinal mucosa, TRPA1 is a molecule of interest in studies that attempt to uncover the mechanisms triggering mucositis.

The aim of this study was to investigate whether carvacryl acetate is a TRPA1 receptor agonist. We also aimed to verify whether this interaction between carvacryl acetate and TRPA1 could reduce inflammation and increase mucosal protection in the gut of mice with CPT-11-induced intestinal mucositis.

Materials and Methods

Molecular docking

Structures of the agonist carvacryl acetate and TRPA1 (PDB ID: 3J9P), determined using cryo-electronic microscopy at a resolution of 4.24 Å, were obtained from the Research Collaboratory for Structural Bioinformatics protein databank. A docking simulation was used to study the binding of TRPA1 and carvacryl acetate. As previously mentioned,[7] the area of the binding site was considered the most desirable region for fitting the agonist. The binding ability of carvacryl acetate and its corresponding binding affinity scores were used to determine better molecular interactions. The results were visualized and analysed using ADT, Maestro, PyMOL and Discovery Studio.

Preparation of carvacryl acetate

Carvacryl acetate (98% purity; Figure 1a) was obtained via acetylation of carvacrol, using acetic anhydride for the acetylation and pyridine as a catalyst. Carvacryl acetate was obtained (4.779 g; 0.025 mol) at a 76% yield.[8,9] The structural identification of carvacryl acetate was performed by spectroscopic techniques (1H and 13C NMR, IR) and by comparison with the literature data.[1]

Animals

Female Swiss mice (25–30 g) were randomly maintained in appropriate cages at 25 ± 2 °C under a 12-h light/dark cycle with food and water provided *ad libitum*. All experimental procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Institute of Health, Bethesda, MD, USA) and were approved by the local ethics committee (protocol no. 080/15). The animals were divided into experimental groups of 5–7 animals each.

Effect of carvacryl acetate on intestinal mucositis: experimental protocol

The experimental protocol to induce intestinal mucositis was based on a previously described protocol with modifications.[10,11] In this study, which lasted 8 days, the mice in the experimental groups received only carvacryl acetate (25, 75, or 150 mg/kg, i.p.) on the first day.[1,12] Between the second and fifth days the mice received CPT-11 (75 mg/kg, i.p.), followed by carvacryl acetate 30 min later. From days 6–8, the mice received either carvacryl acetate or DMSO (2%, i.p.). To verify the putative involvement of TRPA1 receptors in the carvacryl acetate effect on mucositis, a different group of mice was administered HC-030031 (0.025 mg/kg, i.p.) (4), a TRPA1 antagonist, 30 min before carvacryl acetate from day 2 onwards. Negative and positive controls received only DMSO (2%, i.p.) or only CPT-11 (75 mg/kg, i.p.), respectively.

In the experimental period, the mass and survival of the mice were measured daily. The mice were then killed with an overdose of ketamine/xylazine (>100/10 mg/kg, s.c.). Blood samples were obtained by cardiac puncture to perform leucogram and bacterial counts, and segments of the jejunum (80–100 mg) were removed for further analysis described below.

Blood leucocyte and bacteria counts

Blood samples obtained by cardiac puncture were transferred to test tubes, which were then heparinized and diluted with Turk's solution (380 µl of blood and 20 µl of diluent solution). The leucocytes were counted in a Neubauer Chamber with a light microscope.

To count the bacteria, blood samples obtained by cardiac puncture were quantified according to a previously developed methodology[13] with modifications.

Histological and morphometric evaluation

Segments of jejunum were prepared according to.[12] The severity of intestinal mucositis was determined by grades.[11,14] The length of the intestinal villi and depth of the crypts were measured (ImageJ Software, version 1.4, NIH, USA). Between 5 and 10 villi and crypts were measured per slice, and a range of 5–8 slices were analysed per group.[15]

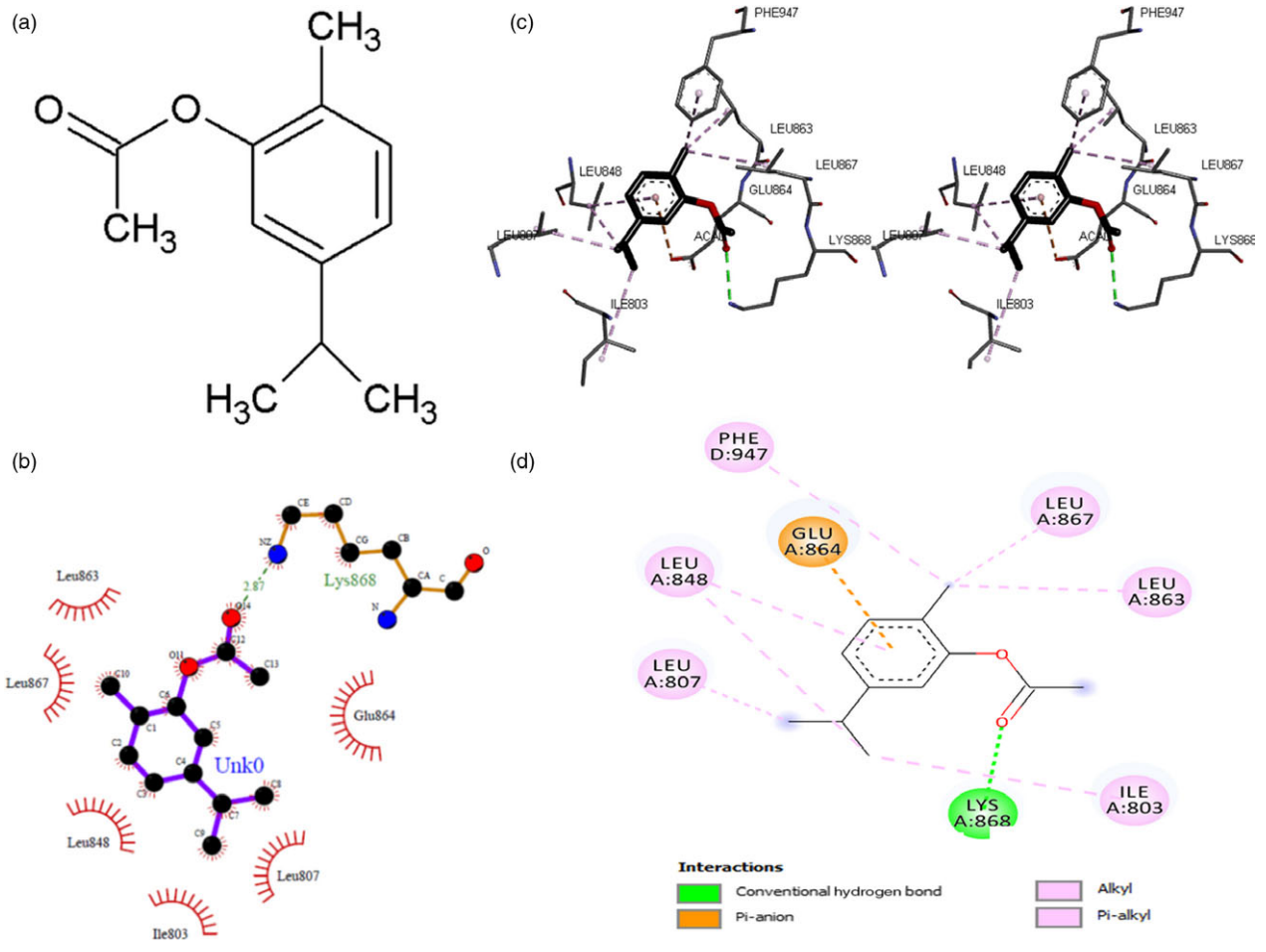

Figure 1 Chemical structure of carvacryl acetate (5-isopropyl-2-methylphenyl acetate, $C_{12}H_{16}O_2$). Models of molecular docking of carvacryl acetate onto TRPA1 (a); Details of interactions between carvacryl acetate and the activation site of TRPA1 (b); 3D stereo view of carvacryl acetate docking in the activation site, showing interactions with neighbouring residues through hydrophobic interactions (c); 2-D illustration of the types of interactions between carvacryl acetate and TRPA1, containing a legend with the various interaction types (d).

Determination of Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity of enterocytes was measured in segments of mouse jejunum according to a previously described methodology with modifications.[16] Results are expressed as a relation between the inorganic phosphate and protein concentration (μmol P_i/mg protein/h).[17]

Effect of carvacryl acetate on inflammatory and oxidative stress markers in intestinal mucositis

Myeloperoxidase activity was assessed according to.[18] Cytokine levels (TNF-α, IL-1β and KC) were measured using an enzyme-linked immunosorbent assay (ELISA) according to[15] and the manufacturer's instructions (DuoSet ELISA Development kit R&D Systems, Minneapolis, MN, USA).

Glutathione (GSH) and superoxide dismutase (SOD) levels were determined according to the method described by[19] and,[20] respectively. The malondialdehyde (MDA) was used as an indicator of lipid peroxidation. The MDA concentration for jejunum tissue was measured using the method described previously by.[21]

The Griess reaction[22] was used to quantification of the nitric oxide (NO) metabolites, nitrate (NO_3^-) and nitrite (NO_2^-) (collectively NOx), in mouse small intestine according to.[22]

Immunohistochemical assay for NF-κB and COX-2

Segments of mouse jejunum were incubated with goat anti-COX-2 (1:200, SC-1747; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-NF-kB p50 NLS antibodies

(1:200, SC-114; Santa Cruz Biotechnology). The quantitative estimation of DAB products from immunostaining was performed using digital images of at least ten different areas of each section.[23]

Statistical analysis

Results are expressed as mean ± SEM of 5–8 animals per group, and statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Newman–Keuls *post hoc* test, when appropriate. Statistical threshold was set at $P < 0.05$.

Results

Molecular docking

The chemical structure of carvacryl acetate is shown in Figure 1a. The protein–ligand complexes of carvacryl acetate were inserted at the TRPA1 cavity through Autodock 4.2 software, as shown in Figure 1c. Interactions between carvacryl acetate and the TRPA1 receptor occurred at eight amino acids residues at the active site of TRPA1, (ILE803, LEU807, LEU848, LEU863, GLU864, LEU867, LYS868 and PHE947) (Figure 1b). A hydrogen bond formed from the interaction between the acetylate ring and LYS868. GLU864 interacted with carvacryl acetate through a pi-anionic interaction. The other amino acid residues in TRPA1 interacted with carvacryl acetate via alkyl interactions. The binding energy that resulted in the carvacryl acetate-TRPA1 interaction was negative (−6.1 kcal/mol), in a single conformation with an inhibition constant of 36.8 μM. These results show that carvacryl acetate binds tightly to the active site of TRPA1. All molecular docking models of interactions between carvacryl acetate and the TRPA1 receptor are illustrated in Figures 1b–d.

Effect of carvacryl acetate on survival and body mass variation

The mouse survival rate and changes in body mass were observed throughout the intestinal mucositis experiment (Figure 2). Mice treated with CPT-11 (75 mg/kg, i.p.) had the lowest survival rate (62.5%) compared to that of mice treated with DMSO 2% (negative control, 100% survival) or those treated with carvacryl acetate at any concentration (25, 75, or 150 mg/kg, i.p., 87.5%, 100% and 87.5% survival, respectively). The only dose at which there were no deaths was 75 mg/kg of carvacryl acetate (100% survival).

Body mass increased only in mice treated with DMSO 2% (2 ± 0.9 g at 7 days), while those treated with CPT-11 lost body mass (5.4 ± 2.1 g at 7 days). Mice treated with carvacryl acetate (25, 75 and 150 mg/kg, i.p.) lost less mass

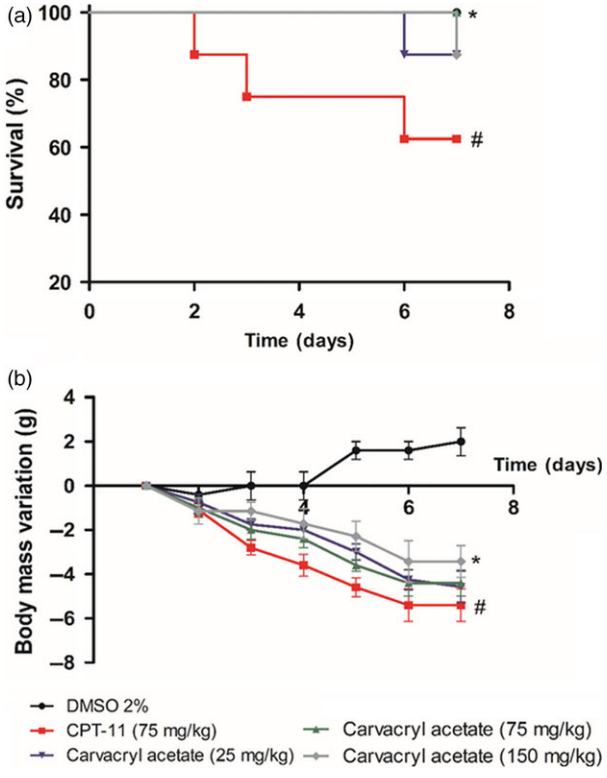

Figure 2 Carvacryl acetate effect on survival and change in body mass in mice. Carvacryl acetate treatment (75 mg/kg) in mice increased the survival (a) and decreased body mass (b) of mice with induced mucositis compared to CPT-11 (75 mg/kg) treatment. Rows represent the mean ± standard error of the mean expressed as change in body mass and were analysed using one-way analysis of variance (ANOVA) followed by a Newman–Keuls test. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs CPT-11 (75 mg/kg) group.

compared to that observed for the CPT-11 group (4.6 ± 1.7 g, 4.4 ± 1.7 g and 3.4 ± 1.3 g, respectively). Therefore, as carvacryl acetate (75 mg/kg) caused no deaths and caused a small change in body mass, this dose was chosen for all further experimental procedures. Moreover, this dose and its route of administration have been previously reported as effective in other mice models of inflammation by our research group (1).

Effect of carvacryl acetate on leucogram and bacterial counts

After intestinal mucositis induction, leucograms were determined. CPT-11 treatment caused a decrease in total leucocyte number (3382 ± 613.7 leucocytes/mm^3) relative to that observed for DMSO 2% treatment (4967 ± 1145 leucocytes/mm^3). Conversely, the density of leucocytes in mice treated with carvacryl acetate was higher (5710 ± 660.9 leucocytes/mm^3) than that in the CPT-11

and DMSO groups. Mice treated with HC-030031 (0.025 mg/kg) had the lowest density of leucocytes (2570 ± 1142), as shown in Figure 3a.

Carvacryl acetate effectively reduced bacterial growth (Figure 3b) relative to that reported with CPT-11. The levels of bacterial growth in mice treated with carvacryl acetate were similar to those in mice treated with DMSO. Pretreatment with HC-030031 increased the bacterial count to the level observed after CPT-11 treatment.

Effect of carvacryl acetate on morphological and morphometric parameters

As determined by degrees of mucositis, mice treated with CPT-11 showed extensive loss of crypt architecture, including necrotic, shortened and flattened villi (Figure 4b). Mucositis of degree 3–4, necrotic cells and infiltration of inflammatory cells such as neutrophils into more than 20% of the lamina propria were observed. The injury scores in mice treated with carvacryl acetate (Figure 4c) were significantly lower (0–2) than those for the CPT-11 group, suggesting that carvacryl acetate significantly restored the histopathological alterations caused by intestinal mucositis. This result is corroborated by the observation of a reduction in infiltration of inflammatory cells and the general alteration of architecture, largely restoring normal tissue structure of villi and crypts. Figure 4a shows that there was no injury to mouse jejunum in samples treated with DMSO 2% (0–1). Moreover, Figure 4d shows that pretreatment with HC-030031 nullified the beneficial action of carvacryl acetate on intestinal mucosa, resulting in mucositis of degrees 2–4; this shows that carvacryl acetate likely acts through TRPA1 receptors.

Morphometric analysis also showed that treatment with carvacryl acetate restored villi length (185 ± 37.2 μm) and enlarged crypt depths (86.5 ± 17.34 μm), as shown in Figure 5a,b. CPT-11 caused a significant shortening of the villi height (104.5 ± 12.63 μm) and a reduction in crypt depth (33 ± 12 μm), while DMSO left the intestinal architecture intact (villi height, 199 ± 13.4 μm; crypt depth, 89.6 ± 14.9 μm). Pretreatment with HC-030031 reduced villi height (77.26 ± 8 μm) and crypt depth (40.79 ± 5.9 μm) to levels similar to those observed after CPT-11 treatment.

Na^+/K^+-ATPase activity

Treatment with CPT-11 reduced the activity of Na^+/K^+-ATPase enzyme (1027 ± 299 μmol/mg/h) compared to that reported with DMSO treatment (1894 ± 514.6 μmol/mg/h). However, after treatment with carvacryl acetate, Na^+/K^+-ATPase activity returned to baseline values (2156 ± 884.3 μmol/mg/h), as shown in Figure 6.

Effect of carvacryl acetate on inflammatory and oxidative stress parameters

Results related to the effects of carvacryl acetate (75 mg/kg) on MPO, GSH, MDA, NOx and cytokines (TNF-α, IL-1β and KC) are shown in Table 1. Higher levels of MPO were

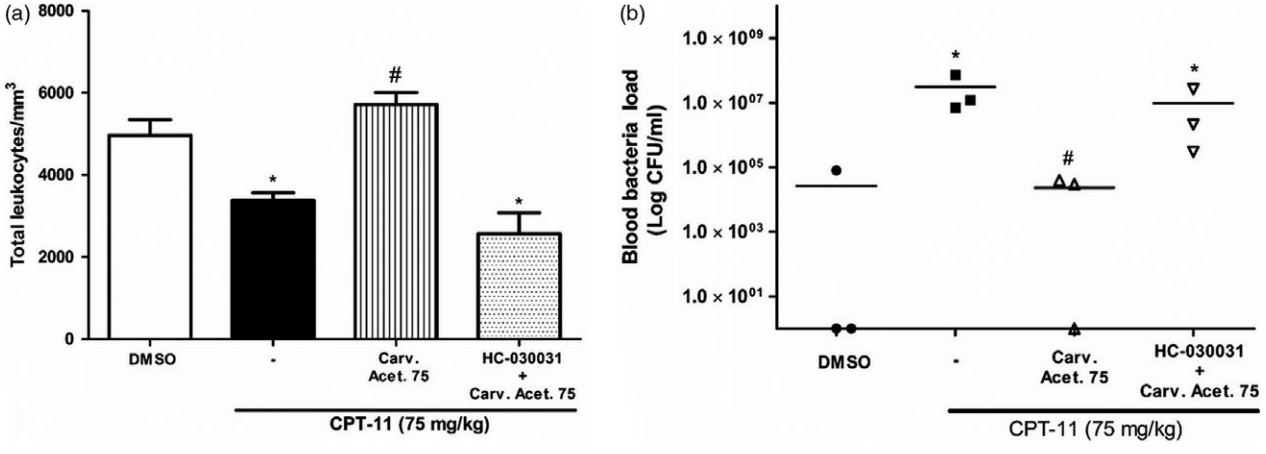

Figure 3 Carvacryl acetate effect on leucocyte levels and bacterial load in blood of mice. CPT-11 (75 mg/kg) treatment decreased the total number of leucocytes in mice with induced intestinal mucositis, and treatment with carvacryl acetate (75 mg/kg) restored leucocyte levels to normal (a). Bacterial load, expressed as a logarithm of the number of bacterial colonies, was also measured in blood samples. In mice treated with CPT-11 (75 mg/kg), an increase in bacteremia was observed, which was reduced by treatment with carvacryl acetate (75 mg/kg) (b). In both cases, pretreatment with HC-030031 (0.025 mg/kg) prevented the action of carvacryl acetate. Bars and dots represent the mean ± standard error of the mean and were analysed using one-way analysis of variance (ANOVA) followed by a Newman–Keuls test. $^*P < 0.05$ vs control group; $^{\#}P < 0.05$ vs CPT-11 (75 mg/kg) group.

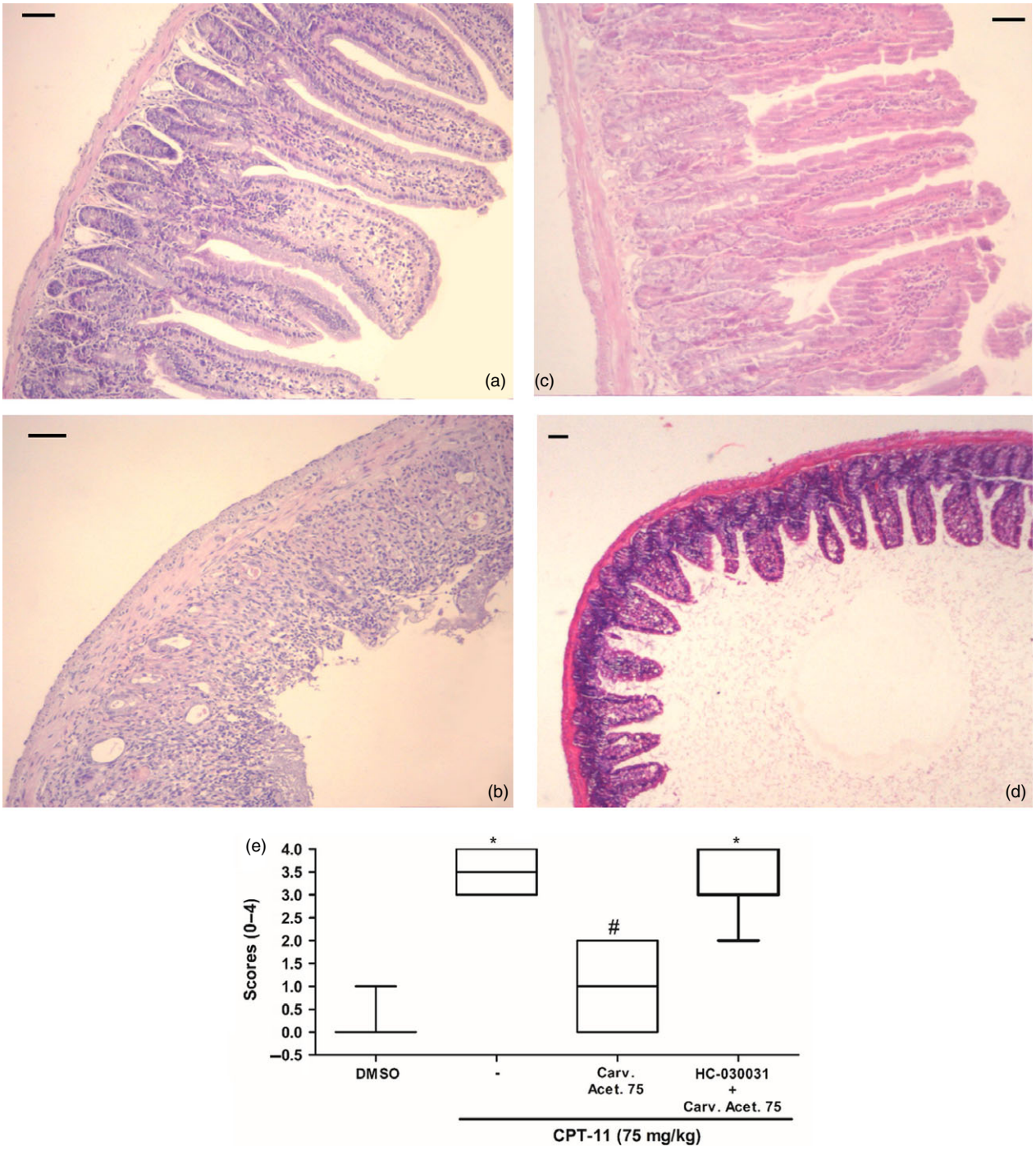

Figure 4 Photomicrographs from mouse jejunum mucosa stained with haematoxylin and eosin. (Panel a) DMSO 2% group (negative control) exhibits normal histological features, with intact tissue architecture (preservation of villi and crypts) and no evidence of mucositis. (Panel b) CPT-11 (75 mg/kg) group shows pronounced mucositis with shortened villi caused by vacuolated cells, loss of crypt architecture, and infiltration of inflammatory cells into the lamina propria. (Panel c) Carvacryl acetate (75 mg/kg) partly restored the tissue architecture after induction of mucositis with some preservation of villi height and crypt depth. (Panel d) HC-030031 (0.025 mg/kg) pretreatment group shows prevention of the carvacryl acetate action, with villi shortening and loss of crypt architecture. Scale bars: 50 mm. (Panel e) Histological grading of mucositis for mouse jejunum treated with carvacryl acetate (75 mg/kg), which significantly reduced the grade of CPT-11-induced intestinal mucositis. Data represent median values of scores and were analysed using a Kruskal–Wallis test. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs CPT-11 (75 mg/kg) group.

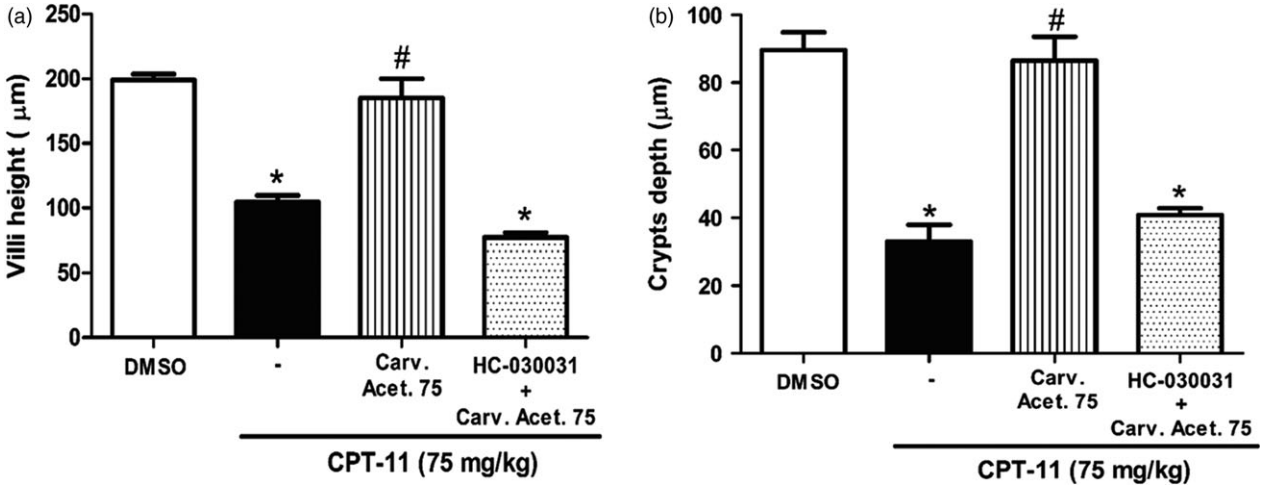

Figure 5 Villi height (a) and crypt depth (b) of mouse jejunum mucosa showing villi shortening and decrease of crypt depth in mice subjected to induced intestinal mucositis. Carvacryl acetate (75 mg/kg) reverted the effects of CPT-11 (75 mg/kg) and pretreatment with HC-030031 (0.025 mg/kg) showed similar effects as CPT-11. Bars represent the mean ± standard error of the mean and were analysed using one-way analysis of variance (ANOVA) followed by a Newman–Keuls test. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs CPT-11 (75 mg/kg) group.

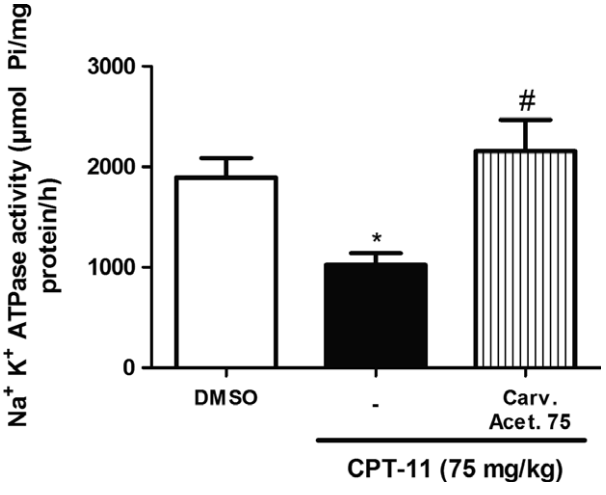

Figure 6 The Na^+/K^+-ATPase activity in the mouse jejunum was measured by determining the release of inorganic phosphate from the hydrolysis of ATP. Pretreatment with carvacryl acetate (75 mg/kg) increased the activity of Na^+/K^+-ATPase significantly as compared to the CPT-11 group. Bars represent the mean ± standard error of the mean and were analysed using one-way analysis of variance (ANOVA) followed by a Newman–Keuls test. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs CPT-11 (75 mg/kg) group.

observed in mice that received CPT-11 (4.08 ± 2.1 U MPO/mg of tissue) than in mice treated with DMSO (0.95 ± 1.4 U MPO/mg of tissue). Treatment with carvacryl acetate decreased the levels of MPO in the jejunum (1.16 ± 1.12 U MPO/mg of tissue) while pretreatment with HC-030031 induced a significant increase in MPO levels (4.01 ± 1.6 U MPO/mg of tissue).

Treatment with CPT-11 significantly increased the levels of the cytokines IL-1β, KC and TNF-α (447.9 ± 53.8 pg/ml, 1024 ± 147.9 pg/ml and 144.5 ± 51 pg/ml, respectively) compared to that reported for the DMSO group (12.57 ± 6.2 pg/ml, 8 ± 9.98 pg/ml and 9.83 ± 8.42 pg/ml, for IL-1β, KC and TNF-α, respectively). Carvacryl acetate reduced the CPT-11-induced increase in cytokine levels (148.4 ± 51.12 pg/ml, 497.9 ± 151.4 pg/ml and 46.24 ± 42.38 pg/ml for IL-1β, KC and TNF-α, respectively). Pretreatment with HC-030031 increased the cytokines levels (426 ± 54.9 pg/ml, 810.9 ± 133.3 pg/ml, 139.1 ± 40.91 pg/ml, for IL-1β, KC and TNF-α, respectively), close to those observed after CPT-11 treatment, clearly preventing carvacryl acetate action.

The presence of GSH, a cellular defence agent, is inferred by hepatic non-protein sulfhydryl (NPSH) levels. Thus, after intestinal mucositis induction with CPT-11, a reduction of NPSH levels (165 ± 90.91 μg/ml) was seen compared to that with DMSO treatment (385.8 ± 77.59 μg/ml). Treatment with carvacryl acetate increased NPSH levels (610.5 ± 95.09 μg/ml), and pretreatment with HC-030031 prevented this increase (216 ± 92.96 μg/ml).

The levels of SOD, another cellular defence agent, after intestinal mucositis induction with CPT-11 was reduced (2.72 ± 0.49 U SOD/μg) if compared to that observed after DMSO treatment (3.92 ± 0.18 U SOD/μg). Treatment with carvacryl acetate increased SOD levels (5.04 ± 0.31 U SOD/μg), and pretreatment with HC-030031 prevented this increase (3.83 ± 0.20 U SOD/μg).

Another commonly used marker of oxidative stress is MDA, a product of lipidic peroxidation. Mice treated with CPT-11 had higher MDA levels (280.1 ± 119.7 nmol/ml

Table 1 Effects of carvacryl acetate (75 mg/kg) at biochemical dosages of MPO, GSH, SOD, MDA, NOx and cytokines (TNF-α, IL-1β and KC)

Experimental group	MPO (U/mg)	MDA (nmol/ml)	GSH (μg/ml)	SOD (U/μg)	NOx (μM)	TNF-α (pg/ml)	IL-1β (pg/ml)	KC (pg/ml)
DMSO 2%	0.95 ± 1.38	161.40 ± 83.02	385.80 ± 77.59	3.92 ± 0.18	0.24 ± 0.03	9.833 ± 8.424	12.57 ± 6.18	8.00 ± 9.98
CPT-11 (75 mg/kg)	4.08 ± 2.10*	280.10 ± 119.70*	165.00 ± 90.91*	2.72 ± 0.49*	0.47 ± 0.16*	144.5 ± 51.01*	447.9 ± 53.77*	1024.00 ± 147.90*
Carv. Acet. (75 mg/kg)	1.16 ± 1.12†	111.80 ± 81.85†	610.50 ± 95.09†	5.04 ± 0.31†	0.24 ± 0.15†	46.24 ± 42.38†	148.4 ± 51.12†	497.90 ± 151.40†
HC-030031+ Carv. Acet. (75 mg/kg)	4.01 ± 1.62*	194.10 ± 38.72*	216.00 ± 92.96*	3.83 ± 0.20*	0.48 ± 0.21*	139.1 ± 40.91*	426.00 ± 54.87*	810.90 ± 133.30*

MDA, malondialdehyde; GSH, Glutathione; SOD, superoxide dismutase. Data shown are expressed as mean ± SEM (n = 5–8). Analysis of variance (ANOVA) and Newman–Keuls test. *$P < 0.05$ vs DMSO group; †$P < 0.05$ vs CPT-11 group.

than mice that received DMSO did (161.4 ± 83.02 nmol/ml). Treatment with carvacryl acetate reduced MDA levels (111.8 ± 81.85 nmol/ml) to those observed in the DMSO group, which supports the protective action of carvacryl acetate. Mice pretreated with HC-030031 showed slightly elevated MDA levels (194.1 ± 38.72).

NOx levels represent the amount of oxidative damage in inflamed tissues. Thus, mice that received CPT-11 had higher levels of NOx (0.47 ± 0.16 μM) than mice that received DMSO treatment did (0.24 ± 0.03 μM). Carvacryl acetate lowered NOx levels (0.24 ± 0.15 μM), to those observed in the DMSO group, confirming its protective action on intestinal mucosa. Pretreatment with HC-030031 increased NOx levels (0.48 ± 0.21 μM), to those observed in the CPT-11 group. This result confirms that carvacryl acetate exerts its protective action through interactions with the TRPA1 receptor.

Immunochemical detection of NF-κB and COX-2

Jejunum sections submitted to immunochemical detection of NF-κB and COX-2 showed intensely stained areas in CPT-11-treated samples (33.89 ± 12.66 cells immunostained for NF-κB detection and 47.6 ± 11.14 cells immunostained for COX-2 detection) compared to the results of the DMSO group (NF-κB, 7.26 ± 3.63 cells immunostained; COX-2, 19.5 ± 6.9 cells immunostained). Treatment with carvacryl acetate significantly reduced these immunostained areas (NF-κB, 23.58 ± 12.62 cells immunostained; COX-2, 12.14 ± 3.6 cells immunostained); in the case of COX-2, the staining intensity decreased to levels close to those of the DMSO group. In the DMSO and carvacryl acetate groups, there were few immunostained cells in the epithelium. However, in the CPT-11 group, the epithelium and muscle layers showed intense immunostaining, as shown in Figures 7 and 8, respectively.

Discussion

Results showed that carvacryl acetate stably binds to TRPA1, both because of the number and the type of interactions between them. Carvacryl acetate interacted with TRPA1 and activates, acting as an agonist, through eight amino acids residues, which contributed to the high stability in this interaction. It bound to the TRPA1 receptor via hydrogen bonds and pi-anionic interactions, both of which are among the strongest kind of chemical interactions, which also contributed to the strength of the interaction. Hydrogen bonds are very strong due to the presence of high hydrogen electropositivity and high oxygen, nitrogen and fluorine electronegativity.[24]

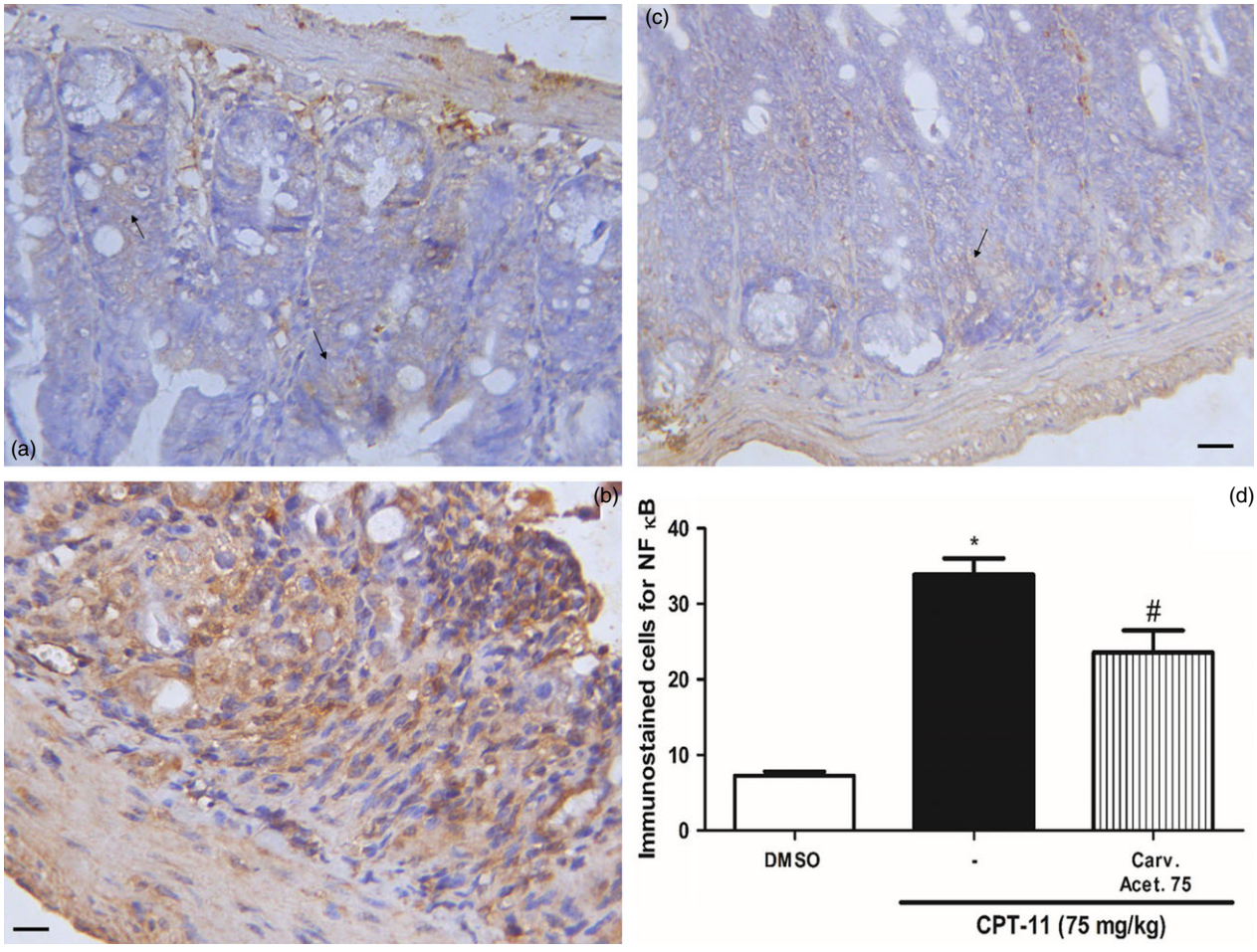

Figure 7 Photomicrographs from immunohistochemical staining for NF-κB in jejunum of mouse with induced intestinal mucositis. Moderate NF-κB staining was seen in normal jejunum (a). A jejunum from a mouse treated with CPT-11 showed intense immunostaining for NF-κB in the epithelium and muscular layer (b). Treatment with carvacryl acetate considerably reduced the immunostaining in the surface epithelium (c), which was demonstrated by a reduction in the number of immunostained cells following carvacryl acetate (75 mg/kg) treatment (d). Bars represent the mean ± standard error of the mean and were analysed using one-way analysis of variance (ANOVA) followed by a Newman–Keuls test. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs CPT-11 (75 mg/kg) group.

Comparison of the molecular docking models shows that the interactions between carvacryl acetate and TRPA1 are more stable and lasting than those between carvacrol and TRPA1.[12] This is because carvacrol and TRPA1 interacted through only six amino acids residues using bonds weaker than hydrogen bonds.[12] Some molecules can show a large conformational variation, with even a few rotatable bonds creating many different conformations.[25] In this way, carvacrol can bind to TRPA1 through seven different conformations.[12] Carvacryl acetate, however, only showed a single conformation in the TRPA1 receptor-binding site.

This stable and lasting interaction between carvacryl acetate and TRPA1 is also corroborated by observation of the inhibition constant, which is a parameter associated with the dissociation constant. Interactions between carvacryl acetate and TRPA1 showed a smaller inhibition constant than that reported for the carvacrol-TRPA1 interaction.[12]

To demonstrate that the interaction between carvacryl acetate and TRPA1 could culminate in a relevant biological effect against intestinal mucositis, we observed some clinical parameters. Carvacryl acetate did not affect the mice's survival and induced a loss in body mass.

Some of the main side effects of CPT-11 are neutropenia due to myelosuppression,[26] and the increase in bacteria in the blood due to tissue damage.[13,27] Results demonstrated that carvacryl acetate restored leucocyte levels in the blood and minimized bacteremia, which were again increased through HC-030031 pretreatment. Bacteremia may have been triggered by the disruption of the mucus barrier in the small intestine. Normally, bacteria are located in the loose mucus and cannot penetrate the inner mucus layer.[27,28]

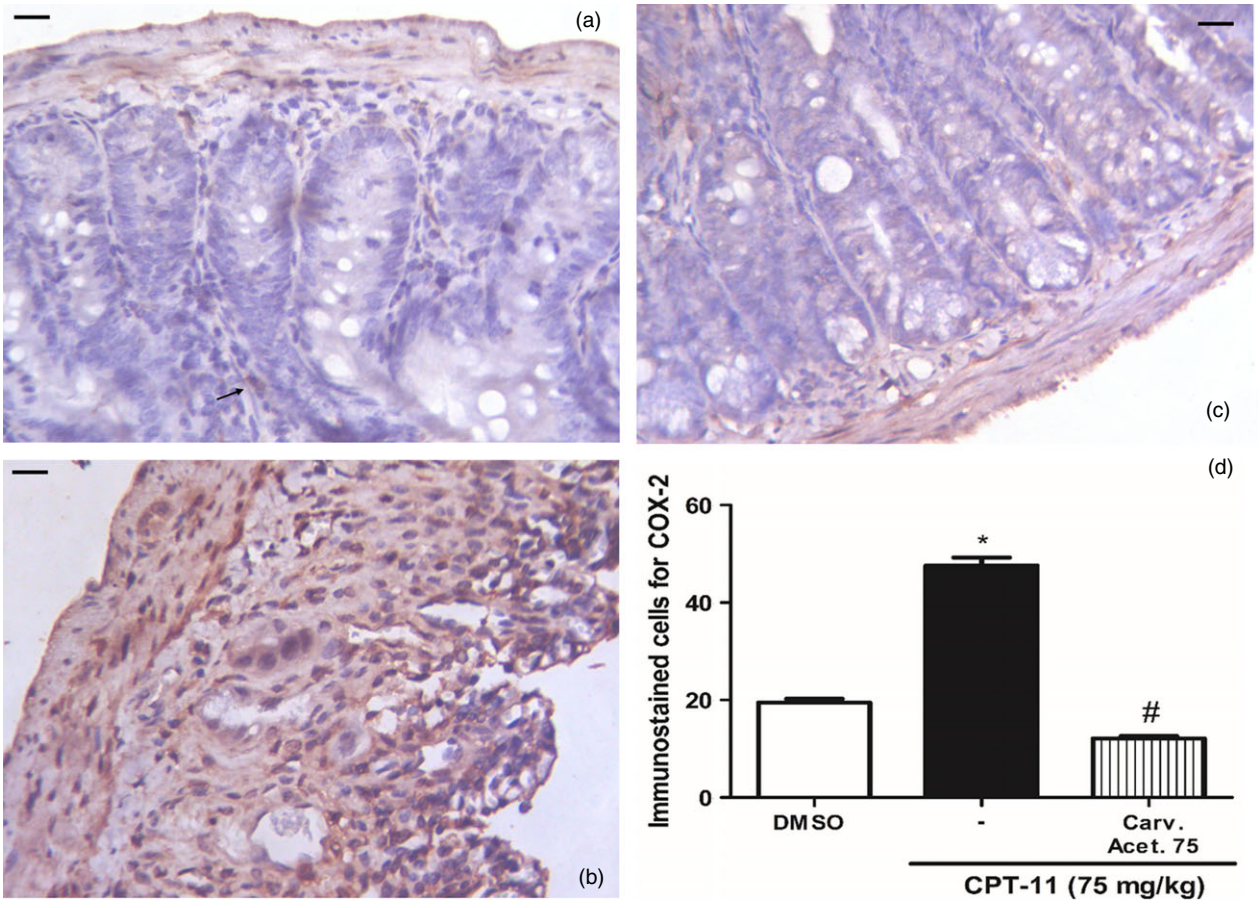

Figure 8 Photomicrographs from immunohistochemical staining for COX-2 in jejunum mucosa in a mouse with induced intestinal mucositis treated with DMSO 2% (a). The jejunum of a mouse that received CPT-11 showed intense immunostaining for COX-2 in the epithelium and muscular layer (b). Treatment with carvacryl acetate considerably reduced the immunostaining present in the surface epithelium (arrow, c). This was demonstrated by a reduction in the number of immunostained cells following carvacryl acetate (75 mg/kg) treatment (d). Bars represent the mean ± standard error of the mean and were analysed using one-way analysis of variance (ANOVA) followed by a Newman–Keuls test. $^{*}P < 0.05$ vs control group; $^{\#}P < 0.05$ vs CPT-11 (75 mg/kg) group.

However, in intestinal mucositis, because of the tissue destruction caused by CPT-11 treatment, there was damage to the mucus layer, which may have allowed bacteria to reach the bloodstream through contact with injured epithelial cells.

Furthermore, in intestinal mucositis, we observed high levels of MPO, indicating a large amount of neutrophil migration to the injured tissue. Treatment with carvacryl acetate reduced MPO levels in the jejunum tissue, although there was an increase in the systemic levels of leucocytes. This is because CPT-11 causes systemic neutropenia, along with an increase in neutrophil migration to the injured tissue to defend against inflammation. Oxidative stress, which is common in inflamed tissues, can induce p53 activation resulting in senescence, arrest cycle and cell death in the hematopoietic compartment.[29,30] This could explain the low leucocyte systemic levels after CPT-11 treatment and its reversion after carvacryl acetate treatment. Moreover, as proposed in other studies,[30] the neutrophil migration to the injured site itself may help explain the decrease in leucocyte number in the blood.

To confirm that neutrophils trigger the release of inflammatory mediators,[31] we observed the levels of some pro-inflammatory cytokines (IL-1β, KC – an ortholog of human IL-8 in mouse and TNF-α), which decreased after treatment with carvacryl acetate. It is well established that these cytokines have important functions in the development of intestinal mucositis by regulating and amplifying the immune response, contributing to tissue damage.[11,32] These actions are related to the stimulation of cytokine release by IL-1β; induction of metabolite generation from arachidonic acid by IL-1β, which can act as a trigger for inflammation; and activation by TNF-α of some proteases released by epithelial cells, fibroblasts and neutrophils,

related to tissue injury.[11,31] Moreover, KC acts as a relevant chemokine recruiting neutrophils to the inflammation site[11,33] and, thus, acting as a feedback agent in this process, as more neutrophils causes more cytokine activation.

TNF-α, through interactions with TNF-R1, is also associated with activation of the transcription factor NF-κB.[34] Additionally, the presence of bacteria in injured mucosa, as observed in intestinal mucositis, can lead to the activation of NF-κB.[35] The transcription factor NF-κB is associated with an increase in pro-inflammatory cytokine levels, such as IL-1β and TNF-α, and can induces apoptosis, contributing to the pathology of intestinal mucositis. Furthermore, NF-κB activates the expression of cell surface receptors to molecules related to the inflammation, cell adhesion molecules and molecules related to the response to oxidative stress.[34]

Another pro-inflammatory mediator shown to be highly expressed in CPT-11-induced intestinal mucositis was COX-2, which was also decreased by carvacryl acetate treatment. COX-2 is involved in the synthesis of prostaglandins, which are responsible for the development of inflammation.[34,36] As in other studies, we noted that COX-2 and NF-κB levels seemed to follow a similar pattern of expression; both increased after CPT-11 treatment and decreased after carvacryl acetate treatment. This agrees with the understanding that NF-κB upregulates COX-2 expression, which can also be increased by IL-1β.[37]

Results also showed a concomitant reduction in oxidative stress parameters (MDA and NOx) and increase in cellular defence agents (GSH and SOD) after treatment with carvacryl acetate. This is consistent with the fact that increasing levels of reactive oxygen and nitrogen species can activate many pro-inflammatory pathways and upregulate pro-inflammatory cytokines.[29,36] The mechanisms by which carvacryl acetate reduced MDA levels and increased GSH and SOD levels may have been related to the decrease in neutrophil migration to the small intestine mucosa, or by the reduction in the direct toxic action of CPT-11 on the epithelial cells. The increase in GSH levels may also be secondary to the consumption of other reactive oxygen species.[36]

The enzyme Na^+/K^+-ATPase is responsible for the active transport of many electrolytes in the small intestine. Its inhibition prevents the absorption of Na^+ and K^+ in the small intestine and, thus, induces intestinal fluid accumulation, which contributes to diarrhoea,[17,38] one of the side effects of CPT-11 therapy.[11] Information about Na^+/K^+-ATPase activity in intestinal mucositis is scarce. This study showed that Na^+/K^+-ATPase activity was diminished with CPT-11 treatment and returned after carvacryl acetate treatment. In this manner, carvacryl acetate may also help prevent diarrhoea found in intestinal mucositis, encouraging survival.

Based on these results, it is possible to infer that TRPA1 act as a sensitive channel for cell damage. Other authors showed this and the fact of its sensitivity are increased under inflammatory conditions in mouse sensory neurons, as demonstrated by other authors.[3,39] Through interactions with carvacryl acetate, which act as an agonist for this receptor, TRPA1 could act in the modulation of the cells responses to the damage made by ROS and stimulated for other inflammation related factors. When it is used an antagonist of TRPA1, it is possible to notice that this results are reverted and there is no response to the inflammation and cell damage, which is in concordance with the action of carvacryl acetate on TRPA1.

Conclusion

Results show that carvacryl acetate presented a strong and stable interaction with the TRPA1 receptor. This may be responsible for the anti-inflammatory and antioxidant action of carvacryl acetate in intestinal mucositis. Therefore, our results suggest that carvacryl acetate may be an effective drug to treat intestinal mucositis, especially because of its route of action through TRPA1.

Declarations

Conflict of interest

The Authors confirm that there is no conflict of interests.

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References

1. Damasceno SRB *et al.* Carvacryl acetate, a derivative of carvacrol, reduces nociceptive and inflammatory response in mice. *Life Sci* 2014; 94: 58–66.
2. Fortes AC *et al.* Aplicações do acetato de carvacrol em formulações farmacêuticas para o tratamento da esquistossomose. BR 1 O 2012 007004-9 A2, 19 March 2012, 19 November 2013.
3. Nilius B *et al.* The transient receptor potential channel TRPA1: from gene

to pathophysiology. *Pflugers Arch* 2012; 464: 425–458.
4. Kim MJ *et al.* The TRPA1 agonist, methyl syringate suppresses food intake and gastric emptying. *PLoS One* 2013; 8: e71603.
5. Yeoh A *et al.* Radiation therapy-induced mucositis: relationships between fractionated radiation, NF-κB, COX-1, and COX-2. *Cancer Treat Rev* 2006; 32: 645–651.
6. Stringer AM *et al.* Irinotecan-induced mucositis manifesting as diarrhea corresponds with an amended intestinal flora and mucin profile. *Int J Exp Pathol* 2009; 90: 489–499.
7. Takaishi M *et al.* 1,8-cineole, a TRPM8 agonist, is a novel natural antagonist of human TRPA1. *Mol Pain* 2012; 8: 1–12.
8. Vogel AI *et al. Vogel's Text Book of Practical Organic Chemistry*, 5th edn. Englewood Cliffs: Prentice-Hall, 1996.
9. Moraes J *et al.* Anthelmintic activity of carvacryl acetate against *Schistosoma mansoni*. *Parasitol Res* 2013; 112: 603–610.
10. Ikuno N *et al.* Irinotecan (CPT-11) and characteristic mucosal changes in the mouse ileum and cecum (reports). *J Natl Cancer Inst* 1995; 87: 1876–1883.
11. Melo ML *et al.* Role of cytokines (TNF-α, IL-1α and KC) in the pathogenesis of CPT-11-induced intestinal mucositis in mice: effect of pentoxifylline and thalidomide. *Cancer Chemother Pharmacol* 2008; 61: 775–784.
12. Alvarenga EM *et al.* Carvacrol reduces irinotecan-induced intestinal mucositis through inhibition of inflammation and oxidative damage via TRPA1 receptor activation. *Chem Biol Interact* 2016; 260: 129–140.
13. Wong DVT *et al.* The adaptor protein Myd88 is a key signaling molecule in the pathogenesis of irinotecan-induced intestinal mucositis. *PLoS One* 2015; 10: e0139985.
14. Woo PCY *et al.* Clarithromycin attenuates cyclophosphamide-induced mucositis in mice. *Pharmacol Res* 2000; 41: 526–532.
15. Lima-Júnior RC *et al.* Involvement of nitric oxide on the pathogenesis of irinotecan-induced intestinal mucositis: role of cytokines on inducible nitric oxide synthase activation. *Cancer Chemother Pharmacol* 2012; 69: 931–942.
16. Bewaji CO *et al.* Comparison of the membrane-bound $(Ca^{2+} + Mg^{2+})$-ATPase in erythrocyte ghosts from some mammalian species. *Comp Biochem Physiol* 1985; 82: 117–122.
17. Yakubu MT *et al.* Antidiarrhoeal activity of *Musa paradisiaca* Sap in wistar rats. *Evid Based Complement Alternat Med* 2015; 1: 1–9; Article ID 683726.
18. Bradley PP *et al.* Cellular and extracellular myeloperoxidase in pyogenic inflammation. *Blood* 1982; 60: 618–622.
19. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 1968; 25: 192–205.
20. Das K *et al.* A modified spectrophotometric assay of superoxide dismutase using nitrite formation by superoxide radicals. *Indian J Biochem Biophys* 2000; 37: 201–204.
21. Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 1978; 86: 271–278.
22. Green LC *et al.* Analysis of nitrate, nitrite and (15N) nitrate in biological fluids. *Anal Biochem* 1982; 126: 131–138.
23. Yeoh AS *et al.* Nuclear factor kappa B (NFκB) and cyclooxygenase-2 (Cox-2) expression in the irradiated colorectum is associated with subsequent histopathological changes. *Int J Radiat Oncol Biol Phys* 2005; 63: 1295–1303.
24. Levitt M, Perutz MF. Aromatic rings act as hydrogen bond acceptors. *J Mol Biol* 1988; 201: 751–754.
25. Vieth M *et al.* Do active site conformations of small ligands correspond to low free-energy solution structures. *J Comput Aided Mol Des* 1998; 12: 563–572.
26. Ribeiro RA *et al.* Irinotecan- and 5-fluorouracil-induced intestinal mucositis: insights into pathogenesis and therapeutic perspectives. *Cancer Chemother Pharmacol* 2016; 78: 881–893.
27. Lam W *et al.* The number of intestinal bacteria is not critical for the enhancement of antitumor activity and reduction of intestinal toxicity of irinotecan by the Chinese herbal medicine PHY906 (KD018). *BMC Complement Altern Med* 2014; 14: 490.
28. Johansson ME *et al.* The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci USA* 2008; 105: 15064–15069.
29. Abbas HA *et al.* Mdm2 is required for survival of hematopoietic stemcells/progenitors via dampening of ROS-induced p53 activity. *Cell Stem Cell* 2010; 7: 606–617.
30. Arifa RD *et al.* The reduction of oxidative stress by nanocomposite Fullerol decreases mucositis severity and reverts leukopenia induced by Irinotecan. *Pharmacol Res* 2016; 107: 102–110.
31. Wang J, Arase H. Regulation of immune responses by neutrophils. *Ann N Y Acad Sci* 2014; 1319: 66–81.
32. Sartor RB. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. *Gastroenterology* 1994; 106: 533–539.
33. Reaves TA *et al.* Neutrophil transepithelial migration: role of toll-like receptors in mucosal inflammation. *Mem Inst Oswaldo Cruz* 2005; 100: 191–198.
34. Logan RM *et al.* The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: pathobiology, animal models and cytotoxic drugs. *Cancer Treat Rev* 2007; 33: 448–460.
35. Kanarek N *et al.* Critical role for IL-1β in DNA damage induced mucositis. *Proc Natl Acad Sci USA* 2014; 111: E702–E711.
36. Yeoh AS *et al.* A novel animal model to investigate fractionated radiotherapy-induced alimentary mucositis: the role of apoptosis, p53, nuclear factor-κB, COX-1, and COX-2. *Mol Cancer Ther* 2007; 6: 2319–2327.
37. El-Ghazaly MA *et al.* Protective effect of the herbal preparation, STW 5,

against intestinal damage induced by gamma radiation in rats. *Int J Radiat Biol* 2015;91:150–156.
38. Sousa NA *et al.* The efficacy of a sulphated polysaccharide fraction from *Hypnea musciformis* against diarrhea in rodents. *Int J Biol Macromol* 2016; 86: 865–875.
39. Cattaruzza F *et al.* Transient receptor potential ankyrin-1 has a major role in mediating visceral pain in mice. *Am J Physiol Gastrointest Liver Physiol* 2010; 298: G81–G91.