Novel anti-inflammatory action of edelfosine lacking toxicity with protective effect in experimental colitis

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Number of text pages: 37
Number of tables: 1
Number of figures: 8
Number of references: 40
Abstract word count: 250
Introduction word count: 406
Discussion word count: 727

ABBREVIATIONS: Edelfosine, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH₃); HUVEC, human umbilical vein endothelial cells; IBD, inflammatory bowel disease; MPO, myeloperoxidase; NSAID, nonsteroidal anti-inflammatory drug; PGE₂, prostaglandin E₂; PMA, phorbol-12-myristate-13-acetate; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

Section: Inflammation, Immunopharmacology, and Asthma.
Abstract

Edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; ET-18-OCH₃) is an antitumor alkyl-lysophospholipid analog that binds lipid rafts, altering their protein composition (Gajate et al., 2004). Because L-selectin locates in lipid rafts and plays a crucial role in the recruitment of leukocytes into inflamed tissues, we hypothesized that edelfosine might affect inflammation by modulating L-selectin and inflammatory cell migration. Here, we have found that edelfosine inhibited neutrophil-endothelium interaction through L-selectin shedding. Oral treatment of edelfosine diminished inflammation in two murine animal models. Edelfosine showed a higher anti-inflammatory effect than the non-steroideal anti-inflammatory drug (NSAID) indomethacin in the bentonite mouse-paw edema model. Using a rat model of experimental colitis, edelfosine oral administration ameliorated the clinical and histopathologic severity of the inflammatory colitis with a dramatic decrease in mucosal damage and neutrophil infiltration. Colon sections from edelfosine-treated rats showed a remarkable reduction in ulcer formation, edema and inflammatory cell infiltration. Edelfosine enhanced lipopolysaccharide-induced expression of anti-inflammatory IL-10 in mouse macrophages. Edelfosine oral treatment in rats, at doses 8-fold higher than those displaying anti-inflammatory action, lacked toxicity. Edelfosine treatment showed no any significant cardiotoxicity, hepatotoxicity or renal toxicity. Unlike NSAIDs, edelfosine did not inhibit prostaglandin E₂ synthesis in gastrointestinal mucosal biopsies, and no histological alteration in gastrointestinal tract was detected following drug treatment. Thus, edelfosine shows a potent in vitro and in vivo anti-inflammatory activity while sparing gastric mucosa. Our data identify edelfosine as a novel anti-inflammatory drug by abating neutrophil
infiltration through L-selectin shedding, and may provide a new therapeutic approach for inflammatory bowel disease free from toxicity.
Introduction

Inflammation is a protective response, aimed to eliminate deleterious agents and to repair damaged tissues, but it may result in severe tissue destruction and dysfunction. A rapid influx of blood neutrophils characterizes the onset of the inflammatory response, constituting a hallmark of inflammation. Infiltrating cells are able to secrete a wide variety of inflammatory mediators, implying that a therapeutic potential may lie in preventing cellular-mediated pro-inflammatory processes.

Inflammatory bowel disease (IBD) embodies a spectrum of disorders that affect the gastrointestinal tract, the two major entities being Crohn’s disease and ulcerative colitis. Acute neutrophil influx is common in gastrointestinal diseases, and it is considered to play a causative role in inflammatory mucosal injury in IBD (Chin and Parkos, 2006). Current therapeutic agents used for IBD, including aminosalicylates, corticosteroids and immunosuppressive drugs, are not entirely effective and have multiple adverse side effects (Domenech, 2006). IBD is associated with an increased risk for developing colorectal cancer (Vagefi and Longo, 2005), and there is concern about the long-term use of immunosuppressive agents for IBD treatment that might enhance the chance of generating cancer, particularly lymphoma (Biancone et al., 2007).

Edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; ET-18-OCH₃) is the prototype member of a promising family of antitumor drugs collectively named synthetic alkyl-lysoospholipid analogs (Gajate and Mollinedo, 2002; Mollinedo et al., 2004). Edelfosine induces selectively apoptosis in tumor cells through its rather preferential uptake in cancer cells (Mollinedo et al., 1997; Gajate et al., 2000; Gajate et al., 2004; Gajate and Mollinedo, 2007), followed by co-clustering of Fas/CD95 death receptor in lipid rafts (Gajate and Mollinedo, 2001; Gajate et al., 2004; Gajate and Mollinedo,
Edelfosine binds to cell membranes altering their lipid raft composition (Gajate and Mollinedo, 2001; Gajate et al., 2004; Zaremberg et al., 2005; Ausili et al., 2008). L-selectin (CD62L), which mediates neutrophil-endothelium extravasation and plays a key role in the initial capture and rolling of circulating leukocytes on inflamed endothelium, has been located in lipid rafts in neutrophils (Abbal et al., 2006). Rolling adhesions mediated by L-selectin are sensitive to disruption of cholesterol rafts (Abbal et al., 2006).

On these grounds, we reasoned that edelfosine might affect inflammation through modulation of adhesion cell surface proteins in neutrophils. The aim of this study was to investigate the potential anti-inflammatory action of edelfosine and its putative beneficial use in animal models with special emphasis in inflammatory colitis. In addition, toxicity studies were performed to evaluate the safety of this new therapeutic use of edelfosine.

Methods

Cell Culture and Neutrophil Isolation. Human acute myeloid leukemia HL-60 cells and mouse 3T3 fibroblasts were grown in RPMI-1640 and DMEM culture medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere at 5% CO₂ and 95% air.

Neutrophils were freshly prepared from human peripheral blood by dextran sedimentation and centrifugation on Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) followed by hypotonic lysis of residual erythrocytes as previously described (Mollinedo et al., 2003). Cells were resuspended at 5 x 10⁶ neutrophils/ml in Hepes-glucose buffer (150 mM NaCl, 5 mM KCl, 10 mM Hepes, 1.2 mM MgCl₂, 1.3 mM CaCl₂, 5.5 mM
glucose, pH 7.5), and incubated at 37°C in the absence or presence of different agents for distinct periods of time. Subsequently, cells were collected by centrifugation to determine cell surface antigen expression by flow cytometry or their capacity to adhere to endothelial cells.

**Generation of Bone Marrow-Derived Mouse macrophages.** Bone marrow cells were obtained by flushing the femurs from CBF1 mice, and cultured as previously described (Munder et al., 1971) in hydrophobic Teflon bags (Biofolie 25, Heraeus, Hanau, Germany) in DMEM supplemented with 10% heat-inactivated FBS, 5% horse serum, 2 mM L-glutamine, 60 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and the supernatant of L929 fibroblasts at a final concentration of 15% (v/v) as a source of colony-stimulating factors, which drive cell proliferation towards a >95% pure population of bone marrow-derived macrophages.

**Fluorescence Labeling of Cells.** Neutrophils were labeled with calcein-AM (Molecular Probes, Eugene, OR) by incubating 5 x 10^6 cells /ml with 5 μM calcein-AM for 30 min at room temperature in calcein labeling buffer (HBSS without Ca^{2+} or Mg^{2+} containing 0.02% BSA). Cells were then washed twice with calcein labeling buffer and resuspended in the desired media.

**Endothelial Cell Adhesion Assay.** Neutrophil adhesion to human umbilical vein endothelial cells (HUVEC) was determined as previously described (Diaz-Gonzalez et al., 1995) with some modifications. HUVEC were isolated and grown in M199 culture medium supplemented with 10% heat-inactivated FBS, endothelial cell growth factor (50 μg/ml)
and porcine intestinal heparin (50 μg/ml) as described previously (Diaz-Gonzalez et al., 1995). HUVEC were grown to confluence on gelatin (0.1%)-coated 24-well plates, and incubated with either TNF-α (20 ng/ml, 6 h) or medium alone at 37ºC. Cells monolayers were washed four times with RPMI-1640 containing 5% heat-inactivated FBS, and 10⁶ untreated and drug-treated calcein-labeled neutrophils in 200 μl were added to each well. The plates were incubated under rotation at 64 rpm at 37ºC for 10 min, then aspirated and washed four times with PBS, and the fluorescence was quantitated with a fluorescence plate reader using excitation wavelength of 485 nm and an emission wavelength of 530 nm. Inhibition of neutrophil adhesion was calculated using the level of attachment of neutrophils to unstimulated HUVEC as the baseline, and the level of the untreated neutrophil attachment to TNF-α-stimulated HUVEC as the maximal value.

**Immunofluorescence Flow Cytometry.** Cell surface expression of leukocyte antigens was analyzed by immunofluorescence flow cytometry as previously described (Mollinedo et al., 1991; Gajate and Mollinedo, 2007) in a Becton Dickinson (San Jose, CA) fluorescence-activated cell sorting (FACS)Calibur™ flow cytometer. The following mouse monoclonal antibodies were used: TS1/11 anti-CD11a, Bear-1 anti-CD11b, TP1/36.1 anti-CD43, HP2/9 anti-CD44, Leu-8 anti-L-selectin, and W6/32, specific for a monomorphic determinant on HLA-A,B molecules, provided by F. Sánchez-Madrid (Hospital de La Princesa, Madrid, Spain). P3X63 myeloma culture supernatant and an isotype-matched fluorescein isothiocyanate(FITC)-conjugate nonrelevant IgG monoclonal antibody were used as negative controls, leading to virtually identical background values. Mean fluorescence intensity (MFI) in linear scale was obtained from at least 7000 cells in each sample, and the fluorescence produced by the myeloma P3X63 supernatant was considered as background. Results were
expressed as relative mean fluorescence intensity (rMFI), defined as \( \text{rMFI} = \frac{\text{MFI}_{\text{agent}} \times 100}{\text{MFI}_{\text{medium}}} \). Background fluorescence was subtracted from all values.

**Edelfosine Uptake.** Drug uptake was measured as described previously (Mollinedo et al., 1997) after incubating cells \( (10^6) \) in 1 ml RPMI-1640/10% heat-inactivated FBS containing 5 \( \mu \)g edelfosine + 0.05 \( \mu \)Ci \[^3\text{H}\]edelfosine for the indicated times, and subsequent washing (five times) with PBS + 2% BSA.

**Soluble L-Selectin ELISA.** Neutrophils \( (5 \times 10^6 \text{ cells/ml}) \) were incubated alone or with the indicated agents in Hapes-glucose buffer at 37ºC. Then, cells were centrifuged and the cell-free supernatants were tested for soluble L-selectin by an ELISA kit (Bender Medsystem, Vienna, Austria) according to the manufacturer’s instructions.

**Apoptosis Assay.** Quantitation of apoptotic cells was calculated by flow cytometry as the percentage of cells in the sub-G1 region (hypodiploidy) in cell cycle analysis as previously described (Gajate et al., 2000).

**Bentonite-Induced Paw Edema in Mice.** Four random groups of 10 Swiss mice each were set up for experimentation. The diameter of both hind paws was measured at day 0 and set as the basal value. Edema was induced by injecting subcutaneously 13.3 mg/ml bentonite in 0.03 ml PBS into the right hind paw. The same volume of 0.03 ml PBS was injected into the left hind paw as a control. The corresponding mice groups were treated daily with 2 mg/kg body weight of indomethacin or 2.5 and 5 mg/kg body weight of edelfosine, given orally in 0.2 ml PBS for 21 days. Inflammation control group was given 0.2 ml PBS only. The diameter of the
hind paws (in mm), as well as the body weight, was then calculated every other day, and inflamed areas were measured in arbitrary units. Inflammation was assessed by paw thickness measured as right paw thickness – left paw thickness.

**Ulcerative Colitis Model in Rats.** Experimental colitis in Wistar rats was induced by 2,4,6-trinitrobenzenesulphonic acid (TNBS) as described previously (Morris et al., 1989). Body weight, wet colon weight and score macroscopic evaluation were used as reference parameters of the disease and evaluated after 24 h and 7 days of the intraluminal administration of 20 mg TNBS (dissolved in 0.57 ml of 30% ethanol) in the colon. An ethanol group consisting of the intraluminal administration of 30% ethanol was also included. Edelfosine was orally administered at a daily dose 5 mg/kg body weight. Prednisone (5 mg/kg) was injected intramuscularly. Five random groups of Wistar rats were set for experimentation (blank saline control, ethanol control, colitis control, 5 mg/kg edelfosine, 5 mg/kg prednisone). At 24 h and 1 week after intracolonic administration of TNBS, 10 rats from each treatment group were killed by an injection of sodium pentobarbital (100 mg/kg, i.v.). Then, colons were dissected and the distal 9 cm were rinsed with saline, weighed (wet weight), opened by a longitudinal incision and scored for clinical macroscopic damage by two independent observers, using a previously established scale of 0-10 (Wallace et al., 1989). The criteria for scoring of morphologic damage were: 0, no damage; 1, localized hyperemia, but no ulcers; 2, hyperemia and thickening of bowel wall in the absence of ulcers; 3, one ulcer without thickening of the bowel wall; 4, two or more sites of ulceration or inflammation; 5, two or more sites of ulceration and inflammation or one site of ulceration/inflammation extending >1 cm along the length of the colon; 6-10, if damage covered >2 cm along the length of the colon, the score was increased by 1 for each
additional centimeter of involvement. In addition, sections of colon (close to cecum, medium zone and proximal to rectum) were removed and stored in 10% formalin for histopathological analysis. Fixed colonic samples were embedded in paraffin wax, sections were stained with hematoxylin and eosin, photographed, and examined by two independent observers in a blinded fashion. Measurement of myeloperoxidase (MPO) and neutrophil elastase activities were assessed as previously described (Woodruff et al., 2003; Cepinskas et al., 1999) as markers of neutrophil infiltration. Results for colon MPO and neutrophil elastase content were converted to absorbance units (OD at 450 nm, MPO; OD at 405 nm, neutrophil elastase) per gram of tissue.

**Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).** 6 x 10^6 bone marrow-derived mouse macrophages were seeded and stimulated in 6-well plates (Costar, Cambridge, MA) in a final volume of 3 ml/well. Total RNA was extracted from cells as previously described (Mollinedo et al., 1997), and the RNA preparations were carefully checked by gel electrophoresis and found to be free of DNA contamination. Reverse transcription was performed in 50 µl containing 5-10 µg of total RNA, 0.4 mM of each dNTP and 50 mU random hexamers (Promega, Madison, WI), 1 mM DTT (Gibco BRL, Paisley, UK), 50 mM Tris-HCl pH 8.3, 3 mM MgCl2, 62.5 mM KCl. After incubation at 70°C for 1 min, 10 U/µl Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and 2 U/µl RNasin (Promega) were added and incubated at 37°C for 60 min. Finally, the samples were incubated at 95°C for 1 min. One µl of the resulting cDNA (adjusted to a concentration of 50 ng/µl input RNA) was then amplified by PCR by using primers for mouse IL-1α (5’-CAGTTCTGCCATTGACCATC-3’ and 5’-
TCTCACTGAAACTCAGCCGT-3’), IL-1β (5’-TTGACGGACCACAAAAGATG-3’ and 5’-AGAAGGTGCTCATGTCTCA-3’), TNF-α (5’-TCTCATCAGTTCTATGGCCC-3’ and 5’-GGGAGTAGACAAGGTACAAC-3’), IL-10 (5’-AGCCGGGAAGACAATAACTG-3’ and 5’-CATTTCGATAAGGCTTGG-3’), and β-actin (5’-TGGAAATCTGTGGCATACTGAAAC-3’ and 5’-TAAAACGCGAGCTCAGTAGCTCCG-3’). A 50-µL PCR mixture contained 0.2 mM of each dNTP, 200 nM of each primer, 0.04 U Taq polymerase (HT Biotechnology Ltd., Cambridge, UK), 10 mM Tris-HCl pH 9.0, 1.5 mM MgCl₂, 50 mM KCl. PCR amplification was performed in a DNA thermal cycler (Thermocycler, Biometra Gmbh, Göttingen, Germany) for 28 cycles, shown to be at the linear phase of amplification, after an initial denaturation step for 5 min at 95°C with the following parameters: 30 s at 95°C, 30 s at 56°C (IL-1α, IL-1β, TNF-α), 58°C (IL-10) or 60°C (β-actin), and 60 s at 72°C. The PCR products were size fractionated onto a 2% agarose gel in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and stained with ethidium bromide. The expected sizes for the distinct PCR amplicons were: IL-1α (218 bp), IL-1β (204 bp), IL-10 (189 bp), TNF-α (212 bp), and β-actin (348 bp).

**ELISA.** The levels of cytokines in bone marrow-derived mouse macrophages were determined by using mouse ELISA kits from eBioscience (San Diego, CA), according to the manufacturer’s instructions.

**Toxicity Studies.** Wistar rats were separated in three groups and given orally edelfosine (daily dose of 40 mg/kg body weight for 1 and 4 weeks), or vehicle (saline) solution acting as the
untreated control group. An additional group of rats received doxorubicin (3 mg/kg, i.v., weekly for 4 weeks). A wide number of biochemical parameters were analyzed to evaluate the function of distinct organs, including kidney, liver and heart, using different commercial kits and a Hitachi 917 automated biochemistry analyzer (Diamond Diagnostics, Holliston, MA).

To analyze cardiovascular function, animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and placed on a heated animal board. Rectal temperature was monitored and maintained at 37°C. A tracheotomy was performed to facilitate breathing throughout the experiment. Animals were surgically prepared for the study by inserting PE-50 polyethylene catheters in the femoral vein and artery, and a PE-10 catheter in the carotid artery, that was advanced until its tip, was placed into the left ventricle. Then, both arterial catheters were connected to pressure transducers for the continuous recording of digital data (AD Instruments, Bella Vista, Australia), which allowed us to study a series of parameters, including arterial pressure and heart rate from the femoral artery transducer, mean arterial pressure, left ventricle systolic pressure and diastolic pressure from the right carotid intraventricular catheter, and maximal and minimal intraventricular pressures \( \frac{dP}{dt_{\text{max}}} \) and \( \frac{dP}{dt_{\text{min}}} \) from the intraventricular pressure curves (right carotid transducer).

After collecting cardiovascular data, abdominal aorta was cannulated and the animals were perfused with heparinized isotonic saline solution (0.9% NaCl) to wash the blood out, and kidney, liver, heart and stomach were removed for histology studies. The removed organs were fixed by immersion in 4% buffered formalin for 24 h. Blocks were dehydrated in a graded series of ethanol increasing gradation solutions, embedded in paraffin (60°C during 24 h), and 7 µm sections were cut by using a Microm HM-310
microtome (Fronine Laboratory, Riverstone, Australia), mounted on glass slides and stained with hematoxylin-eosin and Van Gieson trichrome.

**Prostaglandin E₂ (PGE₂) Assay.** To measure *ex vivo* PGE₂ synthesis, fresh normal human gastric mucosal biopsies, visually assessed by endoscopy, were washed with Hepes-glucose buffer and cut into 1-mg pieces, which were subsequently weighed and incubated in the absence or presence of distinct agents for 20 min at 37°C in 200 μl Hepes-glucose buffer. Then cells from mucosal gastric samples were pelleted by centrifugation, lysed and intracellular PGE₂ was measured using an enzyme immunoassay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) following the manufacturer’s instructions, and expressed as picograms of PGE₂ synthesized per mg of tissue. PGE₂ was also determined in rat colonic mucosal samples as above.

To measure PGE₂ in 3T3 cells, 1.5 x 10⁵ cells in 100 μl of complete DMEM medium were incubated overnight at 37°C (5% CO₂) in 96-well microtitre plates. Then, the distinct agents were added in 100 μl complete DMEM culture medium for 20 min at 37°C, and intracellular PGE₂ was measured as above.

**Data Analysis.** All values are expressed as means ± SEM. Between-group differences were evaluated by Mann-Whitney test and Student’s *t*-test. The criterion for statistical significance was taken as *p* < 0.05.
Results

Edelfosine Inhibits Neutrophil Adhesion to Endothelium by L-Selectin Shedding.

Edelfosine induces rather selective apoptosis in tumor cells through its favored uptake by cancer cells (Mollinedo et al., 1997; Gajate et al., 2000; Gajate et al., 2004; Gajate and Mollinedo, 2007). Peripheral blood mature neutrophils took up low amounts of edelfosine in comparison to the human myeloid leukemia cell line HL-60 (17 ± 3 ng/10⁶ neutrophils versus 148 ± 25 ng/10⁶ HL-60 cells, after 2 h incubation with 5 μg/ml [³H]edelfosine; n = 5). We found that edelfosine did not induce apoptosis in peripheral blood human neutrophils, end cells that undergo spontaneous apoptosis after 9-12 h incubation in culture medium (Santos-Beneit and Mollinedo, 2000), and conversely the onset of the cell death program was somewhat delayed in the presence of the drug (12 ± 4 % and 42 ± 7 % apoptosis after 12 and 18 h in culture medium versus 7 ± 2 % and 32 ± 4 % apoptosis after 12 and 18 h in culture medium containing 5 μg/ml edelfosine; n = 3). Because edelfosine binds easily to cell membranes (Ausili et al., 2008), we examined whether the low uptake of edelfosine in neutrophils might affect some neutrophil cell surface proteins. An early event in inflammation is L-selectin-mediated adhesion of neutrophils to the endothelium blood vessel lining, followed by neutrophil extravasation into the surrounding tissues (Tedder et al., 1995). Neutrophils adhere to HUVEC stimulated with TNF-α to induce the expression of L-selectin ligand(s) (Diaz-Gonzalez et al., 1995). Neutrophil-endothelial cell interaction was largely mediated by L-selectin, since anti-L-selectin LAM1-3 monoclonal antibody inhibited neutrophil adhesion (Figure 1A). Interestingly, the adhesion of neutrophils to endothelium was dramatically inhibited when human neutrophils were preincubated with increasing concentrations of edelfosine (Fig. 1A). Drug pretreatment showed a similar inhibitory effect as the anti-L-selectin antibody on neutrophil adhesion to
endothelium (Fig. 1A). This effect was not due to a decrease in neutrophil or HUVEC viability (>95% cells remained viable by trypan blue and apoptosis analysis through cell cycle examination by flow cytometry). We found that human neutrophils incubated with edelfosine were not affected in their side versus forward scatter parameters by flow cytometry (Fig. 1B, and data not shown). Side versus forward scatter dot plots of human neutrophils were not modified by edelfosine treatment at distinct concentrations (0.01-5 μg/ml edelfosine) and incubation times (5-30 min) (data not shown). Nevertheless the neutrophil cell surface content of L-selectin and CD43, but not of HLA, was highly decreased following edelfosine treatment (Fig. 1B). Edelfosine induced a concentration-dependent down-regulation of cell surface L-selectin expression on human neutrophils (Fig. 1C), akin to that induced by phorbol-12-myristate-13-acetate (PMA) (Fig. 1C) used as a positive control (Alexander et al., 2000). Edelfosine-induced down-regulation of L-selectin and CD43 was very rapid, reaching a maximum within 15-min incubation (Fig. 1D). This effect was not due to neutrophil activation, since cell surface expression of CD11b was largely unaffected upon drug treatment (Fig. 1C). CD11b cell surface up-regulation is an early event in neutrophil activation with different stimuli, such as PMA (Fig. 1C), as a result of the incorporation of granule membrane CD11b into the plasma membrane due to the prone secretion of tertiary granules upon neutrophil stimulation (Mollinedo et al., 1991). Cell surface expression of CD43 and CD44 adhesion molecules, but not of CD11a and HLA, was also down-regulated by edelfosine (Fig. 1, C and D), indicating that drug treatment does not lead to a general down-regulation of cell surface proteins. Edelfosine-induced L-selectin down-regulation was accompanied by an increase in the amount of soluble L-selectin at the extracellular medium (Fig. 1E). These data indicate that L-selectin
is rapidly shed by edelfosine, thus interfering with initial neutrophil-endothelium adhesion stages.

**Edelfosine Inhibits Paw Edema Model of Inflammation.** We next examined the *in vivo* anti-inflammatory potential of edelfosine in the bentonite mouse-paw edema model. We found that orally administered edelfosine inhibited 34% and 49% inflammation, when given at 2.5 and 5 mg/kg body weight, respectively, whereas the nonsteroidal anti-inflammatory drug (NSAID) indomethacin inhibited 31% (Fig. 2). No body weight loss was observed during drug treatment.

**Edelfosine Protects Against Experimental Colitis.** Next, we analyzed the anti-inflammatory effect of orally administered edelfosine in a rat model of experimental colitis induced by intracolonic administration of TNBS (Morris et al., 1989). This model shares many of the histopathological and clinical features of human chronic IBDs, such as ulcerative colitis and Crohn’s disease (Dieleman et al., 1997). TNBS colitis is characterized by a predominant T helper cell type 1 (Th1)-mediated immune response, involved in cell-mediated immunity and phagocyte-dependent responses (Romagnani, 1999; Kitani et al., 2000). Because Th1 cells are implicated in the pathogenesis of organ-specific autoimmune disorders, including Crohn’s disease, the TNBS colitis model is appropriate to examine the putative role of edelfosine in this disease (Romagnani, 1999; Kitani et al., 2000). The TNBS-induced colitis control group showed an inflammatory response, recognized by the presence of ulcerative fissures and bowel wall inflammation after 24 h of TNBS intraluminal administration. A chronic inflammatory response was observed 7 days after TNBS administration, with additional lineal or punctual ulcers, necrosis of epithelium,
edema, reduction of colonic lumen, granulomas, and extensive mucosal and submucosal infiltration of inflammatory cells. The protective action of edelfosine was assessed by histopathology examination and by a number of disease parameters, including body weight, wet colon weight and colonic mucosal damage score evaluated after 24 h and 7 days of TNBS administration. Because of the solvent used for TNBS, an ethanol-treated control group was also included. This latter showed a transient acute bowel inflammation response following 24 h of intraluminal administration, which decreased thereafter reaching similar values to those corresponding to the saline-instilled control rats after 7-day treatment. We also examined the effect of prednisone (5 mg/kg) as a comparison with the anti-inflammatory activity of edelfosine (molecular weights of predinose and edelfosine are 358.4 and 523.7, respectively). TNBS challenge induced a slight body weight loss, being more pronounced in the prednisone-treated group (Figure 3A). Edelfosine (daily dose of 5 mg/kg body weight) significantly protected against body weight loss (Fig. 3A) and showed a remarkable anti-inflammatory activity, with a dramatic decrease in the severity of mucosal damage (Fig. 3B) and colon weight (Figure 3C) compared to the TNBS-administered drug-free colitis control group. Edelfosine inhibited the damage score associated to this disease by 45.5% (data not shown) and 76.5% (Fig. 3B) after 24 h and 7 days of TNBS administration, respectively. In contrast, prednisone showed a small inhibition of inflammation that was not statistically significant (Fig. 3, B and C). Stained colon sections were examined for signs of pathology (Fig. 4, A-E). At 7 days after TNBS challenge, there was evidence of healing with affected mucosa being replaced by surface epithelium in ethanol- and edelfosine-treated rats (Fig. 4, B and D). Colon sections from drug-free colitis control rats (Fig. 4C) showed a severe pathology with intense mucosal destruction, and mucosal and submucosal inflammatory cell infiltration, mostly neutrophils.
Sections of colon from saline- and ethanol-instilled rats showed minimal signs of damage (Fig. 4, A and B). Colon sections from edelfosine-treated rats ameliorated the pathologic severity of the inflammatory colitis, decreasing ulcer formation, edema, and inflammatory cell infiltration as well as inducing reparative phenomena (Fig. 4D). Sections from prednisone-treated rats showed little improvement in disease pathology (Fig. 4E). Colitis control rats showed widespread submucosal intense inflammatory cell infiltration (Fig. 4C, inset), whereas only a submucosal focal mild inflammatory cell infiltration was observed in edelfosine-treated rats (Fig. 4D, inset). These data indicate that edelfosine exerts an outstanding protective effect in experimental colitis. The beneficial effect of edelfosine contrasts with the lack of improvement of prednisone treatment when used at similar doses. This is in agreement with previous reports showing no significant amelioration in disease pathology when prednisone was added after TNBS addition (Woodruff et al., 2003), likely due to the delayed time it takes for steroids to become effective and to their tendency to inhibit natural healing processes associated with disease pathology. Measurement of MPO activity has been widely used as a reliable index of neutrophil infiltration in colon (Martin et al., 2005). We found a high increase in MPO activity in the colon of TNBS-injected rats (Fig. 5A), and a dramatic reduction in colonic MPO was detected when rats were treated with edelfosine (Fig. 5A). Furthermore, we found that edelfosine prevented the increase in neutrophil elastase activity, an additional well-known marker for neutrophil infiltration, in the TNBS-induced colitis model (Fig. 5A). The low elastase activity detected in rats (Fig. 5A) is likely due to the high anti-neutrophil elastase activity present in this rodent species, 3-fold higher than in humans or hamsters (Takahara et al., 1983). Taken together, these data indicated a high neutrophil infiltration in the colons of TNBS-injected rats, which was well-nigh completely inhibited by edelfosine treatment. This was confirmed by further analysis.
of histological sections showing widespread submucosal intense inflammatory cell infiltration in colitis control rats, whereas only a submucosal focal mild inflammatory cell infiltration was observed in edelfosine-treated rats (Fig. 5B). In addition, as shown in Fig. 4D, large areas of colon were free of inflammatory cell infiltration in edelfosine-treated rats. The biochemical and histopathological data shown in Fig. 5 confirm that edelfosine targets on abating neutrophil infiltration in colonic laminar propria in TNBS-induced colitis. Thus, these data indicate that edelfosine exerts an outstanding protective effect on experimental colitis by inhibiting neutrophil infiltration.

**Effect of Edelfosine on the Generation of Macrophage-Derived Cytokines.** During the course of inflammation, the initially predominant neutrophils at the inflammatory foci are replaced by migrating monocytic cells that differentiate into macrophages. Using mouse bone marrow-derived macrophages as an abundant source of macrophages, we analyzed the effect of edelfosine on the expression of a number of pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α) and the anti-inflammatory cytokine IL-10. Fig. 6A shows that edelfosine by itself does not induce cytokine expression, as assessed by semiquantitative RT-PCR, but potentiates the lipopolysaccharide-induced expression of IL-10, without affecting the expression of the proinflammatory cytokines. In addition, edelfosine diminished the basal expression of IL-1β and TNF-α mRNA (Fig. 6A). These changes in the mRNA levels of cytokines led to alterations of their protein levels, as assessed by ELISA. Edelfosine inhibited the basal protein expression of IL-1β and TNF-α (Fig. 6B). In addition, edelfosine potentiated the lipopolysaccharide-induced protein expression IL-10 (Fig. 6B).
Edelfosine Treatment Lacks Toxicity. Rats were given edelfosine orally at a daily dose of 40 mg/kg body weight, a 8-fold higher dosage than the one exerting the anti-inflammatory effect, for 1 and 4 weeks. No weight loss was observed during the four weeks of treatment and no overt toxicity was detected. Biochemical and functional analyses indicated that edelfosine showed no cardiotoxicity, hepatotoxicity or renal toxicity (Table 1). The toxicological data of edelfosine are in clear contrast with those of doxorubicin, showing cardiotoxicity as well as kidney alterations (Table 1). Histological studies demonstrated no damage in kidney, liver, heart and stomach following edelfosine treatment (Fig. 7). Edelfosine did not elicit hypertension (Table 1) or any histological harm at the myocardium (Fig. 7).

Edelfosine Does Not Inhibit Mucosal PGE2 Synthesis. NSAIDs are widely used as anti-inflammatory agents, but have important gastrointestinal toxic side effects, mostly due to the inhibition of the synthesis of PGE2 required for gastric mucosa regeneration (Parente and Perretti, 2003). We next studied whether edelfosine could interfere with the generation of gastric PGE2 in human mucosal biopsies. Calcium ionophores induce NSAID-inhibitable PGE2 generation as a result of COX upregulation (Horton et al., 1999). We found that ionomycin induced an increase in PGE2 synthesis that was blocked by indomethacin (Fig. 8A), but edelfosine neither affected basal PGE2 production, nor inhibited ionomycin induced-PGE2 generation (Fig. 8A). Similar results were obtained in 3T3 mouse fibroblasts (Fig. 8B). We also found that edelfosine oral treatment did not inhibit basal PGE2 production in rat colonic mucosal samples (78 ± 10 and 83 ± 9 pg PGE2/mg tissue, in control untreated and edelfosine-treated rats, respectively; n = 3). These results indicate that
edelfosine exerts its anti-inflammatory action without inhibiting COX activity, thus sparing gastrointestinal mucosa.

**Discussion**

The *in vivo* and *in vitro* data reported here show a novel role of edelfosine as a potent anti-inflammatory agent. This anti-inflammatory activity results by preventing adhesion of neutrophils to endothelial cells through L-selectin shedding. Interaction of neutrophils with endothelium is a key step in the pathophysiology of inflammation, preceding extravasation of neutrophils into the tissue. Pharmacologically-induced L-selectin shedding from neutrophil plasma membrane has been previously described for different NSAIDs (Diaz-Gonzalez et al., 1995), aceclofenac being the most potent drug in this action (Gonzalez-Alvaro et al., 1996). However, our data show that the IC$_{50}$ of edelfosine for L-selectin down-regulation is 0.29 μM (0.15 μg/ml; molecular weight, 523.7), 53-fold lower than that of aceclofenac (15.33 μM; molecular weight, 354.2) (Gonzalez-Alvaro et al., 1996). The fact that edelfosine induces L-selectin shedding from human neutrophils, despite its low uptake in these cells, suggests drug-induced changes at the cell surface following drug-membrane interaction. Edelfosine alters the biophysical properties of model membranes (Ausili et al., 2008), and accumulates in lipid rafts modifying raft protein and lipid composition (Gajate et al., 2004; Zaremberg et al., 2005; Gajate and Mollinedo, 2007). Lipid rafts have been recently involved in L-selectin-dependent leukocyte rolling (Abbal et al., 2006). Thus, it could be speculated that edelfosine induces L-selectin shedding through its action on lipid rafts. Here we have found that oral administration of edelfosine inhibits neutrophil infiltration and ameliorates the pathologic severity of inflammatory colitis. Neutrophils are the cornerstone cell type in inflammation, and our data indicate that
edelfosine acts on early neutrophil-dominated stages of inflammation. In addition, edelfosine has been reported to inhibit the expression of adhesion molecules in endothelial cells (Bosse et al., 1995), thus further supporting an anti-inflammatory role for this drug by abating neutrophil-endothelium interaction.

The anti-inflammatory activity of edelfosine seems to be underlain by its effects on adhesion molecules, thus preventing inflammatory cells from crossing blood vessel walls to reach affected organs. In this regard, natalizumab, the first commercially available selective adhesion-molecule inhibitor, is a humanized monoclonal antibody against alpha-4 integrin, which was recently approved for the treatment of multiple sclerosis and Crohn’s disease (Fiore, 2007; Honey, 2008). Natalizumab is believed to work by inhibiting the ability of inflammatory immune cells to attach to and pass through the cell layers lining the blood-brain barrier and intestines in multiple sclerosis and Crohn’s disease, since alpha-4 integrin is required for white blood cells to move into organs. Natalizumab is administered by intravenous infusion, but the occurrence of a number of adverse effects, including hepatotoxicity and in some cases the appearance of the rare neurological condition progressive multifocal leukoencephalopathy (Stuve and Bennett, 2007), has raised some doubts over its risk/benefit ratio. In addition, concern has been shown over the risks of infection and cancer following natalizumab treatment (Mullen et al., 2008). Thus, if the in vitro and in vivo data reported here proved to be clinically significant for humans, the putative use of edelfosine would show outstanding traits, including its oral administration (Gajate and Mollinedo, 2002; Mollinedo et al., 2004) and lack of hepatotoxicity, cardiotoxicity and renal toxicity.

In addition, we found that the anti-inflammatory action of edelfosine was further supported by the fact that edelfosine enhanced lipopolysaccharide-induced mRNA and
protein expression of the anti-inflammatory cytokine IL-10 and attenuated the basal mRNA and protein expression of the pro-inflammatory cytokines IL-1β and TNF-α in bone marrow-derived mouse macrophages.

Our data indicate no toxicity following edelfosine oral treatment in rats. The absence of PGE2 inhibition and toxicity following edelfosine treatment makes this compound devoid of the harmful gastrointestinal and/or cardiotoxicity side effects of currently used anti-inflammatory drugs, such as NSAIDs and selective COX-2 inhibitors (Akarca, 2005; Graham et al., 2005). The in vivo anti-inflammatory effect of edelfosine in two experimental animal models, together with the lack of any deleterious effect, even at 8-fold higher concentrations than those displaying an anti-inflammatory effect, indicate that edelfosine could be considered as a novel anti-inflammatory drug without toxic side effects. Ulcerative colitis and Crohn’s disease are chronic inflammatory disorders of the bowel that fall under the banner of IBDs, and for which current medical therapies are not satisfactory. Thus, edelfosine may constitute a novel leading compound to ameliorate IBDs and other inflammatory ailments with no adverse side effects. In addition, the dual benefits of edelfosine in anti-cancer and anti-IBD therapy makes this drug an attractive and promising pharmacologic agent with multiple applications.

Acknowledgments

We thank Rocio Misiego for excellent and expert technical assistance. We are indebted to the Blood Bank, and to the Surgery, Pathological Anatomy and Gynecology Departments of the University Hospital of Salamanca for providing us with human peripheral blood, gastric biopsies and umbilical cords.
References


FOOTNOTES

Financial support

This work was supported in part by grants from the Spanish Ministry of Science and Innovation (SAF2008-02251, SAF2005-04293, RD06/0020/10370 – Red Temática de Investigación Cooperativa en Cáncer, Instituto de Salud Carlos III), Fondo de Investigación Sanitaria and European Commission (FIS-FEDER 06/0813), Fundación “la Caixa” (BM05-30-0), Junta de Castilla y León (CSI01A08, SAN673/SA32/08, and GR15 – Experimental Therapeutics and Translational Oncology Program), and Fundación de Investigación Médica Mutua Madrileña (FMM). C.G. was supported by the Ramón y Cajal Program from the Spanish Ministry of Science and Innovation.

Consuelo Gajate has a U.S. patent (US 6,583,127 B1) related to these studies. The other authors disclose no potential conflicts of interest.

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Legends for Figures

Fig. 1. Edelfosine inhibits human neutrophil adhesion to endothelial cells and induces shedding of L-selectin. (A) Effect of edelfosine on neutrophil attachment to human endothelial cell monolayers. HUVEC, cultured for 6 h in the presence of 20 ng/ml TNF-α, were used for neutrophil adhesion assays. HUVEC were then incubated with calcein-labeled neutrophils in medium alone (control, C), with calcein-labeled neutrophils pretreated with LAM1-3 monoclonal antibody (LAM1-3) or with calcein-labeled neutrophils pretreated for 15 min at 37°C with increasing amounts of edelfosine (EDLF). Then, percent of calcein-labeled neutrophil adhesion to the endothelial layer was determined, considering the fluorescence value corresponding to untreated control calcein-labeled neutrophils as 100%. (B) Isolated peripheral blood human neutrophils, showing an homogenous side versus forward scatter population (upper panel) were incubated for 15 min at 37°C in the absence (Control) and in the presence of 5 μg/ml edelfosine (EDLF), and then analyzed by flow cytometry for the cell surface expression of L-selectin, CD43 and HLA (lower panels). P3X63 myeloma supernatant was used as a negative control. The results are representative of five separate experiments. (C) Surface expression of leukocyte antigens following edelfosine treatment. Neutrophils (5 x 10⁶ cells/ml) were incubated for 15 min at 37°C in the absence (control) or in the presence of 50 ng/ml PMA or of increasing concentrations of edelfosine (EDLF). Then, cell surface expression of the indicated leukocyte antigens was analyzed by flow cytometry, and rMFI values were estimated using P3X63 myeloma supernantant and an isotype-matched FITC-conjugate nonrelevant IgG monoclonal antibody as negative controls. Cell surface expression (rMFI)
of each antigen in untreated control neutrophils was considered as 100. (D) Time-course of the down-regulation of cell surface CD43 and L-selectin in edelfosine-treated neutrophils. Neutrophils (5 x 10^6 cells/ml) were incubated at 37°C for the times indicated in the absence (control) or in the presence of 50 ng/ml PMA or 5 μg/ml edelfosine (EDLF). Then, cell surface antigen expression and rMFI vales were determined as above. (E) Quantification of neutrophil-shed L-selectin induced by edelfosine. Neutrophils (5 x 10^6 cells/ml) were incubated for 15 min at 37°C either in medium alone (control, C) or in the presence of 50 ng/ml PMA or of increasing concentrations of edelfosine. Then, cells were centrifuged, and supernatants were assayed for soluble L-selectin by ELISA. Values were obtained in duplicate determinations for each sample. Data shown are means ± SEM (n = 5). Asterisks denote significant differences with the control group (*, p < 0.05; **, p < 0.01).

Fig. 2. *In vivo* anti-inflammatory effect of edelfosine in a paw edema mouse model. Comparison of paw thickness (in arbitrary units, A.U.) following injection of bentonite into the right hind paw and buffer in the left paw of mice among bentonite drug-free mice (inflamed control, IC), bentonite edelfosine (EDLF)-treated mice (daily dose of 2.5 or 5 mg EDLF/kg body weight for 21 days, EDLF-2.5 and EDLF-5), and bentonite indomethacin-treated mice (daily dose of 2 mg/kg body weight for 21 days, INDO). Inflammation was measured by paw thickness measured as right paw thickness – left paw thickness. Data shown are means ± SEM (n = 10). Asterisks denote significant differences with the IC group (*, p < 0.05; **, p < 0.01).
**Fig. 3.** *In vivo* anti-inflammatory effect of edelfosine in an ulcerative colitis rat model. Effects of edelfosine and prednisone on body weight in grams (A), colonic damage score (B), and colon wet weight (C), one week after intracolonic administration of TNBS. Rats received a colonic injection of either TNBS, or of a saline (saline control, SC) or ethanol solution (ethanol control, EC) for sham animals on day 0. Then, TNBS-injected animals were untreated (colitis control, CC) or treated with edelfosine (EDLF) (5 mg/kg) or prednisone (PRED) (5 mg/kg). Data shown are means ± SEM (*n* = 10). Asterisks denote significant differences with the SC (A) and CC (B, C) groups (*, *p* < 0.05; **, *p* < 0.01).

**Fig. 4.** Histological patterns in the effect of edelfosine on the ulcerative colitis rat model. Photomicrographs of sections of colons from rats stained with hematoxylin and eosin one week after intracolonic administration of TNBS. Rats received a colonic injection of either TNBS, or of a saline or ethanol solution for sham animals on day 0. Then, TNBS-injected animals were untreated or treated with edelfosine (5 mg/kg) or prednisone (5 mg/kg). Colon microscopic images of: (A) saline-instilled, sham-rat with normal histologic characteristics (inset at the right panel shows normal appearance of the mucosal crypts of Lieberkühn, submucosa and muscular layers); (B) ethanol-instilled, sham rat showing focal reparative mucous epithelium after ulcerous lesion (inset at the right panel shows postulcerous loss of the pattern of the colonic mucosal glands and regenerated surface epithelium); (C) TNBS drug-free colitis control with total destruction of the mucosal intestinal layer with intense submucosal and serosal inflammatory infiltration (inset at the right panel shows mucosal destruction, submucosal cellular inflammatory infiltrate and serosal inflammation); (D) TNBS edelfosine-treated rat, showing focal postulcerous reparative processes (inset at the right panel shows a weak desorganization of the glandular pattern, moderate focal mucosal...
and submucosal inflammatory infiltrate and regenerated surface epithelium); (E) TNBS prednisone-treated rat, showing severe mucosal necrosis, submucosal inflammatory infiltrate (inset at the right panel shows mucosal necrosis, submucosal inflammatory infiltrate and serosal inflammatory infiltration). Right panels show an enlarged view of the insets indicated at the left panels. Bar, 1000 μm. Images shown are representative of ten rats treated.

**Fig. 5.** Neutrophil infiltration in the ulcerative colitis rat model. (A) Colon MPO (upper) and neutrophil elastase (lower) activity levels were measured as an estimate for neutrophil infiltration. Rats were intracolonomically administered with either TNBS or a saline/ethanol solution (ethanol control, EC) for sham animals on day 0. Then, TNBS-injected animals were untreated (colitis control, CC) or treated with edelfosine (EDLF) (daily dose of 5 mg/kg). Levels of MPO and neutrophil elastase in the colons of the rats were then measured after 7 days. Data shown are means ± SEM (n = 10 for MPO, n = 3 for neutrophil elastase). Asterisks denote significant differences with the CC group (**, p < 0.01). (B) Photomicrographs of submucosal inflammatory cell infiltration in TNBS-administered rats, showing submucosal intense inflammatory cell infiltration in TNBS drug-free rats (colitis control, CC), and submucosal mild inflammatory cell infiltration in TNBS edelfosine-treated rats (EDLF). Bar, 100 μm.

**Fig. 6.** Effect of edelfosine on macrophage-derived cytokine production. Bone marrow-derived mouse macrophages were incubated in culture medium in the absence or presence of 5 μg/ml edelfosine (EDLF) for 24 h, and then stimulated or not with 0.1 μg/ml
lipopolysaccharide (LPS) for 24 h, and analyzed for the induction of the indicated cytokines by semiquantitative RT-PCR (A) or ELISA (B). Untreated control cells were run in parallel. The results in A are representative of three separate experiments. Data shown in B (% of control) are means ± SEM (n = 3). Asterisks denote significant differences of the EDLF and EDLF + LPS groups with the Control and LPS groups respectively (*, p < 0.05; **, p < 0.01).

**Fig. 7.** Histological examination in toxicology studies of edelfosine in rats. Photomicrographs of sections of kidney (A), liver (B), myocardium (C), and stomach (D) from edelfosine-treated rats (40 mg/kg, 4 weeks) show no histological alterations. Bar, 100 μm.

**Fig. 8.** Edelfosine does not inhibit PGE₂ generation. (A) Effect of edelfosine on human gastric mucosa PGE₂ synthesis. Human mucosal gastric biopsies were incubated with the indicated agents (ionomycin, Iono, 10 μM; edelfosine, EDLF, 5 μg/ml - 9.5 μM; indomethacin, Indo, 10 μM) for 20 min at 37°C, and then the intracellular PGE₂ content was determined. Untreated control samples (C) were run in parallel. (B) 3T3 cells (1.5 x 10⁵) were incubated with the indicated agents as above and then the intracellular content of PGE₂ was determined. Untreated control samples (C) were run in parallel. Data shown are means ± SEM (n = 3). Asterisks denote significant differences with the untreated control group (*, p < 0.05; **, p < 0.01).
Table 1

Toxicity parameters in edelfosine-treated rats

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>UNTREATED</th>
<th>EDLF (1 wk)</th>
<th>EDLF (4 wk)</th>
<th>DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/day)</td>
<td>0.22 ± 0.02</td>
<td>0.36 ± 0.06</td>
<td>0.54 ± 0.08</td>
<td>2.49 ± 0.36*</td>
</tr>
<tr>
<td>GGT (U/day)</td>
<td>0.99 ± 0.16</td>
<td>0.57 ± 0.11</td>
<td>1.15 ± 0.34</td>
<td>13.65 ± 3.03*</td>
</tr>
<tr>
<td>LDH (U/day)</td>
<td>0.10 ± 0.01</td>
<td>0.13 ± 0.04</td>
<td>0.16 ± 0.03</td>
<td>3.65 ± 0.62*</td>
</tr>
<tr>
<td>NAG (U/day)</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>CreatCL (ml/min)</td>
<td>1.35 ± 0.10</td>
<td>0.93 ± 0.08</td>
<td>2.05 ± 0.12</td>
<td>0.83 ± 0.02*</td>
</tr>
<tr>
<td>Alb (mg/day)</td>
<td>0.37 ± 0.01</td>
<td>0.22 ± 0.04</td>
<td>0.36 ± 0.05</td>
<td>2.20 ± 0.05*</td>
</tr>
<tr>
<td>UF (dl/day)</td>
<td>0.12 ± 0.01</td>
<td>0.17 ± 0.03</td>
<td>0.17 ± 0.01</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>PI Creat (mg/dl)</td>
<td>0.43 ± 0.02</td>
<td>0.35 ± 0.02</td>
<td>0.35 ± 0.03</td>
<td>0.86 ± 0.03*</td>
</tr>
<tr>
<td>Cl (mEq/day)</td>
<td>2.94 ± 0.13</td>
<td>1.73 ± 0.26</td>
<td>3.99 ± 0.32</td>
<td>0.45 ± 0.02*</td>
</tr>
<tr>
<td>K (mEq/day)</td>
<td>2.17 ± 0.06</td>
<td>1.31 ± 0.18</td>
<td>2.46 ± 0.15</td>
<td>0.92 ± 0.16*</td>
</tr>
<tr>
<td>Na (mEq/day)</td>
<td>2.43 ± 0.10</td>
<td>1.08 ± 0.23</td>
<td>3.23 ± 0.24</td>
<td>0.42 ± 0.25*</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>201.80 ± 20.92</td>
<td>127.17 ± 8.01</td>
<td>148.80 ± 4.27</td>
<td>ND</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>121.60 ± 8.95</td>
<td>140.5 ± 11.03</td>
<td>98.25 ± 13.46</td>
<td>152.8 ± 3.74</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>21.75 ± 1.61</td>
<td>29.25 ± 5.75</td>
<td>32.25 ± 2.17</td>
<td>26.20 ± 2.44</td>
</tr>
<tr>
<td>CPK (U/L)</td>
<td>444 ± 56</td>
<td>592 ± 85</td>
<td>420 ± 81</td>
<td>755 ± 151*</td>
</tr>
<tr>
<td>CKMB (U/L)</td>
<td>949 ± 108</td>
<td>966 ± 123</td>
<td>821 ± 175</td>
<td>1150 ± 195*</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>433 ± 9</td>
<td>397 ± 14</td>
<td>424 ± 10</td>
<td>348 ± 22*</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>143 ± 13</td>
<td>147 ± 3</td>
<td>166 ± 10</td>
<td>138 ± 9</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>-13.6 ± 3.1</td>
<td>-15.0 ± 2.6</td>
<td>-14.2 ± 1.3</td>
<td>3.7 ± 2.5*</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>122 ± 4</td>
<td>129 ± 5</td>
<td>123 ± 2</td>
<td>116 ± 3</td>
</tr>
<tr>
<td>dP/dt Max (mmHg/min)</td>
<td>2966 ± 221</td>
<td>2796 ± 153</td>
<td>3217 ± 249</td>
<td>1526 ± 66*</td>
</tr>
<tr>
<td>dP/dt Min (mmHg/g/min)</td>
<td>-2950 ± 204</td>
<td>-2837 ± 135</td>
<td>-3216 ± 222</td>
<td>-1725 ± 48*</td>
</tr>
</tbody>
</table>

Biochemical and functional parameters of distinct organs were measured in rats untreated and treated with edelfosine (EDLF) (for 1- or 4-week treatment) or doxorubicin (DOX). Renal function was estimated by measuring: daily excretion of alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG), albumin (Alb), chloride, potassium and sodium in urine, creatinine clearance (CreatCL), urinary flow (UF), and creatinine concentration in plasma (PI Creat). Hepatic function was evaluated by measuring in plasma the enzymatic activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). Cardiovascular function was assessed by measuring in plasma the enzymatic activities of creatine phosphokinase (CPK) and its isoenzyme creatine phosphokinase MB (CKMB), as well as by measuring heart rate (HR), left ventricle systolic pressure (LVSP) and diastolic pressure (LVDP), mean arterial pressure (MAP), maximal (dP/dt Max) and minimal (dP/dt Min) intraventricular pressures. Data shown are means ± SEM of 15 rats. Asterisks denote significant differences with the untreated control group (*, p < 0.05).
Figure 1

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Figure 2

![Bar chart showing paw thickness (A.U.) for IC, EDLF-2.5, EDLF-5, and INDO.](image)

- IC: 10
- EDLF-2.5: 8
- EDLF-5: 6
- INDO: 10

* and ** indicate statistically significant differences.
Figure 3

A

Body weight (g)

1 week

2 weeks

SC  EC  CC  EDLF  PRED

B

Damage score

EC  CC  EDLF  PRED

C

Colon weight (g)

EC  CC  EDLF  PRED

*  **  *
Figure 4
Figure 5

A

B

CC

EDLF

MPO (OD/g tissue)

Elastase (OD/g tissue)/10

EC  CC  EDLF

EC  CC  EDLF

**  **

**  **
Figure 6

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Figure 8

A

B

pg PGE2/mg (weight)

C  lono  EDLF  Indo  EDLF+lono  Indo+lono

PGE2 (pg)

C  lono  EDLF  Indo  EDLF+lono  Indo+lono