Novel Anti-Inflammatory Action of Edelfosine Lacking Toxicity with Protective Effect in Experimental Colitis

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ABSTRACT
Edelfosine (1-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 (J Exp Med 200:335–365). 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 nonsteroidal 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 interleukin-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 histologic alteration in gastrointestinal tract was detected after 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.
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 (Dome- nech, 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-lysophospholipid 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, 2004; Gajate and Mollinedo, 2007), followed by coclustering of Fas/CD95 death receptor in lipid rafts (Gajate and Mollinedo, 2001, 2007; Gajate et al., 2004). 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.

Materials and Methods

Cell Culture and Neutrophil Isolation. Human acute myeloid leukemia HL-60 cells and mouse 3T3 fibroblasts were grown in RPMI 1640 medium and DMEM, 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 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) followed by hypotonic lysis of residual erythrocytes as described previously (Mollinedo et al., 2003). Cells were resuspended at 5 × 106 neutrophils/ml in HEPES-glucose buffer (150 mM NaCl, 5 mM KCl, 10 mM HEPES, 1.2 mM MgCl2, 1.3 mM CaCl2, and 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.

Bone Marrow-Derived Mouse Macrophages. Bone marrow cells were obtained by flushing the femurs from CBF1 mice and cultured as described previously (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% nonessential 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 toward a >95% pure population of bone marrow-derived macrophages.

Fluorescence Labeling of Cells. Neutrophils were labeled with calcine-acetoxyethyl ester (Molecular Probes, Eugene, OR) by incubating 5 × 106 cells/ml with 5 μM calcine-acetoxyethyl ester for 30 min at room temperature in calcine labeling buffer (Hanks’ balanced salt solution without Ca2+ or Mg2+ containing 0.02% bovine serum albumin). Cells were then washed twice with calcine labeling buffer and resuspended in the desired media.

Endothelial Cell Adhesion Assay. Neutrophil adhesion to human umbilical vein endothelial cells (HUVEC) was determined as described previously (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 37°C. Cells monolayers were washed four times with RPMI 1640 medium containing 5% heat-inactivated FBS, and 106 untreated or drug-treated calcine-labeled neutrophils in 200 μl were added to each well. The plates were incubated under rotation at 64 rpm at 37°C for 10 min and 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 described previously (Mollinedo et al., 1991; Gajate and Mollinedo, 2007) in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The following mouse monoclonal antibodies were used: TS1/11 anti-CD11a, Bear-1 anti-CD11b, TPI36/1 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-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 rMFI = (MFIagent × 100)/MFImedium.

Background fluorescence was subtracted from all values.

Edelfosine Uptake. Drug uptake was measured as described previously (Mollinedo et al., 1997) after incubating cells (106 in 1 ml of RPMI 1640 medium/10% heat-inactivated FBS containing 5 μg edelfosine + 0.05 μCi of [3H]edelfosine for the indicated times, and subsequent washing (five times) with PBS + 2% bovine serum albumin.

Soluble L-Selectin ELISA. Neutrophils (5 × 106 cells/ml) were incubated alone or with the indicated agents in HEPES-glucose buffer at 37°C. 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 described previously (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 of PBS into the right hind paw. The same volume of 0.03 ml of 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 of PBS for 21 days. Inflammation control group was given 0.2 ml of PBS only. The diameter of the hind paws (in millimeters) 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 of 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, and 5 mg/kg prednisone). At 24 h and 1 week after intracolonial 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 stained with hematoxylin and eosin, photographed, and examined by two independent observers in a blinded manner. Measurement of myeloperoxidase (MPO) and neutrophil elastase) per gram of tissue.

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Reverse Transcriptase Polymerase Chain Reaction. Bone marrow-derived mouse macrophages (6 × 10⁶) were seeded and stimulated in six-well plates (Corning Life Sciences, Lowell, MA) in a final volume of 3 ml/well. Total RNA was extracted from cells as described previously (Molinello 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 to 10 μg of total RNA, 0.4 mM of each dNTP, and 50 μM random hexamers (Promega, Madison, WI), 1 mM dithiothreitol (Invitrogen, Paisley, UK), 50 mM Tris-HCl, pH 8.3, 3 mM MgCl₂, and 62.5 mM KCl. After incubation at 70°C for 1 min, 10 U/μl Moloney murine leukemia virus reverse transcriptase (Invitrogen) 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 microliter 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’-CAGTTCTGGCATTGCCATC-3’ and 5’-CTCT-ACTGAAAATCCAGCCGT-3’), IL-1β (5’-TGACGGACCCTAAAAG-ATG-3’ and 5’-AGAAGTGCTATGCTCTCA-3’), TNF-α (5’-TCTCATGTTCTATGGCCC-3’ and 5’-GGGAGTAGACAGTGACTAAC-3’), IL-10 (5’-GACCGGAGAACAAATACCTG-3’ and 5’-CATT-TCCGATAGGGCTTG-3’), and β-actin (5’-TGAATCCTGGCATTCAAGACAAA-3’ and 5’-TAAAGGCAGCTGATATGCCCG-3’). A 50-μl PCR mixture contained 0.2 mM each dNTP, 200 nM each primer, 0.04 U of Taq polymerase (HT Biotechnology Ltd., Cambridge, UK), 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂, and 50 mM KCl. PCR amplification was performed in a DNA thermal cycler (Thermocycler; Biometra, 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, and 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× TAE (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) and stained with ethidium bromide. The expected sizes for the distinct PCR amplificons were as follows: 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 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). Many 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 polyethylene-50 catheters in the femoral vein and artery and a polyethylene-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 (ADInstruments, 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 (dP/dmax and dP/dmin) from the intraventricular pressure curves (right carotid transducer).

After collecting cardiovascular data, the abdominal aorta was cannulated, and the animals were perfused with heparinized isotonic saline solution (0.9% NaCl) to wash the blood out. 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 solutions and then embedded in paraffin (60°C during 24 h). We cut 7-μm sections by using a Microm HM-310 microtome (Fronome Laboratory, Riverstone, Australia), and sections were mounted on glass slides and stained with hematoxylin and eosin and Van Gieson trichrome.

Prostaglandin E₂ 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 of HEPES-glucose buffer. Then, cells from mucosal gastric samples were pelleted by centrifugation, lyzed, and intracellular PGE₂ was measured using an enzyme immunoassay kit (Amersham Pharmacia Biotech) following the manufacturer’s instructions. Data are ex-

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pressed as picograms of PGE$_2$ synthesized per milligram of tissue. PGE$_2$ was also determined in rat colonic mucosal samples as described above.

To measure PGE$_2$ in 3T3 cells, 1.5 x 10$^5$ cells in 100 l of complete DMEM was incubated overnight at 37°C (5% CO$_2$) in 96-well microtiter plates. Then, the distinct agents were added in 100 l of complete DMEM for 20 min at 37°C, and intracellular PGE$_2$ was measured as described above.

Data Analysis. All values are expressed as means ± S.E.M. 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, 2004; Gajate and Mollinedo, 2007). Peripheral blood mature neutrophils took up low amounts of edelfosine compared with the human myeloid leukemia cell line HL-60 (17 ± 3 ng/10$^6$ neutrophils versus 148 ± 25 ng/10$^6$ 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, terminally differentiated end cells that undergo spontaneous apoptosis after 9- to 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, because anti-L-selectin LAM1–3 monoclonal antibody inhibited neutrophil adhesion (Fig. 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 because of 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; 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 after 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 because of neutrophil activation, because 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 because of 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 histopathologic 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-mediated immune response, involved in cell-mediated immunity and phagocyte-dependent responses (Romagnani, 1999; Kitani et al., 2000). Because T-helper cell type 1 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 linear 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 many 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 group
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 calcine-labeled neutrophils in medium alone (control), with calcine-labeled neutrophils pretreated with LAM1–3 monoclonal antibody (LAM1–3) or with calcine-labeled neutrophils pretreated for 15 min at 37°C with increasing amounts of edelfosine (EDLF). Then, percentage of calcine-labeled neutrophil adhesion to the endothelial layer was determined, considering the fluorescence value corresponding to untreated control calcine-labeled neutrophils as 100%. B, isolated peripheral blood human neutrophils, showing an homogenous side versus forward scatter population (top) were incubated for 15 min at 37°C in the absence (control) and in the presence of 5 µg/ml EDLF, and then analyzed by flow cytometry for the cell surface expression of L-selectin, CD43, and HLA (bottom). P3X63 myeloma supernatant was used as a negative control. The results are representative of five separate experiments. C, surface expression of leukocyte antigens after edelfosine treatment. Neutrophils (5 × 10^6 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 EDLF. Then, cell surface expression of the indicated leukocyte antigens was analyzed by flow cytometry, and rMFI values were estimated using P3X63 myeloma supernatant and an isotype-matched fluorescein isothiocyanate-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 × 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 of increasing concentrations of EDLF. Then, cell surface antigen expression and rMFI values were determined as described above. E, quantification of neutrophil-shed L-selectin induced by edelfosine. Neutrophils (5 × 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 ± S.E.M. (n = 5). Asterisks denote significant differences with the control group (*, p < 0.05; **, p < 0.01).
matory cell infiltration was observed in edelfosine-treated (Fig. 4C, inset), whereas only a submucosal focal mild inflammatory cell infiltration as inducing reparative phenomena (Fig. 4D). Sections from formation, edema, and inflammatory cell infiltration as well logic severity of the inflammatory colitis, decreasing ulcer sections from edelfosine-treated rats ameliorated the pathology with intense mucosal destruction, and mucosal and TNBS challenge, there was evidence of healing with affected weights of prednisone and edelfosine are 358.4 and 523.7, with the anti-inflammatory activity of edelfosine (molecular showed minimal signs of damage (Fig. 4, A and B). 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 a transient acute bowel inflammation response after 24 h of intraluminal administration, which decreased there after 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 prednisone 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 (Fig. 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 marked decrease in the severity of mucosal damage (Fig. 3B) and colon weight (Fig. 3C) compared with 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 pathology 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), probably because of the delayed time it takes for steroids to become effective and 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 (Martín 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 because of the high antineutrophil elastase activity present in this rodent species, 3-fold higher than in humans or hamsters (Takahara et al., 1983).
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 monocytoic 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 several proinflammatory cytokines (IL-1α, IL-1β, and TNF-α) and the anti-inflammatory cytokine IL-10. Figure 6A shows that edelfosine by itself does not induce cytokine expression, as assessed by semiquantitative reverse transcription-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, an 8-fold higher dosage than the one exerting the anti-inflammatory effect, for 1 and 4 weeks. No weight loss was observed during the 4 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 toxicologic data of edelfosine are in clear contrast with those of doxorubicin, showing cardiotoxicity as well as kidney alterations (Table 1). Histologic studies demonstrated no damage in kidney, liver, heart, and stomach after edelfosine treatment (Fig. 7). Edelfosine did not elicit hypertension (Table 1) or any histologic 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 because of 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 up-regulation (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 described previously for different NSAIDs (Díaz-González 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} value 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). That edelfosine induces L-selectin shedding from human neutrophils, despite its low uptake in these cells, suggests drug-induced changes at the cell surface after 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
TABLE 1
Toxicity parameters in edelfosine-treated rats

Biochemical and functional parameters of distinct organs were measured in rats untreated and treated with EDLF (for 1- or 4-wk 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 (PlCreat). 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), and dP/dt max and dP/dt min intraventricular pressures. Data shown are means ± S.E.M. of 15 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>Kidney 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 ± 0.38*</td>
</tr>
<tr>
<td>Kidney GGT (U/day)</td>
<td>0.99 ± 0.16</td>
<td>0.57 ± 0.14</td>
<td>1.15 ± 0.9</td>
<td>13.65 ± 3.03*</td>
</tr>
<tr>
<td>Kidney LDH (U/day)</td>
<td>0.10 ± 0.03</td>
<td>0.13 ± 0.04</td>
<td>0.16 ± 0.03</td>
<td>3.65 ± 0.62*</td>
</tr>
<tr>
<td>Kidney NAG (U/day)</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>N.D.</td>
</tr>
<tr>
<td>Kidney 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>Kidney 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 ± 0.65*</td>
</tr>
<tr>
<td>Kidney 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>Kidney PlCreat (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>Kidney Chloride (mEq/day)</td>
<td>2.94 ± 0.13</td>
<td>1.73 ± 0.28</td>
<td>3.99 ± 0.32</td>
<td>0.45 ± 0.02*</td>
</tr>
<tr>
<td>Kidney Potassium (mEq/day)</td>
<td>2.17 ± 0.06</td>
<td>1.31 ± 0.18</td>
<td>2.46 ± 0.15</td>
<td>0.92 ± 0.19*</td>
</tr>
<tr>
<td>Kidney Sodium (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>Liver ALP (U/l)</td>
<td>201.80 ± 20.92</td>
<td>127.17 ± 8.01</td>
<td>148.80 ± 4.27</td>
<td>N.D.</td>
</tr>
<tr>
<td>Liver 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>Liver 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>Heart CPK (U/l)</td>
<td>444 ± 56</td>
<td>592 ± 85</td>
<td>420 ± 81</td>
<td>755 ± 151*</td>
</tr>
<tr>
<td>Heart CKMB (U/l)</td>
<td>948 ± 109</td>
<td>966 ± 123</td>
<td>821 ± 175</td>
<td>1150 ± 195*</td>
</tr>
<tr>
<td>Heart HR (beats/min)</td>
<td>433 ± 9</td>
<td>397 ± 14</td>
<td>424 ± 34</td>
<td>348 ± 22*</td>
</tr>
<tr>
<td>Heart LVSP (mm Hg)</td>
<td>143 ± 13</td>
<td>147 ± 3</td>
<td>166 ± 10</td>
<td>138 ± 9</td>
</tr>
<tr>
<td>Heart LVDP (mm Hg)</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>Heart MAP (mm Hg)</td>
<td>122 ± 4</td>
<td>129 ± 5</td>
<td>123 ± 2</td>
<td>116 ± 3</td>
</tr>
<tr>
<td>Heart dP/dt max (mm Hg/min)</td>
<td>2966 ± 221</td>
<td>2799 ± 153</td>
<td>3217 ± 249</td>
<td>1526 ± 56*</td>
</tr>
<tr>
<td>Heart dP/dt min (mm Hg/min)</td>
<td>-2837 ± 222</td>
<td>-3216 ± 222</td>
<td>-1725 ± 48*</td>
<td></td>
</tr>
</tbody>
</table>

N.D., not determined.
*p < 0.05, significant differences with the untreated control group.

Fig. 7. Histologic 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 histologic alterations. Scale bar, 100 μm.

commercially available selective adhesion-molecule inhibitor, is a humanized monoclonal antibody against α-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, because α-4 integrin is required for white blood cells to move into organs. Natalizumab is administered by intravenous infusion, but the occurrence of several adverse effects, including hepatotoxicity and in some cases the appearance of the rare neurologic 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 after natalizumab treat-

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
samples were run in parallel. Data shown are means ± S.E.M. (n = 3). Asterisks denote significant differences with the untreated control group (*, p < 0.05; ***, p < 0.001).

of currently used anti-inflammatory drugs, such as NSAIDs and selective COX-2 inhibitors (Akaraa, 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 of currently used anti-inflammatory drugs, such as NSAIDs and selective COX-2 inhibitors (Akaraa, 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

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References


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