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NEUTROPHIL RECRUITMENT IN THE REPERFUSED-INJURED RAT LIVER WAS EFFECTIVELY ATTENUATED BY REPERTAXIN, A NOVEL ALLOSTERIC NON-COMPETITIVE INHIBITOR OF CXCL8 RECEPTORS: A THERAPEUTIC APPROACH FOR THE TREATMENT OF POST-ISCHEMIC HEPATIC SYNDROMES

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Hepatic reperfusion injury represents a crucial problem in several clinical situations including liver transplantation, extensive hepatectomy and hypovolemic shock with resuscitation. Repertaxin is a new non-competitive allosteric blocker of interleukin-8 (CXCL8) receptors, which by locking CXCR1/R2 in an inactive conformation, prevents receptor signaling and polymorphonuclear leukocyte (PMN) chemotaxis. The present study shows that repertaxin dramatically prevents rat post-ischemic hepatocellular necrosis (80% of inhibition) and PMN infiltration (96% of inhibition) at a clinically-relevant time (24 h) of reperfusion. Treatment with repertaxin by continuous infusion is demonstrated to be the optimal route of administration of the compound especially in view of its clinical therapeutic use. Because repertaxin has proven to be safe and well tolerated in different animal studies and in phase I studies in human volunteers, it is in fact a candidate novel therapeutic agent for the prevention and treatment of hepatic post-ischemic injury.

Hepatic ischemia-reperfusion injury remains an unavoidable problem in liver transplantation surgery, extensive hepatectomy, and hypovolemic shock with resuscitation (1-2). In transplantation, ischemia-reperfusion injury is clearly a major determinant of early graft function. Initial poor function is seen approximately in 10-20% of transplanted livers, and is associated with higher graft failure, re-transplantation, and mortality within the first three months (3-6). Other possible important consequences due to ischemia/reperfusion injury include clinical entities with high incidence of morbidity and mortality such as liver failure, multiorgan dysfunction syndrome and/or adult respiratory distress syndrome, with an elevated rate of mortality (5, 7-8).

Recent studies performed in appropriate experimental models have focused the attention on the involvement of CXC-chemokines and polymorphonuclear neutrophils (PMN) as the major cause of post-ischemic hepatic injury (9-14). Accumulation of activated PMN in the hepatic sinusoids adhere to endothelial cells and hepatocytes causing directly microvascular dysfunction and hepatocyte damage by the generation of reactive oxygen species and myeloperoxidase (MPO)-derived oxidant. Moreover, oxidative stress may also deactivate endogenous anti-proteases facilitating the hepatocellular damage associated with PMN release of proteolytic enzymes (15-19). In addition, infiltrated PMN in hepatic sinusoids can obstruct blood flow and contribute to

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create nutritional perfusion alterations (20).

It is becoming generally well accepted that leukocyte trafficking into the ischemic injured liver is a process likely dependent of local gradients of chemotactic factors, e.g. PMN-CXC chemokines like CINC-1/CXCL1 and MIP-2/CXCL2, the rat equivalents of human interleukin-8 (CXCL8; 10-13). The importance played by the CXC chemokines in the pathogenesis of ischemic/reperfusion damage raises the possibility that PMN recruitment may be attenuated through inhibition of CXCL1 and CXCL2. Consistently, anti-chemokine strategies, including neutralizing antibodies, N-terminal modified chemokines and G-protein coupled receptor (GPCR) molecule antagonists have been used to prevent experimental reperfusion injury (21-22).

We have very recently described repertaxin, a non-competitive allosteric blocker of the CXCL8 receptors CXCR1 and CXCR2. Repertaxin is a new receptor inhibitor with a unique mechanism of action: it binds CXCL8 receptors and disrupts receptor signaling downstream events without affecting CXCL8 binding to its receptors (23). Repertaxin was also proven to be efficacious in preventing PMN recruitment and hepatocellular necrosis in the rat liver exposed to ischemia-reperfusion injury (23).

In order to determine the potential clinical application of repertaxin here we propose to further evaluate the efficacy of this compound in the prevention of post-ischemic damage of the liver. To this aim, the efficacy of repertaxin was evaluated 24 h after liver reperfusion, a crucial reperfusion end point characterized by extensive hepatocellular necrosis and maximum PMN infiltration (24). Along the same line, this time of reperfusion seems to constitute a critical point in patient outcome after a clinical situation of post-ischemic liver injury. Next, the optimal schedule of treatment of repertaxin was determined in the same experimental model. The results reported hereafter show that repertaxin treatment dramatically reduced PMN infiltration and hepatocellular necrosis 24 h after reperfusion. Accordingly, the pharmacokinetic profile of repertaxin and its chemical-physical properties in animal treatment by continuous infusion was observed to be the optimal schedule of administration of the compound.

MATERIALS AND METHODS

Reagents

Repertaxin is R(-)-2-(4-isobutylphenyl) propionyl methansulfonamide L-lysine salt (Dompé S.p.A., L'Aquila, Italy).

Animals

All the procedures were performed in the animal facilities according to ethical guidelines for the conduction of animal research (Authorization from the Italian Ministry of Health N. 271/95-B D.Lvo 116/92; Gazzetta Ufficiale della Repubblica Italiana N. 40, February 18, 1992; EEC Council Directive 86/609 OJ L 358, 1 December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication N. 85-23, 1985). Male Sprague-Dawley rats (Harlan-Nossan, Correzzana, Udine, Italy) were used. They were kept in a temperature-controlled environment with a 12 h light-dark cycle and allowed tap water and standard rat chow pellets ad libitum.

Surgery for hepatic ischemia/reperfusion

After anesthesia with a solution of tiletamina clorohydrate and zolazepam clorohydrate (1:1) in 0.9% sterile NaCl solution (100 μ l/100 g body weight, i.p.), rats were positioned in a heat pad to maintain their body temperature at $37.5 \pm 0.5^\circ\text{C}$ and monitored with a rectal temperature probe. Laparotomy was carried out through a midline incision. The liver was gently exposed and subjected to selective inflow occlusion of the median and left lateral lobes by clamping the corresponding portal triad with a micro vascular clamp (25). At the end of 60 min ischemia, the ischemic lobes were allowed to reperfuse by removing the clamp. At the end of the reperfusion phase (12 and 24 h), the rats were killed under anesthesia by exsanguination and specimens of blood and reperfused lobes were taken for morphological and biochemical determination.

Surgery for blood collection

The day before repertaxin administration, animals were weighed and anaesthetized with a solution of tiletamina clorohydrate and zolazepam clorohydrate (1:1) in 0.9% sterile NaCl solution (100 μ l/100 g body weight, i.p.). Then, the left jugular vein was cannulated using a silicone medical grade catheter (0.28 inches ID, 0.47 inches OD, Silastic[®], Dow Corning, U.S.A.) filled with heparin solution (2.8 mg/ml; 506 US units/ml) and marked 2.5 cm from the tip. The catheter was inserted 2.5 cm along the vein. To verify the patency of the cannula, a small amount of blood was sucked into the catheters with a 1-ml syringe. 100 μ l of heparin solution were then injected into the cannulated vein. The day after the surgery, before

administration of the test compound, 100 μ l heparin solution was injected again into the cannulated vein.

Preparation and implantation of the pumps

The pumps (Alzet Osmotic Pump, Model 2ML1, Alza Corp., Palo Alto, CA) were filled with repertaxin or 0.9% sterile NaCl solutions according to the instructions of the supplier. Animals were weighed and anesthetized. Then, pumps were implanted subcutaneously. A small incision was made in the skin between the scapulae and, using a hemostat, a pocket was formed by spreading apart the subcutaneous connective tissues. The pump was inserted into the pocket with the flow moderator pointing away from the incision. The skin incision was closed with wound clips.

Determination of repertaxin plasma levels

Plasma was obtained from heparinised blood by centrifugation at 2000 x g for 10 minutes. The bioanalytical method based upon a high performance liquid chromatographic (HPLC) procedure involved the extraction of repertaxin and internal standard from aliquots of rat plasma [200 μ l mixed with hydrochloric acid (50 μ l, 5.8 M) into hexane/propan-2-ol (3 ml 9:1, v/v) or ethyl ether (3 ml)]. The extracts were evaporated to dryness under vacuum and the residues were reconstituted in acetonitrile/water (200 μ l, 8:2, v/v) or methanol/water (200 μ l, 1:1, v/v). Aliquots were injected onto a Hypersil C18 BDS, 5 μ m or an X-Terra MS, 2.5 μ m analytical column and repertaxin and internal standard were chromatographed and detected by UV absorbance at 223 nm or ESI-MS in positive ion mode, depending on the expected concentration of the analyte in the biological samples (being the HPLC-MS more sensitive than the HPLC-UV method).

Experimental design: Protocol 1

First aim: to determine an appropriate schedule of treatment to evaluate the efficacy of repertaxin in preventing hepatic post-ischemic injury by repeated administration of the compound, the pharmacokinetic profile of repertaxin was evaluated in rats exposed to liver ischemia/reperfusion injury. Animals received two doses of repertaxin (15 mg/kg), i.v. (through the posterior cava vein) 15 min before reperfusion and s.c. 2 h after reperfusion. Blood samples (about 400 μ l) were withdrawn from the cannulated jugular vein using a 1-ml syringe. The blood samples were collected into Eppendorf tubes at the following times: 60, 120, 130, 240, 360 min, 8 h and 12 h after i.v. injection. The withdrawn blood was replaced with 500 μ l of a heparin solution (0.14 g/ml; 25.3 US units/ml).

Second aim: to evaluate the efficacy of repeated

administrations of repertaxin in preventing post-ischemic injury, the following experimental groups were studied.

Group 1: Sham: Animals were only subjected to laparotomy.

Group 2: Vehicle treated: Animals were submitted to 60 min of ischemia followed by 12h or 24h of reperfusion. Rats were treated with 0.9% sterile NaCl solution. The first treatment was given 15 min before the reperfusion phase (i.v), and then every two hours for three times after re-flow (s.c.)

Group 3: Repertaxin treated: Animals received two (12h reperfusion) or four doses (24h reperfusion) of repertaxin (15 mg/kg) dissolved in 0.9% sterile NaCl. The first dose was given 15 min before the reperfusion phase (i.v), and then every two hours for one time (12h reperfusion) or three (24h reperfusion) times after re-flow (s.c.).

Protocol 2

First aim: to evaluate the possibility to s.c. continuously infused repertaxin by means of osmotic pumps, a pharmacokinetic elaboration was done as follows.

In order to obtain a plasmatic concentration of repertaxin at the steady-state (C_{ss}) of 15 μ g/ml, the rate of infusion (R_0) was calculated. Since repertaxin s.c. bioavailability is almost complete, R_0 was calculated according to the following formula:

$$R_0 = C_{ss} \times K_{el} \times V \quad (26)$$

The apparent volume of distribution (V) and the elimination rate constant (K_{el}) of repertaxin were obtained by previous pharmacokinetic studies.

$$R_0 = 15 \times 1.82 \times 283 = 7726 \mu\text{g/h/kg} \sim 8 \text{ mg/h/kg}$$

The pump rate was 10 μ l/h, to obtain 8 mg/h/kg as R_0 (corresponding to 2 mg/h/rat) the pumps were filled with repertaxin 200 mg/ml. Pumps were implanted at the reperfusion time. In order to consider the time of pump activation and to immediately reach plasmatic C_{ss} , rats were also treated with a single i.v. injection of repertaxin 15 min before reperfusion. Repertaxin i.v. dose was identified by preliminary experiments and established at 15 mg/kg.

Blood samples were collected at the following times: 2, 4, 8 and 24 h after i.v. injection.

Second aim: to evaluate the efficacy of repertaxin administered by continuous infusion, rats were s.c. infused by means of the osmotic pumps implantation starting from the time of reperfusion with three different doses of the compound, as follows.

Group 1: i.v. dose: 4.5 mg/kg. R_0 : 2.4 mg/h/kg.

Group 2: i.v. dose: 2.5 mg/kg. R_0 : 1.2 mg/h/kg.

Group 3: i.v. dose: 0.4 mg/kg. R₀: 0.2 mg/h/kg.

Repertaxin i.v. dose was administered 15 min before reperfusion. In the vehicle group, animals were treated with 0.9% sterile NaCl solution (i.v. injection + pump infusion).

Morphological studies

Fragments from the reperfused left lateral lobe were placed overnight in 4% formaldehyde, 50 mM phosphate buffer solution, pH 7.4. Paraffin wax sections of 5 μ m were stained with Harris hematoxylin and eosin. Hepatocellular morphologic criteria, i.e. nuclear changes consisting in pycnosis, karyorrhexis, or karyolysis; cytoplasmic blanching; extreme vacuolization and loss of distinct cell borders, were used to determine necrosis. PMN leukocytes were identified on 5 μ m paraffin sections by means of the naphthol AS-D chloroacetate technique for esterase, described elsewhere (27). Red stained PMN were counted in 20 non-consecutive, randomly chosen x 400 histological fields. Results were expressed as number of PMN per histological power field (hpf).

Measurement of MPO activity

Total hepatic MPO activity was assayed by measuring the hydrogen peroxide-dependent oxidation of *o*-dianisidine described elsewhere (15). Pellets from snap-frozen samples homogenized in 20 mM potassium phosphate buffer, pH 7.4, were re-suspended in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethyl-ammonium bromide. The suspension was frozen and thawed once, sonicated, incubated at 60°C for 2 h, and then centrifuged again. MPO activity was measured by mixing the supernatant with 0.05 M potassium phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine and 0.24 mM H₂O₂ (final concentration). Changes in absorbance at 460 nm were monitored for 2 min at 37°C. One unit of MPO activity was defined as the amount of enzyme reducing 1 μ mol of hydrogen peroxide/minute. The results are expressed as units of MPO/g wet tissue.

ELISA assay for CINC-1/CXCL1

Tissue and plasma levels of CXCL1 were measured by an enzyme-linked immunosorbent assay using the Rat CXCL1 Immunoassay kit (Immuno-Biological Laboratories, Gunma, Japan). Analysis was performed spectrophotometrically. Tissue levels of CXCL1 production was measured in liver homogenates. Liver tissue was homogenized in 20 volumes of homogenization buffer (w/v) (25 mM Tris-HCl, 2 mM MgCl₂, 50 mM KCl, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml soybean trypsin inhibitor, 1.0 mg/ml bovine

serum albumin, 0.02% sodium azide, and 10 μ l/ml protease inhibitor cocktail [1 mg/ml leupeptin, 1mg/ml aprotinin, and 1 mg/ml pepstatin]). The homogenates were centrifuged at 13,000 g for 20 min. Supernatants were removed, centrifuged again to obtain clear lysates and analysis of samples of plasma and clear lysates was performed spectrophotometrically. Results are expressed in ng (tissue)-pg (plasma)/ml.

Biochemical assessment of liver cytotoxicity

The plasma activity of alanine aminotransferase (ALT) was evaluated using a commercial kit from Sigma Chemical Co. (St. Louis MO). Results are expressed in U/l.

Statistical Analysis

Values are expressed as means \pm S.E.M or S.D. One-way analysis of variance and Tukey-Kramer test adjustment for multiple comparisons were used, unless otherwise stated, the alternative being an unpaired Student's *t* test. *p* values \leq 0.05 were considered statistically significant. The correlation between number of PMN and levels of ALT was tested by the non-parametric (Spearman) correlation coefficient.

RESULTS

Efficacy of repertaxin repeated administrations in preventing hepatic reperfusion injury

We first evaluated the efficacy of repeated administrations of repertaxin in preventing tissue damage in post-ischemic rat liver 24h after reperfusion. To this end, we initially evaluated the pharmacokinetic profile of repertaxin in rats exposed to liver ischemia/reperfusion injury at a dose of the compound (15 mg/kg) and with a schedule of treatment (i.v. 15 min before reperfusion and s.c. 2h after reperfusion) previously reported to protect against hepatocellular necrosis 12h after reperfusion (23). As shown in Fig. 1, repertaxin plasmatic concentration peaked at 2h after reperfusion (34 μ g/ml) and was detectable up to 8h after reperfusion (30 ng/ml). These data are in agreement with the pharmacokinetic profile of repertaxin determined in normal rats (half-life almost 35 min; data not shown), suggesting that additive administrations were necessary to evaluate its efficacy at a longer time (24h) after reperfusion.

Next, we evaluated the efficacy of repertaxin in preventing PMN infiltration and tissue damage in the

post-ischemic hepatic lobes 12h and 24h after re-flow onset. PMN infiltration was quantified through a histochemical method, which allows to identify and count red-stained leukocytes, and by evaluating MPO activity in liver extracts. Liver specimens from rats treated with vehicle alone undergoing 60 min of ischemia followed by 12h and 24h of reperfusion had a significantly increased of PMN content (Fig. 2 A) and MPO activity (Fig. 2 B) versus sham-operated animals. As shown in Figure 2 C, the degree of hepatocellular necrosis, in terms of activity of ALT in plasma, indicated substantial liver damage. As expected, rats treated with two repeated administrations of repertaxin (15 mg/kg; i.v. 15 min before reperfusion and s.c. 2h after reperfusion) were protected against PMN infiltration and hepatocellular damage at 12h after reperfusion (Fig. 2 A-C; 23).

On the contrary, repertaxin administration with the same schedule of treatment was observed to be efficacious at 12h after reperfusion (15 mg/kg; i.v. 15 min before reperfusion and s.c. 2h after reperfusion)

did not reduced PMN infiltration and hepatocellular damage at 24h after reperfusion (data not shown).

According to the pharmacokinetic profile of repertaxin (see Fig. 1), rats exposed to 24h of reperfusion were treated with the compound (15 mg/kg) 15 min before reperfusion and every two hours after reperfusion for an additional three times. With this schedule of treatment rat livers were efficiently protected against PMN infiltration and the deleterious consequence of the post-ischemic inflammatory reaction, as the degree of hepatocellular necrosis was attenuated by 86 % (Fig. 2 A-C). Direct positive correlations were found among the number of red-stained leukocytes and the degree of necrosis, stressing the concept that the extension of hepatocellular damage and death found 24h after reperfusion depends on the degree of PMN infiltration (Table I).

Since CXCL1 plays a central role in recruitment of PMN in the liver and in their transmigration from the vascular space to the close proximity of the

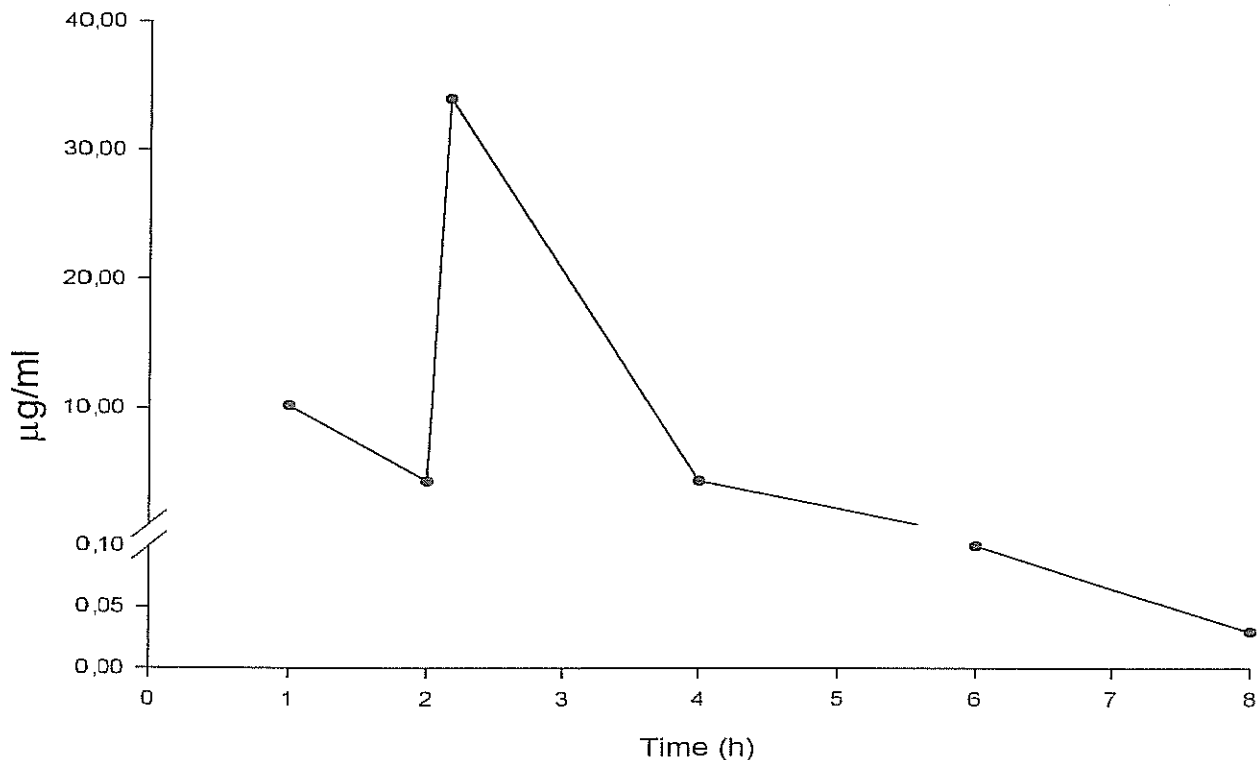


Fig. 1. Plasmatic concentrations of repertaxin after repeated administrations. Animals exposed to liver ischemia/reperfusion were treated with repertaxin (15 mg/kg) 15 min before reperfusion (i.v.) and 2 h (s.c.) after reperfusion. Plasmatic levels of the compound were determined at different times after i.v. injection, as described in Materials and Methods section. Each experimental determination is the mean of three animals.

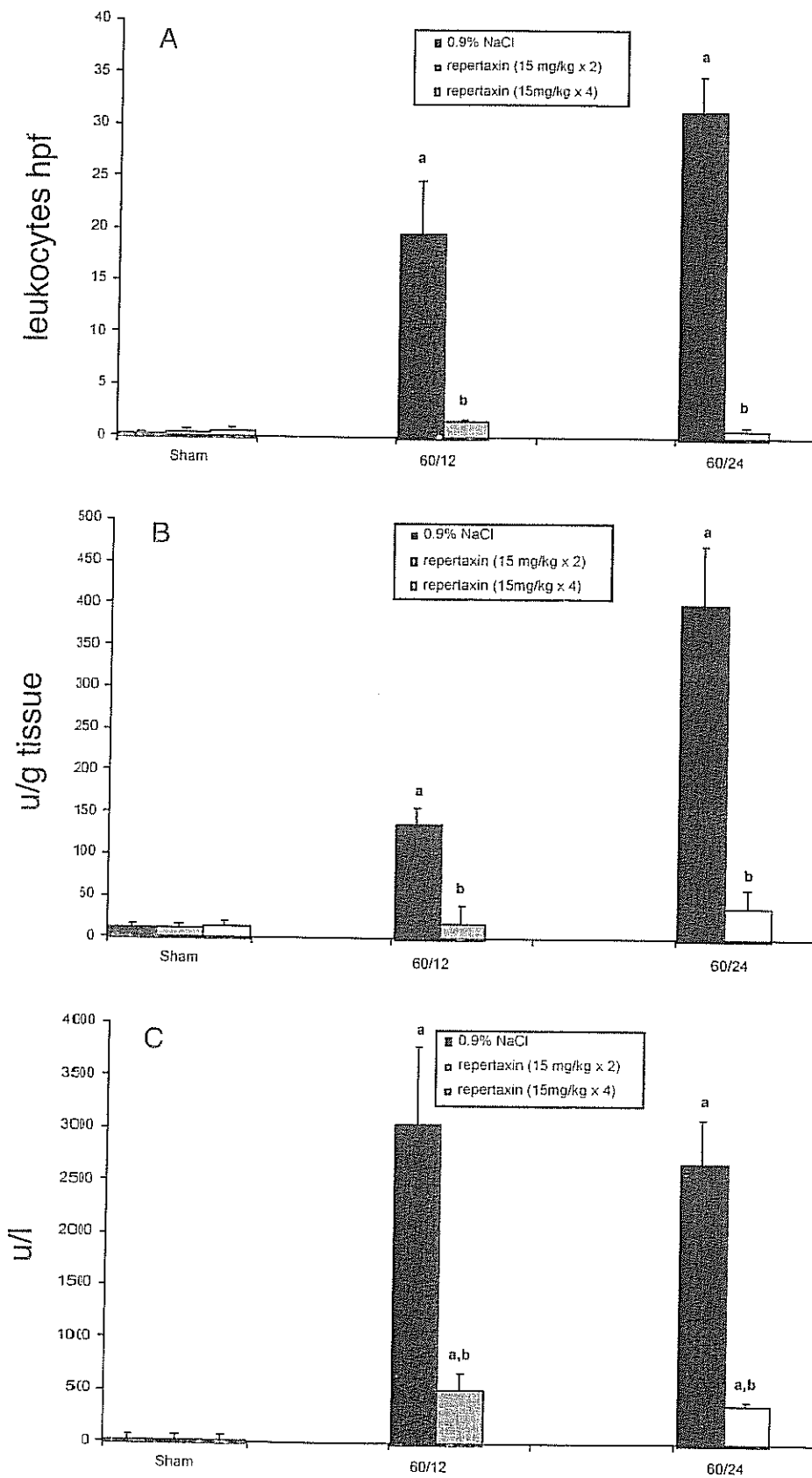


Fig. 2. Efficacy of repeated administrations of reperixin in attenuating PMN infiltration and hepatocellular necrosis in post-ischemic rat livers. PMN recruitment in the post-ischemic liver was assessed by leukocyte counts (A) and by MPO activity (B). Hepatic irreversible damage was evaluated by means of plasma ALT levels (C). 60/12-24: rats submitted to 60 min of ischemia and 12 or 24 h of reperfusion. Rats were treated with vehicle or reperixin (15 mg/kg) as described in Materials and Methods section. Data are expressed as mean \pm S.E.M. (n=5).

a: rats submitted to ischemia/reperfusion injury vs sham. b: reperixin treated rats vs vehicle treated rats.
 A: vehicle and reperixin treated vs sham, $p < 0.01$; vehicle vs reperixin treated, $p < 0.01$.
 B: vehicle and reperixin treated vs sham, $p < 0.05$; vehicle vs reperixin treated, $p < 0.01$.
 C: vehicle and reperixin treated vs sham, $p < 0.01$; vehicle vs reperixin treated, $p < 0.01$.

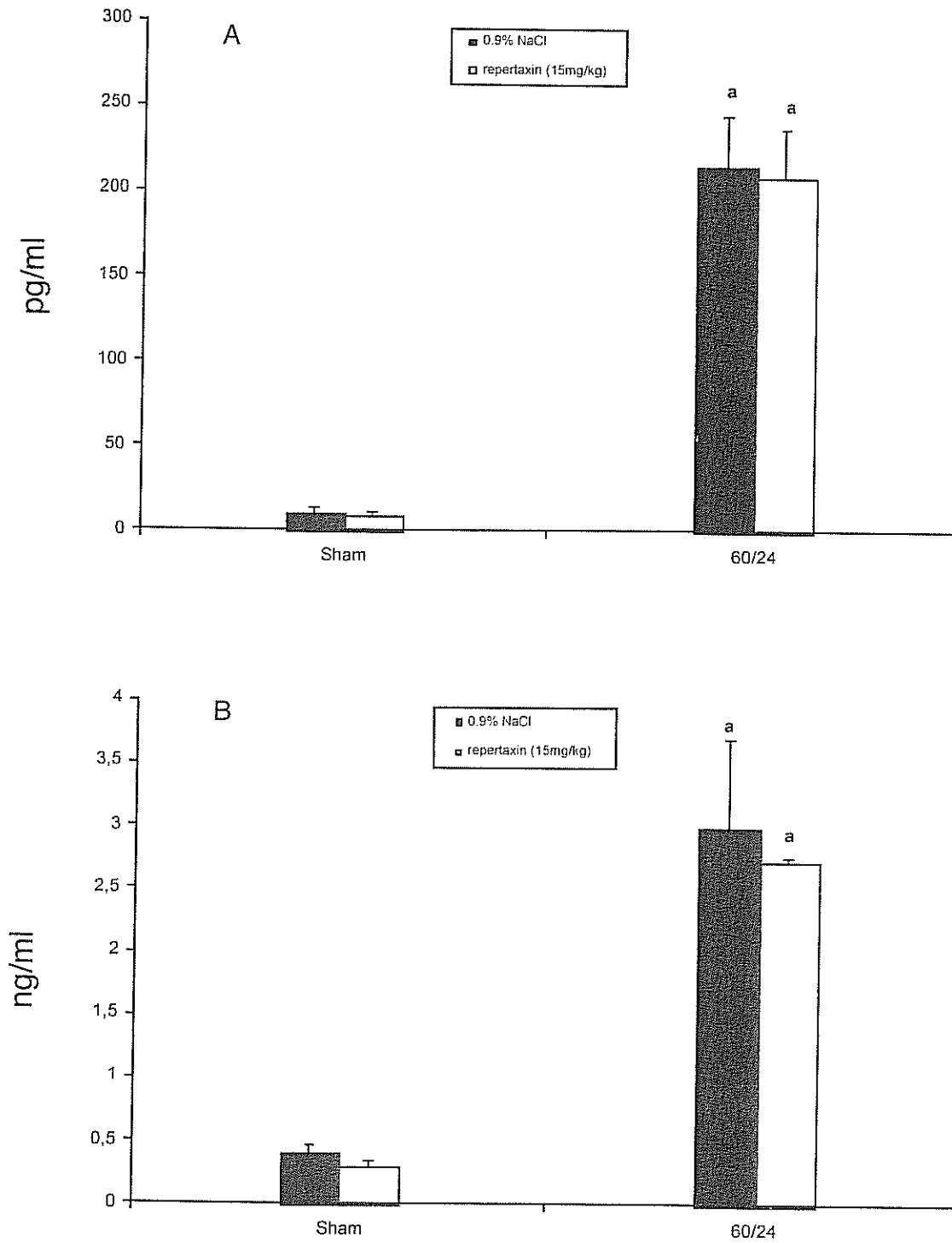


Fig. 3. Repertaxin does not affect CINC-1/CXCL1 production. 60/24: rats were submitted to 60 min of ischemia followed by 24 h of reperfusion. Animals were treated with vehicle or repertaxin (15 mg/kg; four repeated administrations, see Materials and Methods section). Data are expressed as mean \pm S.E.M. (n=5). Plasma (A) and hepatic (B) levels of CINC-1/CXCL1. a: rats submitted to ischemia/reperfusion injury vs sham, $p < 0.01$.

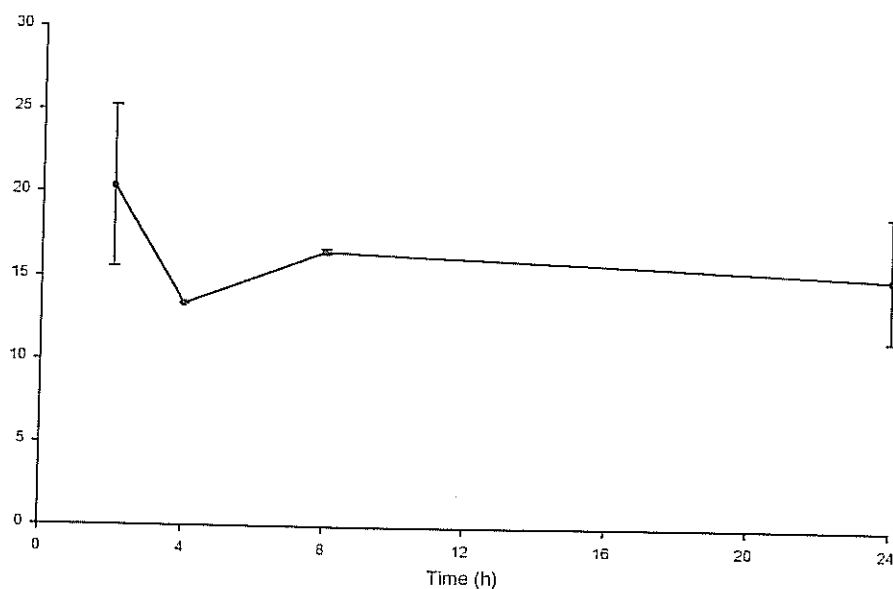


Fig. 4. Plasmatic concentrations of repertaxin after continuous infusion administration. Rats exposed to liver ischemia/reperfusion were treated with repertaxin 15 mg/kg i.v. 15 min before reperfusion and 8 mg/h/kg by s.c. continuous infusion starting from the time of reperfusion. Plasmatic levels of the compound were determined at different times after reperfusion, as described in Materials and Methods section. Each experimental determination is mean \pm S.D. of three animals.

hepatic parenchymal cells (21), levels of this CXC-chemokine were evaluated in plasma and tissue samples. Reperfusion of ischemic liver significantly induced CXCL1 production both in plasma and in tissue (Fig. 3 A-B). In keeping with its molecular mechanism of action (23), repertaxin did not affect CXCL1 production (Fig. 3 A-B).

Repertaxin hepatocellular protection after continuous infusion treatment

The requirement of multiple doses of repertaxin treatment to prevent reperfusion injury suggests that administration by continuous infusion of the compound could be the optimal schedule of treatment to be used, mainly in view of a potential clinical therapeutical use of the compound. To assess the efficacy of repertaxin by continuous infusion in preventing hepatic reperfusion injury, we first evaluated the possibility to continuously infuse repertaxin by s.c. rat implantation of osmotic pumps. Since repertaxin s.c. bioavailability is almost complete (data not shown), rats were treated with repertaxin at a R_0 of 8 mg/h/kg supposed to reach a plasmatic C_{ss} of about 15 μ g/ml (see Material and Methods section, Protocol 2). As shown in Fig. 4, comparable plasmatic levels of repertaxin was observed during the time course of the infusion period. Plasmatic levels of repertaxin were in the range of predicted C_{ss} . Then, we evaluated the efficacy of repertaxin in preventing hepatic

reperfusion injury at three different R_0 (2.4 mg/h/kg, 1.2 mg/h/kg and 0.2 mg/h/kg) of the compound. Repertaxin treatment at R_0 of 2.4 mg/h/kg and 1.2 mg/h/kg practically blocked post-ischemic PMN recruitment (96% of inhibition) at 24h after re-flow (Fig. 5 A). In correspondence, animals also showed a substantial degree of hepatocellular protection, as indicated by the 80% decrease in the extent of necrosis (Fig. 5 B). On the contrary, repertaxin treatment with a R_0 of 0.2 mg/h/kg was less effective (33% and 50% of inhibition of PMN infiltration and ALT plasmatic levels, respectively; Fig. 5 A-B).

The histopathological study of the hepatic lobes after ischemia/reperfusion was evaluated based on PMN infiltration and hepatocellular necrosis. In vehicle treated rats, reperfusion of the ischemic lobes induced multifocal and confluent areas of coagulative necrosis deeply delimited and infiltrated by PMN (Fig. 6 A-B). On the other hand, repertaxin treatment conferred protection against hepatic reperfusion injury, as only scattered foci of hepatocyte necrosis were observed (Fig. 6 C-D). The dramatic reduction observed by repertaxin treatment in the degree of PMN infiltration (Fig. 6 C-D) further stressed the idea of a central role playing by the post-ischemic inflammatory reaction to the extent of irreversible injury to the liver. Plasmatic levels of repertaxin were also determined at the end of the infusion period. As shown in Table II, plasmatic concentration of repertaxin obtained by infusion treatments was in the

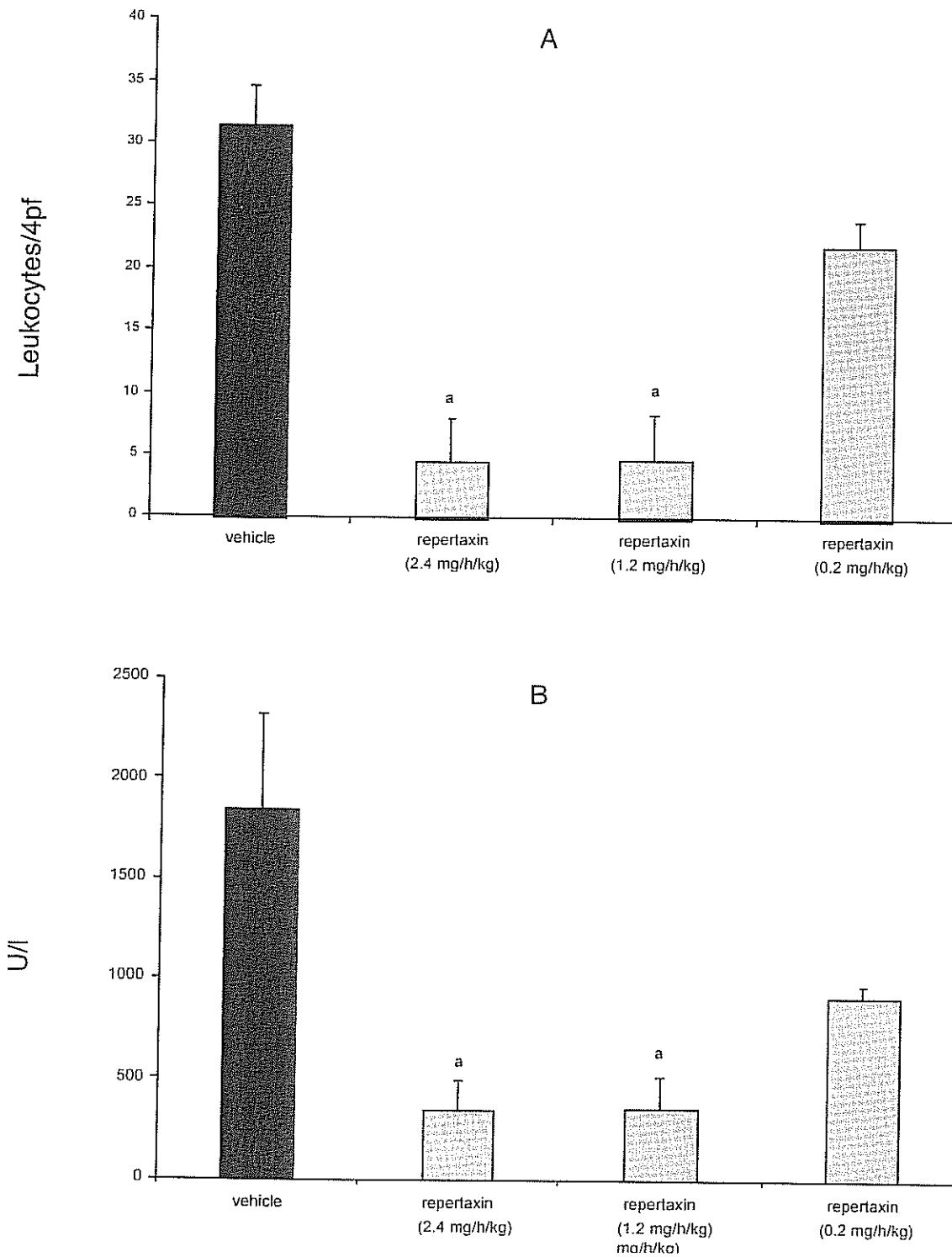


Fig. 5. Reperitaxin hepatocellular protection after continuous infusion treatment. The efficacy of reperitaxin by s.c. continuous infusion was evaluated at three different R0 treatments, as described in Materials and Methods section. (A) PMN recruitment and (B) ALT levels. Data are expressed as mean ± S.E.M. (n=5). a: reperitaxin treated rats vs vehicle treated rats, p: <0.01.

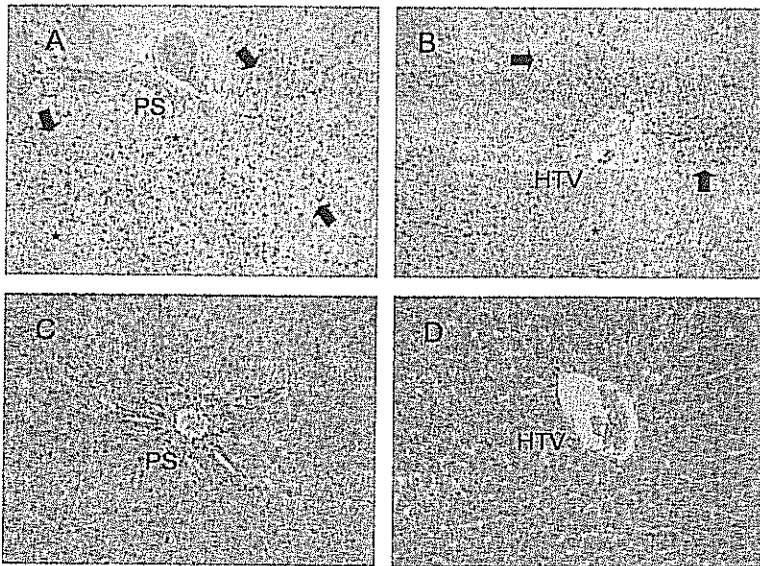


Fig. 6. Histopathology of hepatic lobes after ischemia/reperfusion damage. Efficacy of repertaxin treatment.

A-C: periportal areas; B-D: perivenular areas. Hepatic lobes were submitted to 60 min of ischemia followed by 24 h of reperfusion. Hepatocellular necrosis, sinusoidal dilatation and leukocyte infiltration were evaluated in the liver from vehicle (A and B) or repertaxin (C and D) treated animals. Arrows point PMN infiltration and asterisks indicate necrotic hepatocytes. (Naphthol AS-D chloroacetate technique counterstained with hematoxylin, x200).

range of 0.3-7.2 $\mu\text{g/ml}$. Repertaxin treatment did not affect blood leukocyte levels in all the ranges of R_0 tested (data not shown). No inflammatory reaction was observed around the implanted pumps at the end of the infusion period (data not shown).

DISCUSSION

Liver transplantation has become an accepted therapy for end-stage liver diseases, but graft injury after reperfusion has been one of the critical problems to overcome because of poor immediate graft function, a risk that notably increases when marginal donor liver are used (prolonged cold ischemia storage, severe fatty change), represents a persistent problem and contributes to mortality rates (28-30). During the last years it has become increasingly evident that, at the base of the early graft injury and poor function, the organization of a post-ischemic inflammatory reaction plays a pivotal role (9, 31-32).

Consistent with the physiopathological roles played in reperfusion injury by the hepatic gradient of CXC chemokines and related PMN infiltration, anti-chemokine strategies have proven to be successful (21-22). Repertaxin is the first low molecular weight inhibitor of CXCL8 in clinical development. This molecule is a potent and specific inhibitor of CXCL8-mediated chemotaxis that binds to CXCR1/2 inducing a conformational constrain in the transmembrane region of the receptor, thus preventing receptor-

induced signal transduction (23). Repertaxin exploits a new concept in the pharmacological inhibition of GPCRs, namely the non-competitive allosteric blocking of receptor activation.

In this paper we have initially characterized repertaxin in terms of its pharmacokinetic profile in an experimental model of liver ischemia/reperfusion injury. Furthermore, we have demonstrated repertaxin efficacy in preventing post-ischemic injury 24 hours after reperfusion, a crucial experimental and clinical end point of reperfusion (24). Finally, we have identified the optimal schedule of treatment of repertaxin in the same experimental conditions.

Our results demonstrate that repeated administrations of the compound are necessary to maintain its therapeutic effect. Moreover, in keeping with its chemical reactivity (23), sustained plasmatic levels of repertaxin are necessary to exert its protective action. Since the requirement of multiple doses of repertaxin could be technically inappropriate in view of a therapeutic use of the compound, the finding that treatment by continuous infusion was efficacious in preventing reperfusion injury strongly indicates that repertaxin is in fact a candidate novel therapeutic agent for the prevention and treatment of post-ischemic injury.

Our data prove that treatment with repertaxin by continuous infusion blocked the evolving late phase of reperfusion injury by inhibiting PMN recruitment in the post-ischemic liver (up to 96% of inhibition).

In addition, reperfused livers from rats treated with continuous infusion of repertaxin also had an almost well preserved structure and only scattered foci of hepatocellular necrosis were seen, but no PMN were identified inside or around them. Along the same line, repertaxin administration by continuous infusion has proven safe and well tolerated in different animal studies and in phase I studies in human volunteers (data not shown). Finally, repertaxin experimental efficacy was obtained in a range of plasmatic C_{ss} superimposable to C_{ss} observed in phase I volunteers, showing the possibility to evaluate repertaxin clinical efficacy in the same range of plasmatic C_{ss} proved to be efficacious in preventing experimental reperfusion injury.

The almost complete protection afforded by repertaxin treatment in this experimental rat model of reperfusion liver damage is a main goal, but also stresses, once more, the concept that post-ischemic injury is the consequence of the organization of an acute inflammatory response. Therefore, strategies aimed to block the activation of receptors for chemoattractant receptors, as repertaxin does, appear as new therapeutic approaches in the attempt to control inflammation evoked by ischemia/reperfusion insults.

In summary, we found that repertaxin, a new small-molecule inhibitor of CXCL8 receptor activation, is effective in preventing rat hepatocellular necrosis induced by a clinical-relevant time of reperfusion. According to the pharmacokinetic profile of repertaxin and its chemical-physical properties, treatment by continuous infusion was demonstrated to be the optimal route of administration of the compound. Because repertaxin has been proven safe and well tolerated in different animal studies and in phase I studies in human volunteers, it is in fact a candidate novel therapeutic agent for the prevention and treatment of post-ischemic injury. The protection afforded by repertaxin points to the relevance of CXCL8 as a pharmacological target to prevent lethal reperfusion injury due to PMN infiltration.

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