Lazaroid U-74389G in liver ischemia-reperfusion injury: A swine model

MARIA I. KORONTZI[1], GEORGE THEODOROPOULOS[1], GEORGE AGROGIANNIS[2], IOANNIS FLESSAS[1], DIMOSTHENIS CHRYSIKOS[1], ARISTEA GIOXARI[3], THEODOROS N. SERGENTANIS[4], EFSTRATIOS PATSOURIS[2], GEORGE C. ZOGRAFOS[1] and APOSTOLOS PAPALOIS[5,6]

[1]First Department of Propaedeutic Surgery, Hippocration Hospital; [2]Department of Pathology, School of Medicine, University of Athens, 11527 Athens; [3]Department of Nutrition and Dietetics, Harokopio University, 17676 Athens; [4]Department of Hygiene, Epidemiology and Medical Statistics, School of Medicine, University of Athens, 11527 Athens; [5]Experimental, Educational and Research Center, ELPEN Pharmaceuticals, 19009 Athens, Greece; [6]School of Medicine European University of Cyprus, 2404 Nicosia, Cyprus

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Abstract. Reactive oxygen species have a key role in liver ischemia-reperfusion (I/R) injury. In the present study, the effect of the anti-oxidant compound lazaroid U-74389G in preventing liver I/R injury was investigated in a swine model. Ischemia was produced by portal vein occlusion. Two sets of experiments were performed, each with two groups (n=7 per group). In the first group, the potential protective effect of an intracaval injection of U-74389G after a 30-min ischemia, followed by a 60-min reperfusion period was assessed (biopsies at 0, 15, 30 and 90 min experimental time). In the second set, the effect of intracaval U-74389G injection after 30 min of ischemia, followed by a longer reperfusion period of 120 min was determined (biopsies at 0, 15, 30 and 150 min experimental time). Liver malondialdehyde, hepatocyte vacuolation-degeneration, venous congestion, inflammatory cell infiltration, sinus congestion-dilation and Chiu score of intestinal damage were determined at up to 150 min of reperfusion. In the second set of experiments, the Chiu score of intestinal damage was improved by the administration of U-74389G (3.17±0.40 vs. 4.33±0.21; P=0.030). However, in the two sets of experiments, the liver inflammatory reaction was more pronounced in the U-74389G groups (P=0.017 for the first set, P=0.021 for the second set). No significant effect of U-74389G on any other parameters was detected. In conclusion, intestinal damage due to portal venous congestion and reflow appears to be mitigated by the lazaroid U-74389G; however, intracaval administration of U-74389G does not appear to exert any protective effects against liver I/R-induced inflammation.

Correspondence to: Dr Maria Korontzi, First Department of Propaedeutic Surgery, Hippocration Hospital, School of Medicine, University of Athens, Vas. Sofias 114, 11527 Athens, Greece
E-mail: markorontzi@gmail.com

Present address: Dr Maria Korontzi, 32 Boulevard verd de Saint Julien, 92190 Meudon, France

Abbreviations: I/R injury, ischemia-reperfusion injury, TNF-α, tumor necrosis factor-α, REP, reperfusion

Key words: liver ischemia-reperfusion, lazaroids, U-74389G, malondialdehyde, histopathology

Introduction

Lazaroids are synthetic 21-aminosteroids; as derivatives of methylprednisolone, the carbon 21 of the steroid molecule is replaced by an amino group, thus preserving the anti-inflammatory properties but lacking the steroidal side effects. The effects of lazaroids have been extensively examined in various organs (1-6). The cytoprotective effect of lazaroids is exerted partly by their anti-oxidant properties but also by inhibition of arachidonic acid release, membrane stabilization, suppression of Kuppfer cell activation and downregulation of cytokine expression and release (7-9). Structural differences and different mechanisms of action are responsible for the different efficacy of various lazaroid compounds (10).

The lazaroid U-74389G has been studied in various ischemia-reperfusion (I/R) injury models *in vitro* and *in vivo*, including rat liver lysosomes subjected to exogenously generated oxygen free radicals *in vitro* (8,11), renal I/R injury (12), pancreatic I/R injury in pigs (13,14), intestinal I/R injury in rats (15), orthotopic heart transplantation in mongrel dogs (16), as well as in canine liver preservation (17). The results indicated various mechanisms of action for the lazaroid U-74389G, including inhibition of lipid peroxidation, stabilization of the cellular membrane by incorporation into the lipid bilayer, suppression of pro-inflammatory gene expression by nuclear factor (NF)-κB inhibition, prevention of polymorphonuclear cell infiltration, scavenging of lipid

peroxyl radicals and reduction of tumor necrosis factor-α (TNF-α) release (6,10-12,16-18). Recently, a novel mechanism of action was assigned to U-74389G: inhibition of caspase-1, a cysteine-dependent, inflammatory protease responsible for the production of the pro-inflammatory cytokine interleukin-1b. This anti-inflammatory action was indicated to be time-dependent (19).

In the present study, U-74389G was administered to swine undergoing I/R via the inferior vena cava. A total of four experimental groups (two sets of experiments, comprising reperfusion for 60 or 120 min) were examined. Prior to reperfusion, a 30-min ischemia period was selected, as this appears to best represent the situation in the clinic, particularly in the emergency setting. Apart from liver indices, additional attention was paid to the assessment of intestinal damage, given that occlusion-reperfusion of the portal vein may respectively imply venous congestion-reflow in the small bowel, which is often accompanied by mucosal damage in the small intestine (20). As a whole, the aim of the present study was to evaluate the efficacy of intracaval administration of U-74389G in preventing liver I/R injury in a swine model.

Materials and methods

Experimental protocol. The experiments were performed at ELPEN laboratories (Athens, Greece; license no. EL 09 BIO 03) and were approved by the veterinary authorities of East Attica Region (ref. no. 3217-June 2007) in accordance with Greek legislation (p.d. 160/91) and European Community regulations (directive 309 of 1986; license according to E.U. legislation). This manuscript was written in accordance to the ARRIVE guidelines (21).

The animals used in the present study were Landrace Hellenic Domestic pigs (n=28; weight, 28-35 kg; age, 4-5 months) purchased from the same breeder in Koropi, Greece (E.U. license, EL 090011). Given the study design, the experimental unit was one animal. The animals were acclimatized to the laboratory conditions for 3-4 days with free access to food and water and were fasted during 24 h prior to the experiment, maintaining free access to water throughout. Pigs were housed in steel cages in a temperature-controlled environment, (temperature, 19-23˚C; humidity, 50-60%), on a 12-h light/dark cycle, and were fed with the same diet. All animals received general anesthesia and aseptic techniques were used for the surgical procedure. All procedures were performed at fixed time-points in the morning, to minimize any circadian effects.

A pre-medication injection with midazolam (0.5 mg/kg) and ketamine 15 mg/kg was administered intramuscularly (IM). Atropine (0.045 mg/kg) was administered IM in the neck at 10 min prior to intubation (22-26). Two polyethylene intravenous catheters (18G) were inserted into two peripheral veins in both ears for infusion of crystalloids and anesthetic drugs. Prior to intubation, a bolus of propofol (3 mg/kg) and fentanyl (0.012 mg/kg) was administered. The animals were then intubated and a bolus of cisatracurium (0.5 mg/kg) was administered. General anesthesia was maintained with continuous infusion of propofol at 6-8 mg/kg/h, as well as fentanyl (0.012 mg/kg; Janssen-Cilag International NV; Beerse, Belgium) and cisatracurium (0.5 mg/kg; GlaxoSmithKline Manufacturing SpA, Verona, Italy) and the animals were mechanically ventilated. The femoral vein was also catheterized for blood collection (23,25,26). During the experiment, the animals were continuously monitored by electrocardiography, pulse oximetry and measurement of arterial blood pressure. At the end of each experiment, the animals were euthanized using an intravenous overdose of 200 mg/kg pentobarbital (24).

For the laparotomy, a midline incision and full asepsis were performed prior to the manipulations. The portal vein was isolated and prepared for occlusion immediately prior to its division into the right and left branch at the hepatic hilum. Occlusion was performed with a bulldog clamp. At the end of the 30-min ischemic period, the test drug U-74389G was administered via the inferior vena cava, followed by reperfusion by removal of the clamp. The dose of the lazaroid U-74389G (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was 10 mg/kg.

Two sets of experiments were performed, one adopting reperfusion of 60 min (REP60 groups) and in another, reperfusion was performed 120 min (REP120 groups). In each set, the animals were randomly allocated into either the injection (n=7) or the control group (n=7). In each set, the injection group was compared with the control group. Therefore, two comparisons were made: REP60+U-74389G vs. REP60-controls and REP120+U-74389G vs. REP120-controls.

In the first set (REP60), liver biopsies were obtained at 0, 15, 30 and 90 min experimental time (0, 15 and 30 min of ischemia, and 60 min of reperfusion); at 90 min, small bowel (ileum) biopsies were also obtained. In the second set (REP120), liver biopsies were obtained at 0, 15, 30 and 150 min experimental time (0, 15 and 30 min of ischemia and 120 min of reperfusion); at 150 min, ileum biopsies were also taken. All experiments were acute, meaning that the animals were euthanized by the end of each experiment. The experimental end-points were as follows: liver I/R injury (assessed by determination of hepatocyte vacuolation/degeneration, venous congestion, inflammatory infiltration, sinus dilatation/congestion), liver malondialdehyde (MDA) and intestinal damage, expressed as the Chiu score.

The number of 28 animals (7 animals per group) was based on an a priori power calculation pertaining to the pathology scores. Specifically, assuming a 10% attrition rate, seven animals per group were sufficient for the achievement of 80% statistical power to detect a 0.4-unit change in scores (assuming a standard deviation per group equal to 0.2 units) at a significance level of 0.05. The power calculation was performed with G*Power 3.1.9.2 statistical software (University of Düsseldorf, Düsseldorf, Germany).

Histopathologic evaluation. Tissue specimens were fixed in 4% formalin, embedded in paraffin, sectioned and subjected to hematoxylin-eosin staining. For the histopathologic evaluation of the liver damage hepatocyte vacuolation/degeneration, venous congestion, inflammatory infiltration and sinus dilatation-congestion were rated using grades from 0 (normal) to 3 (severe). These histological findings were graded for morphological changes recognized to be secondary to I/R injury, according to a semi-quantitative scale, depending on the percentage of positive findings in 30 high-power fields for each slide with values assigned as 0 (0% positivity, none),

1 (1-25% positivity, mild), 2 (26-50% positivity, moderate) and 3 (51-100% positivity, severe) (27).

For evaluation of intestinal damage in specimens of ileum, Chiu scoring was used (28). The samples were assessed by two independent pathologists blinded to the experimental groups.

Liver MDA. Tissue samples were rinsed with ice-cold isotonic saline prior to homogenization, which was performed using TBS (20 mmol/; pH 7.4) and an Ultra-Turrax blender (IKA Labortechnik, Staufen, Germany), with 1 ml buffer used for 0.1 g tissue. A total of 10 ml 500 mmol/l butylated hydroxytoluene was added to 1 ml tissue homogenate to prevent sample oxidation. The homogenate was centrifuged at 704 x g and 4˚C for 10 min. and the clear supernatant was used for protein determination. To quantify the MDA content, a commercial kit (Bioxytech®-LPO-586™; OxIS Research™; OXIS Health Products, Inc. BIOXYTECH, Portland, OR, USA) was used according to the manufacturer's protocol. Measurement was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45˚C for 60 min. The stable chromophore product exhibits a maximal absorbance at 586 nm. The results were expressed as μmol/l solution.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. For each set of experiments repeated-measures analysis of variance was performed to assess the differences between groups, in view of the longitudinal nature of data. Given that each set of experiments comprised measurements at different time-points (0, 15, 30, 90 min for the REP60 set and 0, 15, 30, 150 min for the REP120 set), two separate models were constructed, one for each set.

The Chiu score of intestinal damage was not measured longitudinally; therefore, the Mann-Whitney U test for independent samples was used.

Statistical analysis was performed with STATA/SE version 13 statistical software (StataCorp., College Station, TX, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Animal model. Of the 28 randomized animals, 26 were included in the final analysis. A total of two animals, one of the REP120-control group and one of the REP120+U-74389G group, were not finally included in the study due to death of myocardial infarction as confirmed by electrocardiography, which is acknowledged as a common cause of mortality following liver I/R injury (29). The measured parameters in the two sets of experiments are presented in Table I.

Chiu score. In the second set of experiments, the Chiu score of intestinal damage was improved by the administration of U-74389G (3.17±0.40 in the REP120+U-74389G group vs. 4.33±0.21 in the REP120 controls, P=0.030, Mann-Whitney-U test for independent samples). Representative histological images for a Chiu score rating of 0 (REP120+U-74389G group) and 2 (REP120 control group) are provided in Fig. 1A and B, respectively.

Inflammatory reaction. However, in the two sets of experiments, the liver inflammatory reaction was more pronounced in the U-74389G groups (P=0.017 for the REP60+U-74389G vs. REP60 comparison, P=0.021 for the REP120+U-74389G vs. REP120 comparison; repeated-measures ANOVA). Representative histological images for an inflammatory reaction grade of 0 (control group) and 3 (U-74389G group) are provided in Fig. 2A and B, respectively.

Liver MD, hepatocyte vacuolization, venous congestion and sinus dilation. Liver MDA (P=0.056 for the first set and P=0.312 for the second set), hepatocyte vacuolation-degeneration (P=0.636 for the first set and P=0.207 for the second set), venous congestion (P=0.850 for the first set and P=0.223 for the second set) and sinus congestion-dilation (P=0.249 for the first set and P=0.797 for the second set) did not differ between the U-74389 injection and control groups in either set of experiments. Representative images for the histopathological evaluation of liver ischemia are provided in Figs. 3-5, including the presentation of hepatocyte vacuolization-degeneration grade 1 (Fig. 3), venous congestion grade 2 (Fig. 4) and sinus congestion-dilation grade 3 (Fig. 5).

Discussion

In the present study, intracaval administration of the lazaroid U-74389G was not able to favorably modify any of the examined histopathologic variables of hepatic I/R injury; namely hepatocyte vacuolization-degeneration, venous congestion, sinus congestion-dilation, as well as the biochemical markers of liver MDA. However, a protective effect at the level of the ileum was noted, as measured by the improved values of Chiu intestinal damage score. It therefore appears that U-74389G mitigates the damage associated with portal venous congestion-reflow (20).

The protective role of U-74389G at the level of the ileum was in accordance with that previously reported in the literature. Lazaroid U-74389G has been indicated to exert protective effects in rat models of experimental colitis and also on intestinal grafts after heterotopic small bowel transplantation, when administered to donor as well as recipient animals (30,31). Similarly, U-74389G treatment, in addition to cold storage in University of Wisconsin solution, has been indicated to improve the recovery of graft function and to minimize morphological damage to the small intestinal mucosa in a rat model of cold preservation (4). Beneficial effects of U-74389G were also demonstrated in the context of warm ischemia of the small bowel in a rat model (31). In addition, a study evaluating the effect of U-74389G on intestinal recovery after acute mesenteric ischemia and reperfusion in rats indicated that U-74389G was capable of protecting the small intestine from oxidative damage by inhibiting lipid peroxidation (32). Similar results were reported in pig models of bowel and liver ischemia, with U-74389G achieving an attenuation of the tissue levels of MDA and TNF-α, and improvement of the intestinal architecture (33,18).

The result that the inflammatory cell infiltration was more pronounced in the lazaroid group may not be surprising. Lazaroids at high concentrations may be associated with cell injury, although they are still effective radical scavengers (34). Indeed, this potentially detrimental effect was previously

Table I. Parameters measured in the two sets of experiments.[a]

	First set of experiments			Second set of experiments		
Parameter/time of reperfusion (min)	REP60-control (n=7)	REP60+U-74389G (n=7)	P-value	REP120-control (n=6)	REP120+U-74389G (n=6)	P-value
Liver MDA (pmol/mg protein)			0.056			0.312
0	0.88±0.14	0.36±0.05		0.74±0.09	0.52±0.18	
15	0.71±0.12	0.63±0.13		1.67±0.24	1.26±0.33	
30	1.11±0.25	0.62±0.08		0.84±0.25	1.26±0.27	
90	1.26±0.21	0.99±0.09		Not measured	Not measured	
150	Not measured	Not measured		1.14±0.19	0.52±0.18	
Hepatocyte vacuolation-degeneration[b]			0.636			0.207
0	0.00±0.00	0.14±0.14		0.00±0.00	0.00±0.00	
15	0.14±0.14	0.00±0.00		0.00±0.00	0.00±0.00	
30	0.29±0.29	0.00±0.00		0.00±0.00	0.17±0.17	
90	0.43±0.30	0.43±0.20		Not measured	Not measured	
150	Not measured	Not measured		0.33±0.33	0.83±0.31	
Venous congestiona			0.850			0.223
0	0.14±0.14	0.00±0.00		0.50±0.22	0.00±0.00	
15	0.43±0.20	0.29±0.18		0.83±0.17	0.67±0.21	
30	0.43±0.20	0.71±0.18		1.00±0.26	0.83±0.17	
90	1.00±0.38	1.14±0.14		Not measured	Not measured	
150	Not measured	Not measured		1.50±0.34	1.17±0.31	
Inflammatory cell infiltrationa			0.017			0.021
0	0.00±0.00	0.43±0.20		0.00±0.00	0.67±0.33	
15	0.14±0.14	0.71±0.18		0.00±0.00	1.17±0.31	
30	0.57±0.20	1.14±0.14		0.33±0.21	1.17±0.31	
90	1.14±0.26	1.71±0.29		Not measured	Not measured	
150	Not measured	Not measured		1.67±0.42	2.50±0.34	
Sinus congestion-dilationa			0.249			0.797
0	0.29±0.18	0.00±0.00		0.33±0.21	0.00±0.00	
15	0.43±0.20	0.71±0.18		0.50±0.34	1.00±0.26	
30	0.86±0.14	0.86±0.26		1.00±0.45	1.17±0.17	
90	2.00±0.00	1.29±0.18		Not measured	Not measured	
150	Not measured	Not measured		2.00±0.26	2.00±0.45	
Chiu score of intestinal damage	3.43±0.53	2.57±0.37	0.262	4.33±0.21	3.17±0.40	0.030

[a]P-values were derived from repeated-measures analysis of variance, except for Chiu score of intestinal damage, for which the Mann-Whitney-Wilcoxon test for independent samples was performed. Data are expressed as the mean ± standard error of the mean. [b]Histological parameters were evaluated using a semi-quantitative scale, with values assigned as 0 (0% positivity; none), 1 (1-25% positivity; mild), 2 (26-50% positivity; moderate) and 3 (51-100% positivity; severe). MDA, malondialdehyde; REP120, reperfusion for 120 min subsequent to 30 of ischemia.

noted in a swine pancreatic I/R model, where edema was more pronounced in the U-74389G group, in contrary to the protective action that may have been expected (13).

Although the present study did not point to the beneficial effect of U-74389G in liver ischemia, other studies have supported favourable effects of this agent. Todo *et al* (17) used a canine liver transplantation protocol with preservation using lazaroid U-74389G, revealing that MDA increased in all experimental groups during preservation and decreased after reperfusion. Fukuma *et al* (6) assessed the protective effect of U-74389G in an experimental rat model of endotoxin-induced liver injury. They revealed that U-74389G reduced lipid

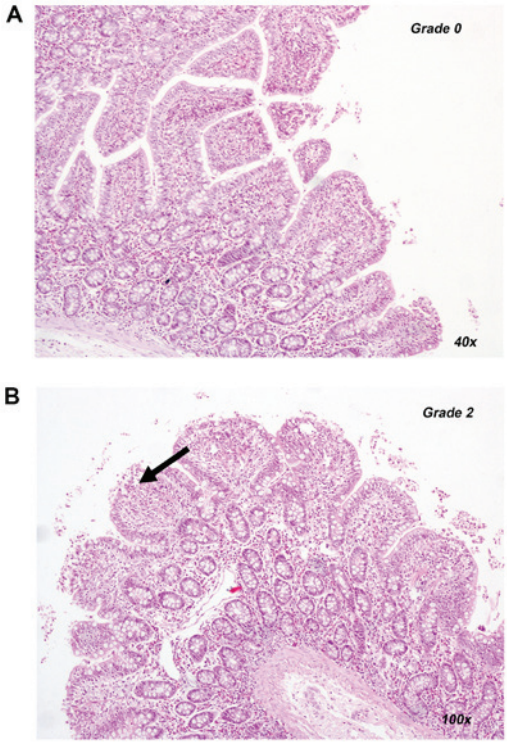

Figure 1. Chui score of ileum specimens. Light microscopy of ileum frozen sections stained with hematoxylin-eosin. Representative images of tissues with (A) a Chiu score rating of 0 in REP120+U-74389G group and (B) a Chiu score rating of 2 in REP120 control group, immediately following reperfusion, at 150 min experimental time (original magnification, x100). In (B), an extension of the subepithelial space with moderate epithelial lifting from the lamina propria is apparent, as indicated by the black arrow.

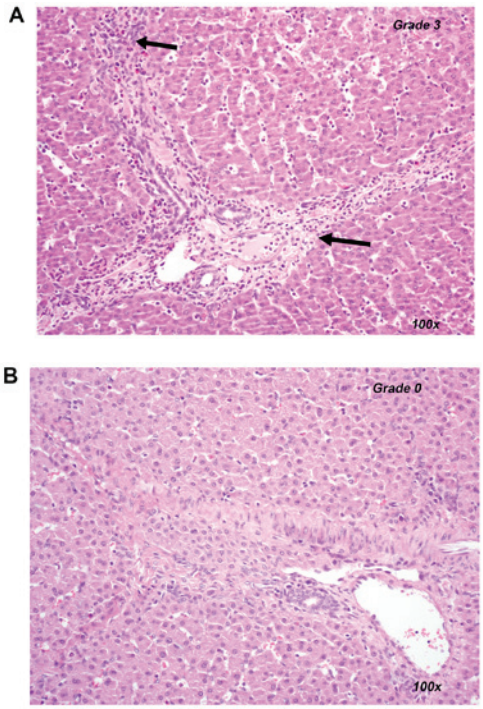

Figure 2. Hepatic inflammatory reaction. Representative light microscopy image of a hepatic frozen section stained with hematoxylin-eosin (original magnification, x100). (A) Inflammatory reaction grade 3 in REP60+U-74389G group, at 60 min of reperfusion (90 min experimental time) and (B) Inflammatory reaction grade 0 in REP60 untreated-control group at 60 min of reperfusion (90 min experimental time). Black arrows indicate acute inflammatory cells (neutrophils).

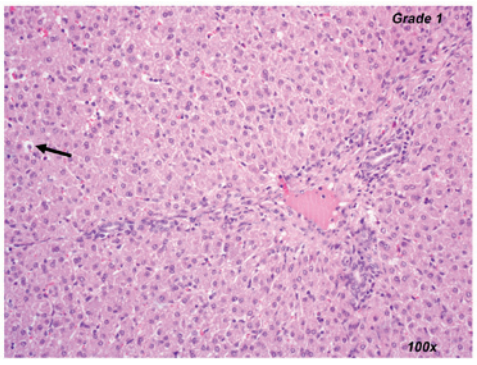

Figure 3. Hepatocyte vacuolization-degeneration grade 1 in U-74389G group, following 120 min reperfusion (150 min experimental time). Representative light microscopy image of hepatic frozen section stained with hematoxylin-eosin (original magnification, x100). The black arrow indicates a vacuolated hepatocyte.

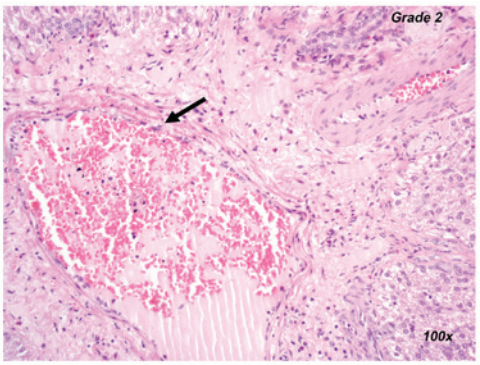

Figure 4. Hepatic venous congestion grade 2 in U-74389G group, following 120 min reperfusion (150 min experimental time). Representative light microscopy image of hepatic frozen section stained with hematoxylin and eosin (original magnification, x100). Black arrow indicates a dilated hepatic vein.

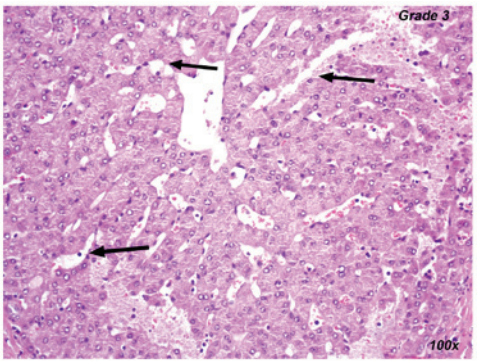

Figure 5. Sinus congestion-dilation grade 3 in U-74389G untreated group, following 60 min reperfusion (90 min experimental time). Representative light microscopy image of hepatic frozen section stained with hematoxylin and eosin (original magnification, x100). Black arrows indicate sinusoidal congestion and dilated sinuses.

peroxidation as indicated by the reduction in MDA (the end product of lipid peroxidation), suppressed pro-inflammatory gene expression by NF-κB inhibition and prevented polymorphonuclear cell infiltration in the liver (6).

In an experimental study on intestinal I/R in swines, Flessas *et al* (5) obtained a statistically significant reduction in

MDA, TNF-α and histopathological scores in tissue treated with U-74389G. In their study, four groups of swine were examined: i) control group-ischemia for 30 min and reperfusion for 60 min; ii) control group-ischemia for 30 min and reperfusion for 120 min; iii) ischemia for 30 min, immediately followed by intravenous injection of lazaroid U-74389G and reperfusion for 60 min; and iv) ischemia for 30 min, immediately followed by intravenous injection of lazaroid U-74389G and reperfusion for 120 min. The present protocol followed the same setting of experimental conditions as the study by Flessas *et al* (5).

Conversely, a recent study using an experimental protocol to induce hemorrhagic shock in swine revealed no protective effect of U-74389G in the small intestine according to the tissue MDA and serum alkaline phosphatase levels. Three groups underwent resuscitation with fluid alone, and in another 3 groups, the lazaroid U-74389G was administered in addition to fluid, while the control group underwent all the surgical procedures without hemorrhagic shock (23).

To the best of our knowledge, only one previous study, namely that by Tsaroucha *et al* (18), has assessed the use of the lazaroid U-74389G to prevent liver I/R injury in a swine model. The study comprised three experimental groups and lazaroid was administered at the end of a 30-min ischemia period, and immediately prior to reperfusion. U-74389G was administered intraportally at a dose of 10 mg/kg. Portal infiltration, MDA and TNF-α levels were significantly lower in the U-74389G-treated groups compared with those in the control (untreated) animals (18).

In the present study, U-74389G was administered by injection into the inferior vena cava; whereas in the study by Tsaroucha *et al* (18), the compound was administered intraportally. This difference may be accountable for a reduced effectiveness, as intraportal administration may maximize the effects of the agent on the liver while systemic distribution is obviated; on the contrary, by intracaval administration, the lazaroid is distributed to the systemic circulation prior to being metabolized in the liver. Furthermore, in most experimental protocols used in the past, the administration of the lazaroid was performed prior to the onset of ischemia (35,36), a situation that cannot be encountered in real clinical practice. In the present study, the compound was administered at the end of ischemia and prior to reperfusion.

The appropriate dose of lazaroids is a topic under investigation. The dose of 10 mg/kg appears to be the most effective in various studies with liver I/R injury (13-15). The mechanism of action of U-74389G in liver or intestinal ischemia in swine is based on the inhibition of lipid peroxidation (as evidenced by the attenuation of MDA), the reduction of TNF-α tissue levels and the improvement of intestinal and liver histopathological parameters The reduction in the serum levels of TNF-α may also contribute to the reduction of leukocyte infiltration (36).

Another limitation of the present study is the application of two separate repeated-measures ANOVA models instead of one; this was deemed necessary, given that each set of experiments comprised measurements at different time-points (0, 15, 30 and 90 min for the REP60 set and 0, 15, 30 and 150 min for the REP120 set). Indeed, the inherently missing values at the last two time-points (for 50% of the whole dataset) would have introduced bias in the calculations. It is possible that a future meta-analysis of the data may answer if the difference in the last two time points is responsible for bias in the present results.

There are also certain technical limitations to the present study, given the lack of more elaborate biochemical end-points, e.g., the levels of NF-κB or TNF-α, which may be mediators of the mechanism of action of U-74389G. Although the focus of the present study was on whether U-74389G has a beneficial or no effect on liver I/R injury in swine, further studies are required to determine the exact biochemical pathways involved in this effect.

In conclusion, intracaval administration of U-74389G does not appear to exert any protective effects on liver I/R-associated injury. However, the intestinal architecture appears to be protected by the lazaroid U-74389G. Further studies that also evaluate intrahepatic tissue concentrations of the agent appear to be required for the optimal protection of the liver in the context of I/R injury.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MK, AP and GZ conceived the study, TS and GA analyzed and interpreted the data and TS performed the statistical analysis. AG, GA and EP performed the histological examination, the laboratory tests, and were major contributors in writing the manuscript. AG, AP and EP provided biological materials, tools and intruments vital for the experimental results. MK, IF and DC reviewed the literature, performed the experiments and took responsibility for data management and reporting. MK and GT took responsibility for the logical interpretation and presentation of the results. GT, GZ and AP reviewed the article prior to submission and gave the final aproval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were performed at ELPEN laboratories (license no. EL 09 BIO 03) and were approved by the veterinary authorities of East Attica Region (ref. no. 3217-June 2007) in accordance with Greek legislation (p.d. 160/91) and European Community regulations (directive 309 of 1986; license according to E.U. legislation).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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