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Kristian Riesbeck
Hanna Schatz
Öyivind Östraat
Gunnar Tufveson
Arne Forsgren
Henrik Ekberg

Enhancement of the immunosuppressive effect of cyclosporin A by ciprofloxacin in a rat cardiac allograft transplantation model

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K. Riesbeck (✉) · A. Forsgren
Department of Medical Microbiology,
Lund University,
Malmö General Hospital,
S-214 01 Malmö, Sweden
Fax: +46 40 336 234

H. Schatz · Ö. Östraat · H. Ekberg
Transplantation Unit,
Department of Surgery,
Lund University,
Malmö General Hospital,
S-214 01 Malmö, Sweden

G. Tufveson
Transplantation Unit,
Department of Surgery,
Gothenburg University,
Sahlgrenska Hospital,
S-413 45 Gothenburg, Sweden

Abstract Ciprofloxacin hyperinduces interleukin-2 production in stimulated human and mouse lymphocytes. In this study, an enhanced and prolonged interleukin-2 response was also detected in polyclonally stimulated rat splenocytes in the presence of ciprofloxacin (5–80 μg/ml) compared to control cells without any antibiotic. Ciprofloxacin was able to counteract the immunosuppressive effect of 10 ng/ml cyclosporin A (CyA) but did not interfere with higher CyA concentrations. In parallel, ciprofloxacin did not influence thymidine uptake in mixed lymphocyte reactions in the presence of CyA. To obtain an *in vivo* application of these findings, graft survival was studied by performing rat cardiac allograft transplantations in the presence or absence of CyA. Brown Norway rats served as donors and Wistar Furth rats as recipients. Ciprofloxacin was injected intraperitoneally either at a high-dose regimen (240 mg/kg per 24 h) into rats every 8th h starting 1 day before transplantation until day 21 or graft loss, or it was injected at a low and clinically relevant dose regimen (45 mg/kg per 24 h) until day 9. CyA was administered orally (10 mg/kg per 24 h) from day 1 through day 9. Ciprofloxacin given alone at a high-dose regimen resulted in a median graft survival of 14.8 days, which was significantly longer than graft survival in rats without treatment (median 8.0 days). A low-dose regimen of ciprofloxacin alone did not affect graft survival. Ciprofloxacin at a high-dose regimen combined with CyA prolonged graft survival to a median of 24.0 days compared to 20.5 days with CyA alone. Ciprofloxacin administered in the drinking water (200 mg/kg per 24 h) until day 9 in addition to CyA did not affect graft survival. However, when the same dose regimen was used in experiments with PVG rats as donors and Wistar/Kyoto as recipients, graft survival was significantly prolonged to a median of 45 days. Ciprofloxacin, given orally without the addition of CyA, did not influence graft survival in either of the two strain combinations. Thus, our data show that ciprofloxacin has no negative impact on heart graft survival rats. It remains to be clarified whether ciprofloxacin influences graft survival in humans.

Key words Cyclosporin A, ciprofloxacin, rat · Ciprofloxacin, cyclosporin A, rat · Heart transplantation, rat, immunosuppression
Immunosuppression, ciprofloxacin

Introduction

The fluoroquinolone antibiotic ciprofloxacin has a broad antibacterial spectrum and is widely used to combat severe infections [37]. In bacteria, the site of action is the DNA-gyrase-DNA complex [21, 31, 36]. During recent years, several reports have been published demonstrating ciprofloxacin as a biological response modifier, in addition to its antibacterial activity [30].

Ciprofloxacin has various effects on lymphocytes and monocytes *in vitro*. At high experimental concentrations (> 50 μg/ml), ciprofloxacin inhibits DNA replication and cell cycle progression, and it induces DNA strand breaks in human lymphocytes [6, 13, 14]. In addition, the synthesis of human tumor necrosis factor-α (TNF-α) [2], IL-1 [29], lymphotoxin (LT), and granulocyte-macrophage colony-stimulating factor (GM-CSF) is inhibited [25]. Ciprofloxacin at low concentrations (< 50 μg/ml) stimulates various immune parameters. Examples of stimulatory effects of ciprofloxacin are increased thymidine incorporation in phytohemagglutinin (PHA)-stimulated lymphocytes [6, 13] and an upregulated IL-1 production demonstrated in lipopolysaccharide (LPS)-stimulated murine monocytes [16, 22, 35].

Interestingly, ciprofloxacin, at both low and high concentrations (5–80 μg/ml), induces the hyperproduction of IL-2 [23–25, 27, 28, 35, 38] and IFN-γ [17, 24, 27, 38] at the mRNA and protein levels in mitogen-stimulated human lymphocyte cultures. Ciprofloxacin (20–80 μg/ml) has also been shown to increase the levels of mRNA for IL-1 α, IL-2 receptor, IFN-γ, IL-3, IL-4, GM-CSF, TNF-α, and LT [28]. Experiments with the human T-cell lymphoma cell line Jurkat or the murine equivalent EL-4, transiently transfected with a plasmid containing the IL-2 promoter region, show ciprofloxacin to enhance IL-2 promoter activation [24, 28]. This would appear to be due to an earlier and stronger ciprofloxacin-dependent activation of the transcriptional regulation factors NFAT-1 (nuclear factor of activated T cells) and AP-1 (activator protein-1). In accordance with these findings, under certain *in vitro* conditions, ciprofloxacin counteracts the effect of the immunosuppressive agent cyclosporin A (CyA), resulting in increased IL-2 and IFN-γ production [27].

Only a few reports exist on ciprofloxacin influencing immune functions *in vivo*. Ciprofloxacin enhances repopulation of murine hematopoietic organs in sublethally irradiated mice, possibly through a stimulating effect on IL-3 and GM-CSF production [20]. Furthermore, repeated subcutaneous treatment of mice with ciprofloxacin (20 mg/kg per 24 h) for 1 week, following isolation and PHA stimulation of splenocytes *ex vivo*, shows an increased thymidine uptake compared to untreated control mice [35]. In parallel with the increased murine thymidine incorporation, another report has recently been published showing that ciprofloxacin treatment *in vivo* increases the *ex vivo* capacity of LPS-stimulated human monocytes to produce IL-1, IL-6, and TNF-α [3].

One aim of this investigation was to determine whether ciprofloxacin enhances IL-2 expression in rat lymphocytes. We also wished to reveal the influence of ciprofloxacin on graft survival in a rat cardiac allograft transplantation model in the presence or absence of the immunosuppressant CyA [34]. In the present study, evidence is shown that ciprofloxacin upregulates IL-2 production in rat lymphocytes. Quite unexpectedly, ciprofloxacin prolongs graft survival in our cardiac transplantation model.

Materials and methods

Animals and drugs

In the first transplantation experiment, isogenic strains of Brown Norway (BN) rats, weighing 100–150 g, served as donors and Wistar Furth (WF) rats, weighing 100–120 g, as recipients. Ciprofloxacin (45 mg/kg per 24 h) was injected intraperitoneally every 8th h, starting 1 day before transplantation (day 0) until day 9. Ciprofloxacin (240 mg/kg per 24 h) was injected intraperitoneally every 8th h, from day 0 until day 21 or graft loss. In the second transplantation experiment, ciprofloxacin was given in the drinking water (2 mg/ml; 200 mg/kg per 24 h) until day 9. CyA was administered (10 mg/kg per 24 h) orally from day 1 through day 9 in all experiments where indicated. Cefuroxime (40 mg/kg per 24 h) was injected twice daily until day 9 or graft rejection. In one set of experiments with ciprofloxacin administered orally, inbred male PVG ($RT1^c$) rats, weighing 100–150 g, served as donors and Wistar/Kyoto (Wi/Ky) rats ($RT1^l$), weighing 180–220 g, as recipients. BN and WF rats were bred at our laboratory and PVG and Wi/Ky rats were obtained from Möllegard Farm (Skensved, Denmark). Ciprofloxacin powder and injection solution (2 or 10 mg/ml) was kindly provided by Bayer (Wuppertal, Germany). CyA was purchased from Sandoz (Basel, Switzerland) as a stock solution of 50 mg/ml. Cefuroxime was obtained from Glaxo (Greenford, England). PHA (Wellcome, Dartford, England) was dissolved in RPMI 1640 with Hepes buffer (Gibco, Paisley, Scotland) and used at a final concentration of 1 μg/ml.

Surgical procedure

Heterotopic rat heart transplantations were performed using BN or PVG rats as donors and WF or Wi/Ky rats as recipients. Hearts were implanted to the right cervical vessels using a nonsuture cuff technique [18]. Graft survival was studied and graft rejection was defined as cessation of palpable heart beats at twice daily examinations.

Determination of serum levels of ciprofloxacin

Drug levels in serum were analyzed in duplicate using an agar diffusion technique (indicator bacterium: *Escherichia coli*). After incubation for 18–20 h at 37 °C, the inhibition zones were measured and the drug concentration calculated using a calibration standard on the same plate as reference.

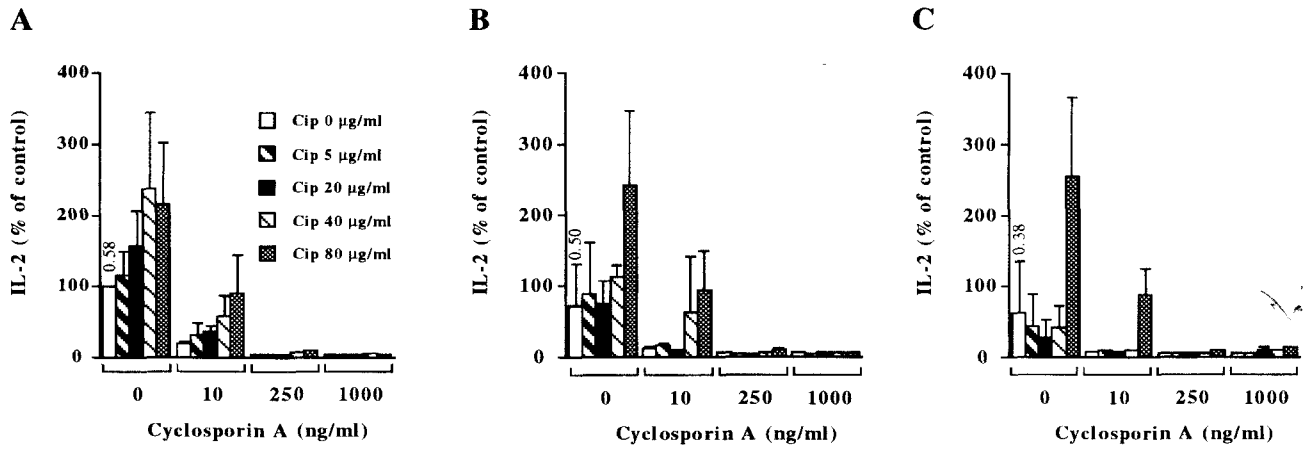

Fig. 1 A–C Effects of ciprofloxacin on IL-2 production in rat lymphocytes after **A** 24 h **B** 48 h and **C** 72 h of incubation. At initiation of cultures, rat splenocytes (10^6/ml) were incubated with PHA (1 µg/ml) and ciprofloxacin (Cip). A dose-response analysis with CyA (10–1000 ng/ml) was performed. Cells were harvested at the times indicated and analyzed for IL-2 biological activity. The means of experiments with lymphocytes from four different rats are presented. Mean IL-2 values (units/ml) for the controls are also indicated. *Error bars* indicate standard deviations. IL-2 concentrations with ciprofloxacin at 24 h were significantly separable ($P < 0.05$ or less) from control cultures without any drugs

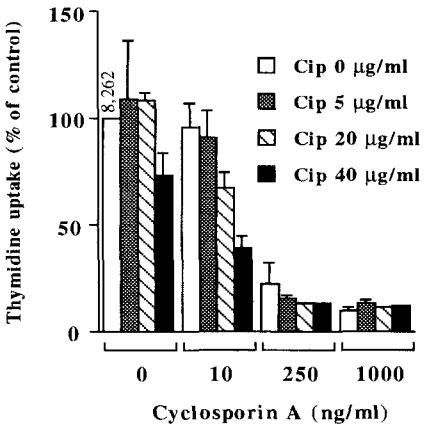

Fig. 2 Effects of ciprofloxacin and CyA on thymidine uptake in rat mixed lymphocyte reactions (MLR). Ciprofloxacin (Cip) and CyA (10–1000 ng/ml) were supplemented at initiation of culture. MLR consisted of BN (10^6/ml) and WF (10^6/ml) splenocytes, which were pulsed with [^{3}H]thymidine during the last 18 h of culture. At 96 h of incubation, cells were harvested and incorporated thymidine measured in a scintillation counter. The incorporation of [^{3}H]thymidine in controls was, on the average, 8.262 cpm. *Error bars* indicate standard deviations

Cell cultures and IL-2 analysis

Lymphocytes from rat spleens were obtained by mechanical disruption and were incubated in RPMI 1640 (10^6/ml in triplicate wells) supplemented with 8 % fetal calf serum, glutamine, gentamicin (12 µg/ml), and 10^{-5} M mercaptoethanol. Ciprofloxacin and CyA were introduced into cell cultures simultaneously with mitogen (PHA). In "two-way" mixed lymphocyte reactions (MLR), equal amounts ($10^6 + 10^6$ cells/ml) of splenocytes from WF and BN rats were incubated for 96 h. To quantify DNA synthesis, lymphocytes were pulsed with [*methyl*-^{3}H]thymidine (1 µCi; TRK 120; specific activity 5 Ci/mmol; Amersham, Buckinghamshire, England) during the last 18-h period. Radioactivity was measured in a scintillation counter. IL-2 biological activity in supernatants was analyzed by the IL-2-dependent stimulation of proliferation of the murine cytolytic T-lymphocyte line CTLL-2, as previously described [25].

Statistics

Wilcoxon's rank sum test and log-rank statistics were used. A P level below 0.05 was considered statistically significant.

Results

Enhancement of IL-2 production in mitogen-stimulated rat lymphocyte cultures

As can be interpreted from Fig. 1, the antibiotic ciprofloxacin caused an increased and prolonged IL-2 response to PHA. At 24 h of incubation, ciprofloxacin (5–80 µg/ml) significantly increased IL-2 concentrations compared to control cells. An enhanced IL-2 production by ciprofloxacin (80 µ/ml) was detected up to 72 h. CyA was also included to investigate whether ciprofloxacin interfered with CyA-dependent inhibition of IL-2 production. In supernatants analyzed for IL-2 biological activity, a total inhibition of IL-2 synthesis by CyA alone (10–1000 ng/ml) was observed. When rat splenocytes were incubated with ciprofloxacin in the presence of CyA, only ciprofloxacin at 80 µg/ml was able to counteract the immunosuppressive effects of CyA at 10 ng/ml.

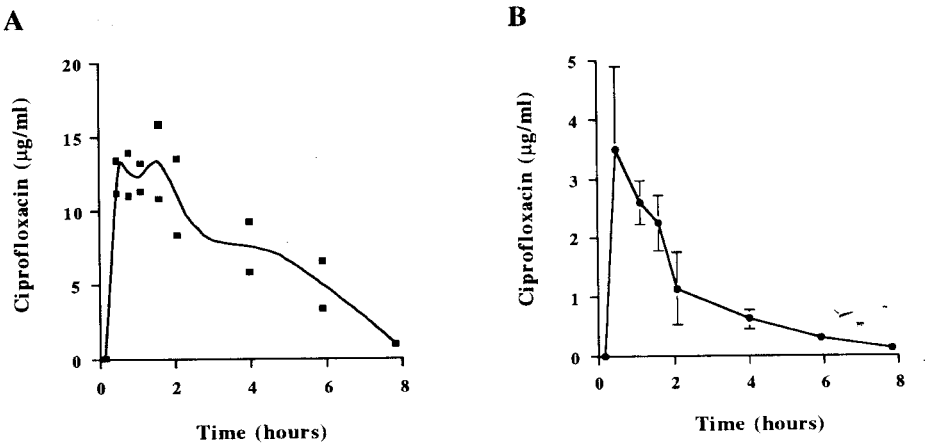

Fig. 3 A, B Serum pharmacokinetics in rats during treatment with ciprofloxacin. **A** A single dose of ciprofloxacin, 240 mg/kg, in two different rats. The curve with mean values is indicated. **B** Mean values of a single dose of ciprofloxacin, 45 mg/kg, in six different rats. Serum samples were obtained at the times indicated and analyzed for ciprofloxacin activity in an agar diffusion test. *Error bars* indicate standard deviations

The next step in our analysis was to elucidate whether ciprofloxacin and CyA interfered with primary mixed lymphocyte reactions (MLR) during allogeneic stimulation. Thymidine incorporation was measured at 96 h. In this experimental model, ciprofloxacin (5 and 20 μg/ml) slightly increased thymidine uptake (Fig. 2) However, ciprofloxacin at low concentrations did not interfere with the inhibitory effects of CyA.

Prolongation of graft survival in a rat cardiac allograft transplantation model

In order to elucidate whether the IL-2 upregulation by ciprofloxacin had any relevance in a solid organ transplant model, cardiac transplantations were performed with BN rats as donors and WF rats as recipients. Ciprofloxacin (240 mg/kg per 24 h) was administered intraperitoneally every 8th h on day 1 until day 21 or graft loss. Serum pharmacokinetics of ciprofloxacin from these experiments is shown in Fig. 3 A. Peak serum levels were 13.3 (10.8–15.8) μg/ml (days 1 and 3) and trough levels were 0.92 (0.89–0.95) μg/ml (day 1) and 2.32 (2.30–2.35) μg/ml (day 3). Graft survival was significantly longer in rats receiving ciprofloxacin (median 14.8 days) compared to control rats without any treatment (median 8.0 days; $P = 0.01$; Table 1, groups 1 and 2). To investigate whether ciprofloxacin and CyA had antagonistic action on graft survival, rats were given CyA, 10 mg/kg per day, orally from day 1 through day 9. Ciprofloxacin and CyA combined prolonged graft survival to 24.0 days compared to 20.5 days with CyA alone (Table 1, groups 3 and 8). To exclude an antibacterial protection by ciprofloxacin, transplanted rats were given cefuroxime intraperitoneally for 9 days (group 9). However, cefuroxime did not influence graft survival.

In other transplantation experiments ciprofloxacin was administered intraperitoneally at a low, clinically relevant dose regimen (45 mg/kg per 24 h) every 8th h on days-1 until day 9. Peak serum levels were 3.5 (1.4–4.4) μg/ml and trough levels were 0.12 (0.07–0.17) μg/ml (Fig. 3 B). Ciprofloxacin or ciprofloxacin in combination with CyA did not significantly prolong graft survival compared to CyA alone (Table 1, groups 4, 5, and 8). A tendency toward prolonged graft survival was detected in transplanted rats treated with both ciprofloxacin (low-dose) and CyA (group 5).

Since an extended graft survival was observed with ciprofloxacin treatment (240 mg/kg per 24 h), a third set of transplantations (BN-to-WF) was performed with ciprofloxacin administered in the drinking water (2 mg/ml; 200 mg/kg per 24 h) from day 1 until day 9. Mean serum trough levels of ciprofloxacin were 0.87 (0.2–1.9) μg/ml. Ciprofloxacin did not significantly influence graft survival in the absence or presence of CyA (Table 1, groups 6 and 7). However, ciprofloxacin induced tolerance (graft survival > 150 days) in one out of six rats when administered in combination with CyA (group 7). Interestingly, when PVG rats were used as donors and WKy rats as recipients, ciprofloxacin in combination with CyA prolonged median graft survival to 45 days (group 12). In this group, ciprofloxacin and CyA induced tolerance in two out of six rats and five out of six heart allografts survived for more than 40 days. As with the BN-to-WF combination, ciprofloxacin given in the drinking water without CyA did not influence graft survival in WKy rats transplanted with PVG hearts (group 11).

Discussion

Our results show that ciprofloxacin enhanced IL-2 synthesis in stimulated rat lymphocytes in a manner similar to that observed in both murine and human experimental models [23–25, 27, 28, 35, 38]. Evidence has recently been presented that ciprofloxacin (20–80 μg/ml) is able to counteract the immunosuppressive effect of CyA-dependent inhibition of IL-2 and IFN-γ produc-

Table 1 Cardiac graft survival after ciprofloxacin treatment in the presence or absence of CyA. Different combinations (numbers 1–13) of rat strains and drugs are indicated. Ciprofloxacin high and low i.p. indicate intraperitoneal administration of 240 mg/kg per 24 h and 45 mg/kg per 24 h, respectively. Ciprofloxacincin orally specifies a dosage regimen of 200 mg/kg per 24 h. Hearts were implanted to the right cervical vessels using a nonsuture cuff technique [18]. Graft rejection was defined as cessation of palpable heart beats

Treatment and strain combination	Graft survival (days)			
BN-to-WF	Median	Individual values	*P*	value
1. No treatment	8.0	6.5, 7.5, 7.5, 8.5, 9.5, 13.5		
2. Cipro (high, i.p.)	14.8	9, 9.5, 14.5, 15, 17, 19	0.01	2 vs 1
3. CyA + cipro (high, i.p.)	24.0	11, 20, 24, 24, 25, 34	0.002	3 vs 1
			NS	3 vs 8
4. Cipro (low, i.p.)	9.0	8, 9, 11		
5. CyA + cipro (low, i.p.)	22.0	15, 18, 19, 25, 35, 55	0.001	5 vs 1
			NS	5 vs 8
6. Cipro (orally)	9.0	7, 8, 9, 9, 10, 15		
7. CyA + cipro (orally)	14.3	11, 12, 14, 14.5, 14, > 150	0.005	7 vs 1
			NS	7 vs 8
8. CyA	20.5	15, 16, 20, 21, 21.5, 26	0.001	8 vs 1
9. Cefuroxime (i.p.)	9.0	6, 9, 11		
PVG-to-WKy				
10. No treatment	8.0	7, 8, 8, 8, 9, 9		
11. Cipro (orally)	9.0	8, 9, 9		
12. CyA + cipro (orally)	45.0	27, 42, 44, 46, > 100, > 100	0.001	12 vs 10
			0.001	12 vs 13
13. CyA	19.5	16, 17, 19, 20, 21, 27	0.001	13 vs 10

tion in human PHA-stimulated lymphocytes *in vitro* [27]. In this study, ciprofloxacin at a high concentration only (80 μg/ml) was able to interfere with CyA in rat splenocytes (Fig. 1). Thus, in the presence of CyA, the influence of ciprofloxacin on IL-2 production in rat splenocytes differed from the effects on IL-2 synthesis observed in human lymphocytes [27]. In parallel, ciprofloxacin did not interfere with CyA in allogeneic stimulated rat splenocytes (Fig. 2).

In spite of indications of an activated immune system, increased IL-2 synthesis (Fig. 1) and transcription [23–25, 27, 28], ciprofloxacin at high-dose regimens extended graft survival in our rat cardiac transplant model (Table 1). It has been shown that IL-2 is strongly expressed and secreted in both human and mouse tissue during an antigraft response [9–11, 32]. Indeed, IL-2 accumulates in tolerated heart grafts [8]. This evidence of IL-2 expression in transplanted tissue thus indicates that we could have expected an increased IL-2 production and, consequently, a negative impact on graft survival in rats treated with the IL-2-enhancing drug ciprofloxacin. However, CyA, at clinically achievable serum concentrations, almost completely inhibited the ciprofloxacin-dependent IL-2 increase *in vitro* (Fig. 1).

Many possible explanations for prolonged graft survival in rats on ciprofloxacin medication may be considered. Results from our laboratory reveal that ciprofloxacin (> 5 μg/ml) inhibits murine IFN-γ production *in vitro* [24]. This is in contrast to human IFN-γ production, which is strongly upregulated by ciprofloxacin (5–80 μg/ml) [17, 27, 38]. The level of expression of the IFN-γ gene is higher in allogeneic than in syngeneic grafts in mice, as demonstrated by Dallman and colleagues [12]. Therefore, a putative ciprofloxacin-dependent inhibition of IFN-γ synthesis in rodents may lead to a diminished expression of IFN-γ-inducible HLA class II antigens [5], which is crucial in graft rejection [1, 4] and, in turn, promote tolerance.

It is important to take into account that eukaryotic cells accumulate fluoroquinolones intracellularly [15]. In bacteria, fluoroquinolones inhibit the supercoiling enzyme DNA gyrase (a type II topoisomerase) [36] but have, in general, been found to be only weak inhibitors of the eukaryotic enzyme in *in vitro* assays [19]. However, evidence of an intracellular effect by ciprofloxacin (80 μg/ml) on topoisomerase II activity in human lymphoblastoid cells has been described [7]. Ciprofloxacin (100 μg/ml) has also been reported to inhibit the oxidative phosporylation by 50 % in isolated rat liver mitochondria [26]. Thus, in lymphocytes, a mitochondrial and/or topoisomerase inhibition may be envisaged by high concentrations of ciprofloxacin, resulting in prolonged graft survival.

In the presence of ciprofloxacin, lymphocyte cell cycle progression (> 20 μg/ml of ciprofloxacin) and thymidine uptake (> 50 μg/ml of ciprofloxacin) are inhibited [13]. Inhibitory effects of ciprofloxacin on the proliferation of Lewis lung carcinoma, a murine bladder carcinoma cell line (25–250 μg/ml) *in vitro,* and on local and metastatic tumor development in mice (60 mg/kg per 24 h) have been demonstrated by Zehavi-Willner [39, 40]. For comparision, peak serum levels of ciprofloxacin in our transplant model did not exceed 15 μg/ml (intraperitoneal administration; 240 mg/kg per 24 h) and 2.0 μ/ml (oral administration; 200 mg/kg per 24 h; Fig. 3). A decrease in bone marrow graft uptake in mice treated with ciprofloxacin (100 mg/kg per 24 h) has also been described [33]. Furthermore, Kletter and collaborators reported inhibition of repopulation of murine hematopoietic organs after treatment with ciprofloxacin (> 90 mg/kg per 24 h) [20]. Thus, the data presented in this report confirms earlier observations of a ciprofloxacin-dependent decrease in cell proliferation *in vivo.* However, further investigations are needed to determine whether ciprofloxacin influences graft survival in human recipients.

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