Regulatory Toxicology and Pharmacology 74 (2016) 161–169

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Safety study of Ciprofloxacin in newborn mice

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ARTICLE INFO

Article history:
Received 3 November 2015
Accepted 5 November 2015
Available online 26 November 2015

Keywords:
Fluoroquinolone
Neonate
Neurodevelopment
Arthrotoxicity
Nephrotoxicity
Hepatotoxicity

ABSTRACT

Ciprofloxacin, a broad-spectrum antimicrobial agent belonging to the fluoroquinolone family, is prescribed off-label in infants less than one year of age. Ciprofloxacin is included in the European Medicines Agency priority list of off-patent medicinal products requiring evaluation in neonates. This evaluation is undergoing within the TINN (Treat Infections in Neonates) FP7 EU project. As part of the TINN project, the present preclinical study was designed to assess the potential adverse effects of Ciprofloxacin on neurodevelopment, liver and joints in mice. Newborn mice received subcutaneous Ciprofloxacin at 10, 30 and 100 mg/kg/day from 2 to 12 postnatal days. Peak plasma levels of Ciprofloxacin were in the range of levels measured in human neonates. We examined vital functions *in vivo*, including cardiorespiratory parameters and temperature, psychomotor development, exploratory behavior, arthro-, nephro- and hepato-toxic effects. We found no effect of Ciprofloxacin at 10 and 30 mg/kg/day. In contrast, administration at 100 mg/kg/day delayed weight gain, impaired cardiorespiratory and psychomotor development, caused inflammatory infiltrates in the connective tissues surrounding the knee joint, and moderately increased extramedullary hematopoiesis. The present study pleads for careful watching of cardiorespiratory and motor development in neonates treated with Ciprofloxacin, in addition to the standard surveillance of arthrotoxicity.

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1. Introduction

Ciprofloxacin is a synthetic broad-spectrum antimicrobial agent, belonging to the fluoroquinolone family, and considered as effective against a wide range of Gram-positive and Gram-negative organisms. Ciprofloxacin interferes with DNA function by inhibiting bacterial DNA gyrate and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair and recombination. Ciprofloxacin is the only fluoroquinolone to be included on the list of "Essential Medicines for Children" by WHO.[1] Ciprofloxacin was approved for use in children 1 through 17 years of age in 2004, but

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[1] http://www.who.int/medicines/publications/TRS958June2010.pdf. Accessed July 28 2014.

http://dx.doi.org/10.1016/j.yrtph.2015.11.002
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not in neonates (Bradley et al., 2011). However, Ciprofloxacin may be prescribed "off-label" for the treatment of suspected or proven gram negative infections in neonates, especially if premature. In such context, data evaluating potential neurodevelopmental toxicity as well as joint and hepatic toxicity are missing (Bradley et al., 2011; Forsythe and Ernst, 2007; Kaguelidou et al., 2011; Sendzik et al., 2009).

In order to gather information on the possible short- and long-term adverse effects of Ciprofloxacin, the European Medicines Agency (EMA) has included Ciprofloxacin in its priority list of off-patent medicinal products for evaluation in the pediatric population.[2] The evaluation of Ciprofloxacin is currently ongoing by the TINN EU project (Treat Infections in Neonates[3]), in view of the submission of a Pediatric Use Marketing Authorization (PUMA) for Ciprofloxacin in neonates (Jacqz-Aigrain, 2011). The present preclinical study was conducted as part of the TINN project to assess the potential adverse effects of Ciprofloxacin on development in juvenile mice.

In order to assess the developmental effects of Ciprofloxacin, we exposed pre-weanling mice to Ciprofloxacin from 2 to 12 days of postnatal age (PND2 to PND12). Firstly, we examined the possible neurotoxic effects of Ciprofloxacin chronic administration on neurodevelopment with a special focus on breathing and psychomotor developments, which are both particularly critical in preterm infants. Then, we looked for effects of Ciprofloxacin on kidneys and liver development, considering previous reports that Ciprofloxacin caused a higher overall risk of hepatotoxicity (Alshammari et al., 2014). Finally, we examined whether Ciprofloxacin produced anarthrotoxic effect, as previously observed in weight bearing joints of juvenile animals and also in pediatric populations (Adefurin et al., 2011).

2. Material and methods

2.1. Test substance

Solutions (40 mL) of 1 mg/mL, 3 mg/mL and 10 mg/mL Ciprofloxacin and a control solution without Ciprofloxacin were prepared in sterile water for injection (5% glucose, Freeflex®, Fresenius Kabi, France) with Ciprofloxacin powder (Sigma–Aldrich, Germany), lactic acid (8, 24, 80 and 20 mg, respectively) and chloride acid (40 μL). The pH was adjusted to pH 5 with sodium hydroxide in all solutions, except the 10 mg/mL solution in which it was adjusted to pH 4. Afterwards, solutions were transferred into sterile vials through 0.2 μm syringe filters (Pall PharmAssure, Pall Medical, France). These solutions were prepared under a laminar airflow cabinet and controlled by the Pharmacy Department of Robert Debré Hospital in Paris.

2.2. Animals

Mouse pups ($N = 230$ for blood sampling, $N = 150$ for physiological and locomotion tests, and $N = 150$ for psychomotor tests and organs collection) were obtained from outbred Swiss female mice (Charles River Lab, France) housed at 20 °C ± 1 °C with a 12 h light/dark cycle and fed *ad libitum* (SAFE, France). All litters were born between 8 pm and 8 am. The first day following birth was denoted Postnatal Day 0 (PND0). Unless mentioned otherwise, litters were culled the day of birth to obtain 10 pups per litter, and housed individually in standard Plexiglas cages (Tecniplast, France). The

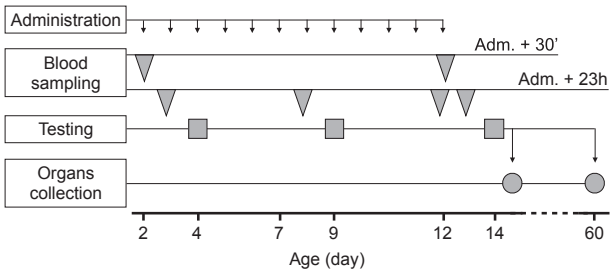


Fig. 1. Overview of our safety study of Ciprofloxacin in newborn mice. Arrows: All animals received a daily SC administration of Ciprofloxacin (0, 10, 30 or 100 mg/kg) from PND2 to PND12 (11 injections). Triangles: Animals (N = 230) were sacrificed to collect blood samples. Squares: Physiological and behavioral testing (N = 120), and psychomotor testing (N = 120) were carried out at PND4, PND9 and PND14. Circles: joints, kidneys and liver were collected at PND14 (N = 60) and PND60 (N = 56) to assess arthro-, nephro- and hepato-toxic effects of Ciprofloxacin.

animals were weighted daily from PND2 to PND14, and at PND16, PND21, PND30, PND41 and PND60. Animals were killed by decapitation at PND14 or by cervical dislocation at PND60. The overview of Ciprofloxacin safety testing in newborn mice is indicated in Fig. 1.

The present study complies with the EMA guidelines on the need for non-clinical testing in juvenile animals of pharmaceuticals for pediatric indications.[4] All the protocols were performed in accordance with the European Communities Council Directive (2010/63/UE) regarding the care and use of animals for experimental procedures, in compliance with the French regulations of the *Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale* (permission # A 94-028-21) and were approved by the Institutional Ethics Committee (Bichat-Robert-Debré) and national committees (Ministère de l'Enseignement Supérieur et de la Recherche – Direction Générale pour la Recherche et l'Innovation). All efforts were made to minimize animal suffering, especially by using non-invasive functional tests specially designed for newborn rodents.

2.3. Administration-route and doses

Ciprofloxacin was administered subcutaneously (SC). In newborn mice, intraperitoneal (IP) administration is poorly suited to chronic administration due to the risk of infection; oral administration is potentially harmful before PND12; intramuscular administration is difficult because of the paucity of muscle bulk; and intravenous (IV) administration is technically problematic because of the small size of blood vessels. We used a maximum dose of 100 mg/kg/day and intermediate doses of 10 and 30 mg/kg/day.

Ciprofloxacin solutions were administered once daily from PND2 to PND12 (11 injections per animal) at doses of 10 or 100 mg/kg/day in the blood sampling study, or at doses of 0, 10, 30 or 100 mg/kg/day in the physiological, psychomotor and behavioral studies. SC injections were alternatively performed at three different sites in the back of the mice, in order to minimize skin lesions.

2.4. Plasma levels

Blood samples were obtained from decapitation of three to five mice, pooled in an EDTA tube and centrifuged (4200 rpm at 4 °C) to obtain one plasma sample of 150 μL approximately and refrigerated

[2] EMEA/197972/2007; http://www.phytonetzwerk.de/Veranstaltungen/Kind_in_der_Apotheke/PNM_Sickmueller-EMEA_Stoffe_Off%20patent_May_2007en.pdf.

[3] http://www.tinn-project.org/.

[4] http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003306.pdf.

at −20 °C. Ciprofloxacin administration was performed daily from PND2 to PND12 at doses of 10 and 100 mg/kg SC. Blood was collected 30 min and 23 h after the first injection at PND2, 23 h after injection at PND6 and PND11, and 30 min and 23 h after injection at PND12. Five samples were collected at each time point and for both doses. The analytical method of Ciprofloxacin has been reported previously (Grondin et al., 2011). Briefly, Ciprofloxacin concentrations were determined using high-performance liquid chromatography with mass spectrometry with Ciprofloxacin-d_8 as internal standard. The calibration curve ranged from 25 to 3000 ng/mL. The inter- and intra-day coefficients of variation (CVs) of controls were 4.1% and 2.4%, respectively. The lower limit of quantification was 25 ng/mL.

2.5. Physiological testing

Physiological testing was carried out at PND4, PND9 and PND14 in the four dose groups (30 animals per dose group). We measured vital functions (breathing and activity, heart rate and body temperature) simultaneously in a plethysmographic chamber, in baseline conditions (10 min) and in response to chemical stimuli (hypoxic and hypercapnic challenges). The total duration of physiological testing for each mouse was 30 min.

Breathing variables were measured non-invasively using a battery of four whole-body flow barometric plethysmographs, as previously described (Miot et al., 2012). Plethysmograph chambers were immersed in a thermoregulated water-bath that maintained their temperature at 33 °C, which is close to thermoneutrality in newborn rodents (Blumberg and Sokoloff, 1998). Because of the limitations of flow barometric plethysmography (Enhorning et al., 1998; Mortola and Frappell, 1998), the absolute values of tidal volume (V_T) and ventilation (V_E) are indicative only, whereas breathing frequency absolute values (f) and apnea durations are reliable. Breathing frequency (f, breaths/min), tidal volume (V_T, µl/g), and minute ventilation (V_E, calculated as (V_T x f/60) and expressed in µl/s/g) were calculated on apnea-free periods (see apnea determination below). Each mouse was exposed to an hypoxic and an hypercapnic challenge to test for respiratory reflexes. After 10-min normoxia, hypoxia was achieved by switching the airflow through the plethysmograph to 10% O_2 + 90% N_2 at the same flow rate (200 ml/min per chamber) for 3 min, after which the flow was switched back to normoxia for 7 min. Similarly, hypercapnia was achieved by switching the airflow to 8% CO_2 + 21% O_2 + 71% N_2.

The baseline (i.e. normoxic/normocapnic) levels for breathing variables were calculated as the mean value over the 3-min of air breathing preceding the hypoxia or hypercapnia challenge. Breath-by-breath values for V_E, V_T, and f were averaged over consecutive 30-sec periods for the peak determination. Each individual peak V_E value was calculated as the maximum mean value over a 3-min period starting 30 s after the gas switch, to take into account possible interindividual differences in time to reach the peak V_E response to hypoxia or hypercapnia and washout of the chamber. V_E response to hypercapnia was expressed as the percentage V_E change relative to baseline average V_E, using the formula 100 x (peak V_E - baseline V_E)/baseline V_E. We determined V_T and f responses to hypoxia and hypercapnia using the same formula, knowing that peak values were calculated as the mean of V_T and f over the 30 s period during which V_E was found to be maximum.

Apneas were defined as ventilatory pauses longer than 0.7 s, which is approximately twice the duration of a normal breath in PND4, PND9 and PND14 Swiss mice pups. Apneas were determined using an automatic classification method (Matrot et al., 2005). Activity was detected based on large disturbances in the respiratory signal caused by the combined effects of positional changes inside the chamber and changes in breathing pattern. These disturbances in the respiratory signal were detected using the following criterion: {(Vi-Ve)/(Vi + Ve)}, where Vi and Ve are the magnitudes of the inspiratory and expiratory limbs of the volume signal, respectively. This automatic detection method was previously validated against visual scoring of movements (Matrot et al., 2005). Apnea duration and activity were expressed as a percentage of recording time during the 10-min period preceding the hypoxic challenge.

Each chamber was equipped with an electrocardiographic (ECG) recording platform composed of four rectangular gold electrodes insulated from one another and embedded in the floor of the chamber, as previously described (Ramanantsoa et al., 2007). An ECG signal was obtained when at least three paws contacted three electrodes and, occasionally, when the pup laid on the floor over three electrodes. Heart rate (HR in beats/min, BPM) was determined from the R-R wave peaks detected automatically by a custom program (Christov, 2004). The baseline HR was calculated for each individual during the 10-min normoxia period at the beginning of the procedure.

We measured body temperature non-invasively in PND4 pups in the plethysmograph chamber. This was done by measuring the emission of infrared (IR) radiations from surface of the interscapular region using an infrared camera (FLIR Systems Thermovision A20, MA) as described (Ramanantsoa et al., 2011). The interscapular region contains the heat-generating brown adipose cells and is the area of highest skin temperature (Blumberg and Sokoloff, 1998). The camera was moved over each of the four animal chambers every 1 min 15 s. IR measurement was done through a special window of the plethysmograph chamber made of Zinc Selenide, to ensure permeability to IR emission. We previously showed that the non-invasive measurements of IR interscapular temperature and oesophageal temperatures were highly correlated (Bollen et al., 2009). IR temperature was not collected at PND9 and PND14 because fur precluded this technique. We measured temperature of all pups in room environment at PND4, PND9 and PND14, before and after physiological testing by placing a thermocouple probe at the level of the interscapular region.

2.6. Psychomotor development

We used three tests from Fox battery (Fox, 1965): righting reflex, cliff-drop aversion, and negative geotaxis. The tests were performed this order, at 26 °C. Righting reflex: each pup was placed on its back on a non-slippery surface. We recorded the time needed by the pup to regain its feet. A maximum time of 30 s was given to complete the task. Pups not able to complete the task were given the maximum time of 30 s. Cliff-drop aversion reflex: each pup was placed on the edge of an elevated platform, with both front paws over the edge. We recorded the time needed for the pup to crawl away from the edge. Negative geotaxis: each pup was placed with the head facing downward with an angle of 30° at PND4 and PND9, and of 45° at PND14. We recorded the time needed for the pup to turn until it faced up the slope. The failure to complete any test was scored 0 (1 otherwise; mean score per group x100 indicated the proportion of mice that completed the test in this group).

2.7. Exploratory behavior

We performed open-field tests at PND4, PND9 and PND14 with a custom, thermoregulated open-field system at 26 °C, as previously described (Dehorter et al., 2011). Each mouse was placed on a transparent acrylic plate (50 cm × 60 cm) covered with a silicone gel and its activity was tracked for 1 min. A camera recorded the trajectory of the pup. We determined the explored zones and the total distance traveled using custom software.

2.8. Arthrotoxicity assessment

Elbow and knee joints were collected at PND14. Limbs were disarticulated from axial skeleton, and soft tissue partially removed. The joints were radiographed using a Faxitron (Hewlett Packard, USA). The lengths of intact humeri, tibiae and fibulae were measured using numerized X-rays. Limbs were fixed in 4% buffered formalin. Decalcification was done in 0.1M EDTA. After paraffin embedding, 4 μm thick longitudinal sections of the entire limbs were cut using a rotary microtome. Routine staining with Haemotoxylin Eosin and Saffron and PAS staining were performed. Histological analysis of the bone and cartilage was performed in order to detect degenerative, necrotic or abnormal bone or cartilage formation.

2.9. Liver and kidney histology

Livers were collected at PND14 (N = 60) and PND60 (N = 56), were fixed in formalin and embedded in paraffin. Sections were stained with HES (hematoxylin, eosin and saffron) and Masson's trichrome to assess epithelial cells, vessels and connective tissues. In addition, we quantified the number of hematopoiesis foci per hepatic lobule. A similar analysis was conducted on kidneys.

2.10. Statistical analysis

All study variables were analyzed using repeated measure analysis of variance (ANOVA) with treatment (0, 10, 30, 100 mg/kg/day Ciprofloxacin), litter and gender as between-subjects factors and age as a repeated factor. Weights from PND2 to PND14 and from PND16 to PND60 were analyzed separately. Unless specified, litter and gender had no significant effects, either as a main effect or in interaction with treatment. When appropriate, pairwise comparisons were done using the Fischer's PLSD with Bonferroni correction. Statistical analyses were conducted using R software (www.r-project.org).

3. Results

3.1. Local tolerance to injections

The mice receiving 0, 10, and 30 mg/kg/day did not show any sign of pain or local irritation at the sites of injection. In contrast, the mice receiving 100 mg/kg/day showed signs of cutaneous inflammation in the region of SC injections (skin rash, hair loss, and skin necrosis) as early as PND5 (i.e. after three injections) and until PND60. These mice also showed defensive responses indicative of irritation after SC injection until PND7 and scratching for about 1 min beyond this age. These responses vanished completely beyond this delay.

3.2. Ciprofloxacin plasma concentration

Peak plasma Ciprofloxacin concentrations (30 min after injection) were similar at PND2 and PND12 for both 10 mg/kg and 100 mg/kg daily injections (Table 1). Trough plasma concentrations at PND3, PND7 and PND13 were below 0.5 mg/L for both doses (Table 1).

3.3. Clinical observation and weight gain

Ciprofloxacin treatment did not influence general appearance, behavior and mortality throughout the study period. However, 100 mg/kg/day Ciprofloxacin significantly reduced weight gain from PND2 to PND14 (weight by age interaction $F = 10.82$, $p < 0.001$, and partial comparisons shown in Fig. 2A). There was no effect of sex over the PND2-PND14 period. From PND16 to PND60, weights were not found significantly different between dose groups, neither in females (N = 60) nor in males (N = 58, Fig. 2B).

3.4. Physiological testing

As a rule, Ciprofloxacin treatment did not significantly affected physiological variables and activity (Table 2 and Supplementary Fig. 1A–C). However, breathing frequency (f) responses to hypoxia at PND4 displayed a marginally significant effect of dose ($F = 2.805$, $p = 0.043$, all pairwise comparisons between dose groups were not significant). This difference did not significantly impact V_E and was of limited biological significance. Also, the V_E-response to hypercapnia displayed a marginally significant interaction between sex and dose ($F = 2.88$, $p = 0.039$). Examination of individual data revealed that this effect is linked to the high V_E-response to hypercapnia in 3 out of 13 female pups in the 100 mg/kg/day group. Ciprofloxacin treatment had no significant effect on heart rate (Table 2) and body temperature (Supplementary Fig. 2). In summary, the effect of Ciprofloxacin on physiological variables was limited to modest ventilatory changes in the 100 mg/kg/day group.

3.5. Psychomotor development

On PND4, the righting reflex was significantly impaired in mice receiving 100 mg/kg/day compared to controls ($F = 2.83$, $p = 0.038$ and pairwise comparisons shown in Table 3). This effect born upon the impairments of males, who showed lower scores compared to control ($p = 0.020$) and 10 mg/kg/day groups ($p = 0.044$), whereas females showed similar scores (group by gender interaction: $F = 3.21$, $p = 0.023$). The defect observed in male pups receiving 100 mg/kg/day vanished on PND9, when all the pups performed the test very quickly. This test was not performed on PND14, due to the impossibility to measure the short time needed by all the pups to regain their feet. Cliff-drop aversion scores improved from PND4 to PND9 in all groups but did not reveal significant effects of treatment (Table 3). Finally, negative geotaxis scores did not indicate significant effects of Ciprofloxacin treatment at any dose. In the 100 mg/kg/day group, fewer pups completed the test compared to other groups, although not significantly (Table 3). However, the pups that did complete the test needed significantly shorter times to do so, likely thanks to their smaller weight (Table 3). Thus, the psychomotor testing indicated a transient delay in righting test acquisition in the high dose group.

3.6. Exploratory behavior

The distance traveled increased with age (as a normal effect of development) with no significant effect of Ciprofloxacin treatment (Table 4). The explored zones did not show any significant effect of treatment.

3.7. Joints histology and bone length

Histology of humero-ulnar joints with HES staining did not indicate cartilage alteration at any age, and for any dose (Fig. 3A–D). However, the connective tissues surrounding the knee joint displayed moderate inflammatory infiltrates in the 100 mg/kg/day group at PND14 (Fig. 3C and D). Bone lengths were not significantly influenced by Ciprofloxacin treatment (Table 5 and Fig. 3E).

Table 1
Plasma concentration of Ciprofloxacin in mice treated with daily doses of Ciprofloxacin 10 or 100 mg/kg SC from PND2 to PND12 (Adm1 to Adm11).

	Adm. 1 + 30′	Adm. 1 + 23 h	Adm. 5 + 23 h	Adm. 10 + 23 h	Adm. 11 + 30′	Adm. 11 + 23 h
Age	PND2	PND3	PND7	PND12	PND12	PND13
Pooling	5	5	4	3	3	3
Pups remaining per litter	7 to 8	5 to 6	3 to 4	2 to 3	1 to 2	
10 mg/kg/day						
Weight (g)	2.21	2.89	4.05	8.01	7.29	9.25
	(0.17)	(0.26)	(0.55)	(0.69)	(0.41)	(0.66)
Plasma concentration (mg/l)	1.86	0.02	0.02	0.00	2.28	0.00
	(0.32)	(0.02)	(0.01)	(0.00)	(0.25)	(0.00)
100 mg/kg/day						
Weight (g)	2.07	2.48	4.65	8.33	6.74	9.25
	(0.23)	(0.49)	(0.29)	(0.94)	(0.52)	(1.04)
Plasma concentration (mg/l)	10.35	0.47	0.15	0.09	10.57	0.08
	(2.00)	(0.18)	(0.03)	(0.03)	(1.03)	(0.03)

Values are group means (Standard Deviation, SD). Blood samples were collected and pooled 30 min after the first and 11th administration and 23 h after the first, 5th, 10th and 11th administration. Pooling indicates the number of individual samples pooled together. Animals used for sampling were randomly chosen among litters.

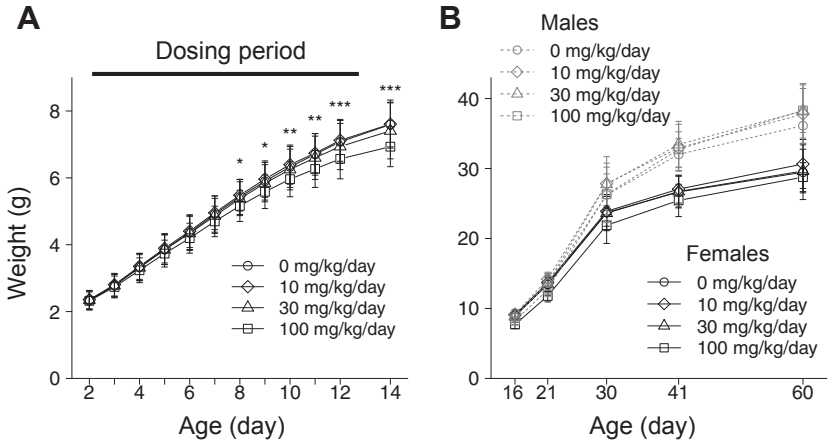


Fig. 2. Weight gain from PND2 to PND14 (A) and from PND16 to PND60 (B). A: weight gain was influenced by treatment (N = 59, N = 58, N = 60, N = 60 for 0, 10, 30 and 100 mg/kg/day, respectively), and from PND8 to PND14, animals of the 100 mg/kg/day group were significantly lighter than controls. There were no significant differences between 0, 10 and 30 mg/kg/day dose groups at any age. Weights are notably smaller than those indicated in Table 1, due to larger litter sizes. B: Weight gain from PND16 to PND60 (N = 18, N = 17, N = 12, N = 13 females, and N = 11, N = 12, N = 18, N = 17 males for control, low, mid and high dose, respectively) was not influenced by treatment in females and males. Note the different scales. Pairwise comparisons differences between 0 and 100 mg/kg/day dose groups; *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$ for partial comparison between 0 and 100 mg/kg/day dose groups. Values are group means. Error bars are SD.

3.8. Liver and kidney histology

The kidneys were histologically normal. In the liver, no architectural modification, fibrosis, necrosis, steatosis, vascular lesion, bile duct proliferation or granuloma were observed. Two days after the last injection (i.e. at PND14), the number of hematopoiesis foci in the 10 mg/kg/day (4.7 ± 1.9) and in the 30 mg/kg/day (5.1 ± 1.9) groups were not different compared to controls (4.5 ± 1.6). In contrast, mice receiving 100 mg/kg/day Ciprofloxacin had significantly more numerous hematopoiesis foci per hepatic lobule (7 ± 2.2) compared to controls ($p = 0.0041$), to the 10 mg/kg/day group ($p = 0.010$) and to the 30 mg/kg/day group ($p = 0.048$). We observed no architectural modification, fibrosis, necrosis or vacuolation of liver cells, steatosis or vascular lesion, bile duct proliferation, nor granuloma.

4. Discussion

The present study assessed potential toxic effects of Ciprofloxacin administered SC once daily at 10, 30 and 100 mg/kg dose from PND2 to PND12 in newborn mice. In addition to weight gain and arthro-, nephro-, and hepato-toxicity, we examined, for the first time, potential neurodevelopmental toxicity affecting cardiorespiratory function, psychomotor development and exploratory behavior. The main results were that, at 10 and 30 mg/kg/day, there were no Ciprofloxacin related effects on any study parameter, while administration of 100 mg/kg/day transiently delayed weight gain, had small effects on physiological variables, and affected joints and liver development. In contrast, psychomotor development and exploratory behavior were spared in this dose group, showing the limits of toxicity of Ciprofloxacin in newborn mice.

4.1. Period of treatment

The study period is a critical aspect of safety studies in juvenile animals, because the animals must be tested at developmental stages corresponding to those of the target pediatric population. To fulfill this requirement, we administered Ciprofloxacin from PND2 to PND12. This period covers the period during which Ciprofloxacin may be administered in preterm infants, considering that, from a neurodevelopmental perspective, P2 in rodents corresponds to about 25–28 weeks of gestation in humans and P12 slightly exceeds full term (Craig et al., 2003; Semple et al., 2013).

4.2. Clinical relevance of the doses

The doses used in the present study (10, 30, 100 mg/kg/day, SC) were akin to those used in neonates, which range from 3 to 94 mg/

Table 2
Cardiorespiratory variables, activity, heart rate and body temperature measured in a thermoregulated plethysmographic chamber.

Dose (mg/kg/day)	PND4				PND9				PND14			
	0	10	30	100	0	10	30	100	0	10	30	100
N	29	28	30	30	29	28	30	30	29	28	30	30
females/males	13/16	11/17	11/19	13/17	13/16	11/17	11/19	13/17	13/16	11/17	11/19	13/17
Weight (g)	2.98	2.95	2.95	2.85	5.56	5.44	5.38	5.15	7.33	7.09	6.94	6.59
	(0.26)	(0.23)	(0.30)	(0.25)	(0.49)	(0.40)	(0.41)	(0.36)	(0.65)	(0.64)	(0.77)	(0.48)
Baseline values												
V_E (μl/s/g)	31.2	31.0	31.2	33.1	22.9	21.6	21.4	20.8	30.9	29.4	32.8	31.6
	(6.4)	(6.9)	(7.6)	(5.9)	(4.5)	(3.9)	(3.8)	(4.1)	(9.9)	(8.2)	(8.6)	(13.3)
V_T (μl/g)	9.7	9.7	9.6	9.5	7.7	7.6	7.6	7.7	10.4	10.1	10.4	10.3
	(1.1)	(1.5)	(1.5)	(1.4)	(1.1)	(1.0)	(1.0)	(1.0)	(1.6)	(1.7)	(2.3)	(1.9)
f (breaths/min)	191.6	193.0	193.5	208.4	179.5	170.9	169.6	163.8	177.5	175.0	197.3	186.3
	(28.3)	(32.4)	(28.3)	(23.6)	(27.5)	(28.3)	(30.8)	(29.9)	(37.9)	(41.2)	(58.3)	(64.7)
Apneas (%)	0.6	0.9	0.6	0.2	0.9	1.1	1.4	1.5	1.0	0.9	0.9	1.2
	(1.0)	(2.6)	(1.3)	(0.4)	(0.9)	(1.4)	(1.9)	(1.7)	(1.6)	(1.6)	(1.9)	(1.5)
Activity (%)	17.9	19.6	17.5	15.8	18.4	14.3	11.6	10.7	65.3	67.8	67.0	64.1
	(15.1)	(15.6)	(9.7)	(10.2)	(16.9)	(11.9)	(12.7)	(9.7)	(28.3)	(25.6)	(23.5)	(26.2)
HR (bpm)	517.3	506.0	518.9	533.6	536.5	520.8	522.5	501.3	583.1	603.9	598.7	588.3
	(41.7)	(33.9)	(36.6)	(26.0)	(46.6)	(44.9)	(38.3)	(51.3)	(35.2)	(37.2)	(61.9)	(48.1)
Temp before (°C)	34.8	34.9	35.0	34.9	35.0	35.0	35.2	35.2	35.9	35.6	36.1	35.8
	(0.8)	(0.7)	(0.7)	(0.6)	(0.7)	(0.9)	(0.6)	(0.7)	(0.6)	(0.9)	(0.4)	(0.7)
Temp after (°C)	34.8	34.9	35.0	35.0	35.6	35.6	35.6	35.6	36.9	36.7	36.7	36.6
	(0.8)	(0.8)	(0.8)	(1.0)	(0.4)	(0.3)	(0.4)	(0.4)	(0.5)	(0.5)	(0.5)	(0.4)
Response to hypoxia												
V_E (%)	78.6	67.8	70.9	60.4	92.6	97.2	102.2	94.8	90.9	89.0	76.9	83.6
	(34.9)	(28.1)	(18.1)	(18.9)	(31.6)	(22.4)	(26.7)	(27.3)	(39.7)	(42.0)	(38.8)	(58.6)
V_T (%)	16.2	12.1	13.4	13.0	11.8	12.8	12.9	10.3	12.7	9.9	12.7	9.8
	(17.5)	(12.3)	(9.4)	(9.5)	(12.7)	(10.5)	(11.3)	(14.1)	(18.6)	(22.1)	(17.0)	(23.6)
f (%)	53.3	49.6	51.8	42.0	72.4	75.8	80.3	78.6	70.8	76.3	57.4	66.5
	(14.7)	(17.0)	(20.6)	(11.7)	(23.0)	(20.6)	(25.2)	(30.0)	(33.8)	(39.3)	(35.8)	(44.9)
Response to hypercapnia												
V_E (%)	138.6	125.2	120.0	111.2	100.7	117.1	123.0	139.3	398.1	331.4	365.1	297.5
	(69.3)	(41.6)	(56.8)	(44.0)	(40.8)	(64.9)	(67.9)	(83.2)	(200.7)	(188.0)	(161.7)	(202.9)
V_T (%)	63.6	53.1	52.7	52.1	50.0	54.9	58.9	58.3	116.2	103.6	108.3	95.5
	(31.4)	(19.1)	(20.7)	(24.5)	(16.8)	(17.8)	(23.2)	(24.2)	(35.9)	(42.4)	(32.9)	(39.3)
f (%)	45.7	47.2	45.4	39.8	34.0	39.5	38.8	49.0	126.0	109.5	120.3	95.5
	(30.3)	(19.5)	(37.6)	(26.8)	(24.1)	(32.7)	(29.2)	(34.1)	(73.5)	(73.2)	(59.2)	(68.1)

Values are group means (SD). Baseline values and ventilatory responses to hypoxic and hypercapnic challenges expressed as percent increases from baseline levels. *Abbreviations:* V_E: ventilation; V_T: tidal volume; f: breathing frequency; Apneas and Activity are expressed as percentage of recording time. HR: heart rate; Temp. before and Temp. after: interscapular temperature before and after plethysmography in room environment, respectively.

Table 3
Psychomotor development assessed with Fox battery tests.

Dose (mg/kg/day)	PND4				PND9				PND14			
	0	10	30	100	0	10	30	100	0	10	30	100
N	30	30	30	30	30	30	30	30	30	30	30	30
females/males	18/12	18/12	12/18	13/17	18/12	18/12	12/18	13/17	18/12	18/12	12/18	13/17
Weight (g)	3.35	3.47	3.42	3.34	5.99	6.20	6.06	5.79	7.80	8.02	7.78	7.21
	(0.42)	(0.33)	(0.34)	(0.29)	(0.55)	(0.37)	(0.51)	(0.38)	(0.59)	(0.47)	(0.70)	(0.52)
Righting reflex												
Score × 100	68	56	59	47*	100	100	100	100	NA	NA	NA	NA
	(47)	(50)	(49)	(50)	(0)	(0)	(0)	(0)				
Time (s)	7.2	5.3	6.6	4.7	<1	<1	<1	<1	NA	NA	NA	NA
	(8.5)	(7.8)	(8.4)	(6.8)								
Cliff-drop aversion												
Score x 100	78	74	68	71	98	96	96	98	NA	NA	NA	NA
	(42)	(44)	(47)	(46)	(15)	(21)	(21)	(15)				
Time (s)	7.1	6.0	5.1	6.3	1.9	1.6	1.6	1.8	NA	NA	NA	NA
	(8.0)	(7.8)	(6.6)	(7.9)	(1.5)	(0.9)	(1.6)	(0.9)				
Negative geotaxis												
Score x 100	70	64	69	64	88	91	84	82	73	64	67	54
	(46)	(48)	(47)	(48)	(33)	(29)	(36)	(38)	(44)	(48)	(47)	(50)
Time (s)	8.2	7.2	7.2	6.3	8.5	8.2	7.3	6.4	4.0	2.9	2.8	1.5*
	(8.0)	(8.0)	(7.2)	(6.7)	(7.1)	(6.9)	(6.2)	(7.1)	(4.9)	(3.6)	(3.1)	(1.8)

Values are group means (SD). Righting reflex, Cliff-drop aversion, Negative geotaxis: success rate and time before success as described in Section 2.6. Pairwise comparison with controls: *: $p < 0.05$.

kg/day (oral) and 3.2–76.9 mg/kg/day (IV). However, the usual dose is 10–30 mg/kg/day in at least 60% of studies, generally administered in two divided doses for both oral and IV routes (Adefurin et al., 2011).

Plasma levels, which are more meaningful than doses for correct comparisons between animal and human studies, confirmed the clinical relevance of the present preclinical protocol. For example, IV Ciprofloxacin at the dose of 10 mg/kg, 12 hourly in preterm

Table 4
Exploratory behavior.

Dose (mg/kg/day)	PND4				PND9				PND14			
	0	10	30	100	0	10	30	100	0	10	30	100
N	29	28	30	30	29	28	30	30	29	28	30	30
females/males	13/16	11/17	11/19	13/17	13/16	11/17	11/19	13/17	13/16	11/17	11/19	13/17
Weight (g)	3.00	2.92	2.93	2.87	5.58	5.46	5.41	5.17	7.31	7.13	6.97	6.62
	(0.26)	(0.22)	(0.26)	(0.25)	(0.50)	(0.42)	(0.43)	(0.38)	(0.67)	(0.67)	(0.80)	(0.52)
Open-field test												
Distance traveled (AU)	40.4	45.3	38.7	45.3	46.9	59.0	43.5	67.9	76.0	73.8	74.5	85.3
	(28.3)	(26.8)	(37.6)	(31.3)	(28.2)	(33.2)	(23.7)	(32.3)	(54.0)	(49.4)	(46.3)	(59.2)

Values are group means (SD). Distance traveled (in arbitrary units, AU) during an open field test for 1-min.

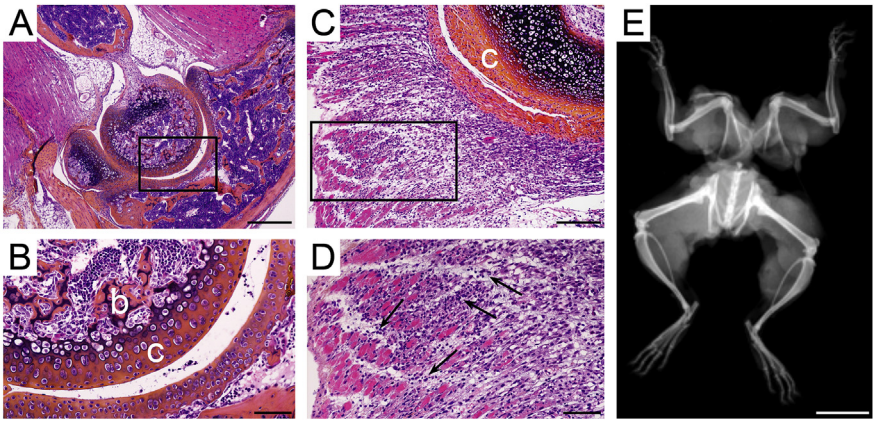


Fig. 3. Cartilage joints of juvenile (PND14) and adult (PND60) mice treated with daily doses of Ciprofloxacin 0, 10, 30 or 100 mg/kg/day SC from PND2 to PND12. A: histological section of humero-ulnar joint, stained with HES at PND14 in one mouse of the 100 mg/kg/day group. Scale bar: 1 mm. B: magnification (x20) of the region delineated in A. No cartilage alteration was observed in any dose group at PND14 and PND60. Scale bar: 150 μm. C: Histological section of muscular and connective tissues surrounded the knee joint, stained with HES at PND14 in one animal of the 100 mg/kg/day group. Scale bar: 1 mm. D: magnification (x20) of the region delineated in C. Note the presence of moderate inflammatory infiltrates (arrows) in these tissues, found only in the 100 mg/kg/day dose group at PND14. Scale bar: 150 μm. E: X-ray image of the skeleton of an animal of the 100 mg/kg/day group at PND14. No anomalies of bone length and maturation were detected in any dose group at PND14 and PND60. b: bone; c: cartilage. Scale bar: 10 mm.

Table 5
Skeletal imaging in juvenile (PND14) and adult (PND60) mice.

Dose (mg/kg/day)	PND14				PND60			
	0	10	30	100	0	10	30	100
N	15	15	15	15	14	13	14	15
females/males	7/8	6/9	3/12	4/11	6/8	5/8	7/7	9/6
Weight (g)	7.25	6.90	6.91	6.50	36.67	36.92	36.00	33.34
	(0.73)	(0.63)	(0.56)	(0.55)	(4.71)	(4.92)	(6.24)	(4.06)
Bone Length								
Humerus	53.7	54.2	54.9	53.1	115.3	113.4	114.9	115.0
	(10.5)	(11.5)	(12.4)	(11.2)	(17.6)	(18.8)	(17.9)	(20.8)
Radius-cubitus	71.2	72.1	73.8	70.9	126.5	123.3	125.9	126.5
	(11.9)	(12.7)	(14.6)	(13.2)	(18.7)	(21.2)	(19.3)	(22.1)
Femur	52.5	53.0	52.9	51.4	146.6	142.1	145.7	144.2
	(8.7)	(8.2)	(10.6)	(10.0)	(22.1)	(23.7)	(22.1)	(24.0)
Tibia-fibula	83.8	83.3	84.3	81.9	165.1	158.9	163.4	162.5
	(16.2)	(16.5)	(17.4)	(17.4)	(25.5)	(26.1)	(25.8)	(26.7)

Values are group means (SD).

neonates with sepsis resulted in peak Ciprofloxacin concentrations ranged from 2.3 to 3.0 μg/mL (Aggarwal et al., 2004; Kaguelidou et al., 2011) and trough concentrations ranged from 0.7 to 1.0 μg/mL. These values are of the same order of magnitude as levels measured in newborn mice in the present study (1.86 and 2.28 μg/mL), 30 min after administration in the 10 mg/kg/day group. Recently, a population pharmacokinetics study conducted at doses of 20–30 mg/kg/day in neonates and young infants of less than three months found ciprofloxacin levels ranging from 0.5 to 16.0 μg/mL (Zhao et al., 2014). This study showed that babies treated with ciprofloxacin can be exposed to high plasma levels, which can be compared with those measured in newborn mice in the present study, 30 min after administration in the high dose group (100 mg/kg/day, 10.35 and 10.57 μg/mL). Taken together, the doses and resulting plasma levels support the design of the present neurodevelopmental toxicity study.

4.3. Delayed weight gain at 100 mg/kg/day

Weight gain delay is a common expression of developmental

toxicity. No significant adverse effects on weight gain were observed at 10 and 30 mg/kg/day, in keeping with previous reports that weight gain was not affected in juvenile mice treated with 50 mg/kg/day from PND7 for 7 or 14 days (Linseman et al., 1995). The present results extend these previous observations by indicating that Ciprofloxacin at usual doses does not affect weight gain even when administered at an earlier stage of development (i.e. from PND2), period which roughly corresponds to prematurity in human infants. In contrast, adverse effects on weight gain were observed here at the high dose of 100 mg/kg/day, suggesting that the limit of safety lies between 50 and 100 mg/kg/day. Because plasma levels measured in the 100 mg/kg/day group correspond to those measured in neonates treated with ciprofloxacin at doses of 20–30 mg/kg/day (as noted above), our data suggest that Ciprofloxacin might affect weight gain in humans. However, Ciprofloxacin administered at a lower dose of 10 mg/kg/dose 12-hourly during the treatment period to very low weight babies did not affect linear growth until 12 months corrected age (Dutta et al., 2006).

4.4. No adverse effects on cardiorespiratory variables

The possible adverse effects of Ciprofloxacin on breathing are a particularly critical issue in preterm infants, due to their specific risk of respiratory failure. Furthermore, breathing pattern is also relevant because it is a highly sensitive marker of arousal level and emotions, all reflecting the influence of higher processes, including in newborn mice (Durand et al., 2003). As a rule, the cardiorespiratory component of safety assessment has largely not been studied in neurodevelopmental toxicity studies. As a matter of fact, the possible adverse effects of Ciprofloxacin on these vital functions were not addressed in neither preclinical or clinical studies (Kaguelidou et al., 2011). In the present study, we assessed the cardiorespiratory effects of Ciprofloxacin using an innovative platform allowing simultaneous non-invasive measurements in controlled environmental conditions (Ramanantsoa et al., 2013). This methodology avoided disturbances of breathing pattern by experimental conditions and measurement devices. We found that cardiorespiratory variables were not influenced by Ciprofloxacin treatment at any dose nor age, although marginal effects, of uncertain biological significance, were observed at 100 mg/kg/day. In line with previous preclinical studies, this latter observation confirmed that the dose of 100 mg/kg/day may present a risk for development.

4.5. Psychomotor development

Neurobehavioral assessment is the gold standard of drug safety analysis in animal studies. In the present study, this assessment was based on three tests from the Fox battery (Fox, 1965), which is a robust method to evaluate psychomotor development that has been in use for over 30 years. The Fox battery is currently the only standardized battery of tests in newborn rodents. The three tests used in the present study (righting reflex, cliff-drop aversion, and negative geotaxis) were chosen for their reproducibility and their relevance with respect to the study period (Fox, 1965). The righting reflex revealed a transient development delay in the 100 mg/kg/day Ciprofloxacin group at PND4, which extended the effects observed in breathing pattern to psychomotor development.

4.6. Joints histology and bone growth

Detailed histological analysis of joints confirmed that 10 and 30 mg/kg/day did not cause arthrotoxicity. In contrast, we observed moderate inflammatory infiltrates in the connective tissues surrounding knee joint in the 100 mg/kg/day dose group at PND14. The effect of Ciprofloxacin on bone and joints in juvenile mammals (mice, rats, beagle dogs) has been extensively investigated in numerous studies (Sendzik et al., 2009), although only few of them addressed the early postnatal period (Supplementary Table 1). Taken together, these studies showed the lack of adverse effects of Ciprofloxacin at mild doses, e.g. ~10 mg/kg (Bradley et al., 2011) and showed arthrotoxicity above this dose (Supplementary Table 1). In mice, arthrotoxicity of Ciprofloxacin was reported at 50 mg/kg/day SC for 7 or 17 days from PND7, and at 200 mg/kg/day SC for 5 days (Linseman et al., 1995). In young beagle puppies (13–16 week old, i.e. after weaning), arthrotoxicity was observed during a 14-day treatment course (oral) at 90 mg/kg/day but not at 30 mg/kg/day (von Keutz et al., 2004). Of note, in this latter study, arthrotoxic concentrations of the drugs in plasma corresponded to plasma levels achieved during therapy in humans (Sendzik et al., 2009). In lambs, which are felt to approximate human growth rates and activity more closely than juvenile beagle dogs or rats (Bradley et al., 2011), administration of parenteral Ciprofloxacin at 15 mg/kg twice daily for 14 consecutive days in 6–8 week old weanlings did not impair nor bone growth nor articular development (Sansone et al., 2009).

Despite species differences in susceptibility to joint lesion and bone growth impairments caused by Ciprofloxacin, the present results confirm and extend previous results on Ciprofloxacin arthrotoxicity (Bradley et al., 2011; Sendzik et al., 2009). The present data are congruent with previous studies in preterm infants (mean gestational age 33.2 ± 3.83 weeks) showing that Ciprofloxacin at a dose of 20 mg/kg in two divided doses IV for a period of 14 days did not show arthrotoxicity (Chaudhari et al., 2004). However, a limitation of the present study is that ultrastructural effects of Ciprofloxacin on joints were not analyzed. Such toxic effects have been detected in Achilles tendons using electron microscopy in rats at PND30 treated with a very high dose of Ciprofloxacin 600 mg/kg/day for five days by oral gavage (Bae et al., 2006). Therefore, watchful waiting of joint development in treated neonates seems mandatory, even at low doses of Ciprofloxacin.

4.7. Liver toxicity

The liver has been identified as a target organ of Ciprofloxacin toxicity in both animal and humans, as recently reviewed (Adikwu and Brambaifa, 2012). We observed an increased number of hematopoiesis foci in treated mice in the 100 mg/kg/day group, possibly due to increased immune response, as supported by the moderate inflammatory infiltrates in connective tissues. The present results extend previous results showing fetal toxicity of Ciprofloxacin in rats, with decreased fetal liver weight, and increased degenerative cells in zone 11 and 111 of the fetus liver (Channa and Janjua, 2003).

4.8. Juvenile animal studies

The present study was part of the TINN EU program that aims at assessing the safety of Ciprofloxacin use in neonates, this drug being included in the EMEA priority list for studies into off-patent pediatric medicinal products. Preclinical and clinical results of the TINN project will serve as a basis for a PUMA application. As previously noted, the importance of conducting preclinical safety studies in developing mammals with special consideration for organ systems that undergo significant postnatal development has been stressed by both Food and Drug Administration, and the European Medical Agency (Soellner and Olejniczak, 2013). Despite their limitations, juvenile animal studies are frequently requested by regulatory agencies, for example in the frame of Pediatric

Investigation Plans (Baldrick, 2010). The TINN project aimed at validating new tools to improve the sensitivity of pediatric preclinical studies. The present study shows that the technological limitations to conduct neurodevelopmental studies in newborn rodents during the dosing period are being overcome.

5. Conclusion

This study was conducted to assess the possible adverse effects of Ciprofloxacin on postnatal development in mice. We found that subcutaneous administration of 100 mg/kg/day led to a moderate toxicity mainly characterized by transient decrease in weight gain, modest psychomotor and physiological effects during the dosing period, and moderate impairments of joints and liver development. However, no lasting effects were observed on vital functions, psychomotor development, exploratory behavior, joint cartilage and skeletal development at 10 and 30 mg/kg/day. Extrapolation from mice to humans and even more from newborn mice to neonates should be cautious, but according to the present study, Ciprofloxacin is potentially toxic in neonates when used at therapeutic doses. Thus, the present results plead for careful watching of cardiorespiratory and motor development during and after treatment with Ciprofloxacin in neonates, in addition to the standard surveillance of arthrotoxicity.

Conflicts of interest

Boris Matrot, Estelle Durand and Jorge Gallego own or owned stocks of PhenoPups SAS at the time this work was conducted. Thomas Bourgeois, Anne-Lise Delezoide, Wei Zhao, Fabien Guimiot, Homa Adle-Biassette, Maud Ringot, Thomas Storme, Chantal Le Guellec, Behrouz Kassaï, Mark Turner and Evelyne Jacqz-Aigrain declare no conflict of interest related to this work.

Funding sources statement

This work was supported by a grant from the Commission of the European Union 7th Framework Programme: TINN (Treat Infections in Neonates), FP7-HEALTH-2007-B, N° 223614.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2015.11.002.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2015.11.002.

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