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Research paper

Development of a long-acting injectable formulation with nanoparticles of rilpivirine (TMC278) for HIV treatment

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ABSTRACT

Long-acting parenteral formulations of antiretrovirals could facilitate maintenance and prophylactic treatment in HIV. Using the poorly water- and oil-soluble non-nucleoside reverse transcriptase inhibitor (NNRTI) TMC278 (rilpivirine) as base or hydrochloride (HCl), nanosuspensions were prepared by wet milling (Elan NanoCrystal® technology) in an aqueous carrier. Laser diffraction showed that the average particles size were (1) close to the targeted size proportionality (200–400–800 nm), with increasing distributions the larger the average particle size, and (2) were stable over 6 months. Following single-dose administration, the plasma concentration profiles showed sustained release of TMC278 over 3 months in dogs and 3 weeks in mice. On comparison of intramuscular and subcutaneous injection of 5 mg/kg (200 nm) in dogs, the subcutaneous route resulted in the most stable plasma levels (constant at 25 ng/mL for 20 days, after which levels declined slowly to 1–3 ng/mL at 3 months); 200 nm nanosuspensions achieved higher and less variable plasma concentration profiles than 400 and 800 nm nanosuspensions. In mice, the pharmacokinetic profiles after a single 20 mg/kg dose (200 nm) were similar with two different surfactants used (poloxamer 338, or D-alpha-tocopheryl polyethylene glycol 1000 succinate). In conclusion, this study provides proof-of-concept that 200-nm sized TMC278 nanosuspensions may act as long-acting injectable.

1. Introduction

In spite of major progress made in the past decade to inhibit the replication of HIV-1, thereby preventing the clinical presentation of AIDS, none of the currently available treatments for HIV infection can cure the infection [1,2]. Also HAART, or highly active antiretroviral therapy consisting of at least three antiretroviral drugs, may fail following the development of viral resistance. Factors contributing to the incomplete suppression of HIV and to the development of resistance include insufficient drug potency, non-compliance, restricted tissue penetration, drug resistance and several host factors, such as host genetics [3–7]. Compliance during a life-long treatment is crucial, as establishing minimal inhibitory drug concentrations in the blood inhibits viral growth and the development of resistant strains [3,5,8–10].

A long-acting formulation of anti-HIV medication that generates sustained effective inhibitory concentrations with infrequent dosing may improve adherence to therapy. Next to facilitating maintenance of viral suppression following traditional anti-HIV therapy, a long-acting formulation, may serve as a practical opportunity for pre-exposure prophylaxis.

Long-acting formulations are well-accepted approaches for contraception and to treat psychiatric disorders [11,12]. Most long-acting formulations are based on the active ingredient being incorporated in either an oil-based solution or suspension (such as haloperidol depot formulations), a microfine watery suspension (such as steroid depots) or a suitable matrix from which the active ingredient is slowly released (Risperidone Consta®) [11,12]. The advent of nanotechnology, using suspensions of drug particles in the nanosize range, has provided opportunities to integrate poorly water- and oil-soluble molecules in long-acting injectable formulations: nanosizing allows to influence their release, while offering the advantage of higher mass packing (and thus higher dose) per volume and improved physical stability via use of more monodisperse material and one or more surfactants [13,14].

TMC278 (rilpivirine) is a very poorly water- and oil-soluble diarylpyrimidine derivative and was chosen as a candidate for developing a long-acting therapeutic and prophylactic treatment for the following reasons: (1) as an NNRTI, it interferes early in the cycle of viral HIV replication, preventing the viral DNA synthe-

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sis and subsequent integration of the viral genome in the host's DNA; (2) it is effective against a broad range of wild-type and HIV-mutant strains: its potent antiviral effect has been confirmed in a dose-finding study in treatment-naïve HIV patients [15,16]; (3) TMC278 is well tolerated during long-term administration: in a 48-week study it was found to result in fewer neuropsychiatric and metabolic side effects than efavirenz [17,18]. This paper describes some elementary pharmaceutical characteristics of TMC278 nanosuspensions prepared in an aqueous carrier and with average particle sizes targeted in the 200–800 nm range. In order to provide proof-of-concept of their long-acting release profile, a series of pharmacokinetic pilot experiments were performed in dogs and mice, thereby evaluating the impact of route of administration, chemical form, surfactant and average particle size.

2. Materials and methods

2.1. Test compounds and formulations

The NNRTI rilpivirine (TMC278 (E)-4-[[4-[[4-(2-cyanoethenyl]-2,6-dimethylphenyl]-amino]-2-pyrimidinyl]-amino]-benzonitrile was isolated as the free base or its corresponding HCl salt. Both are stable crystalline polymorphic forms and are largely insoluble in water and oil (less than 2×10^{-5} mg/mL): the physical status is crystalline and solubility in water or in phosphate buffer at pH 7 is <0.1 mg/mL (Tibotec, data on file). Unless specified as TMC278.HCl salt, TMC278 refers in the text to its form as base.

Using Elan's proprietary NanoCrystal® technology (Elan Corporation, Dublin, Ireland) [19], sterile nanosuspensions were prepared in an aqueous carrier containing a hydrophilic surfactant. Two non-ionic surfactants were tested: (1) poloxamer 338 (Pluronic F108, BASF) and (2) D-alpha-tocopheryl polyethylene glycol 1000 succinate (Vit-E TPGS, Eastman Chemical Company). The TMC278 crystals were nanosized by continuous wet milling on a US Stoneware roller mill, using zirconium beads with a diameter of 500 μm (YTZ Balls, Nikkato Co., Japan) during all preparations. Grinding volume, grinding time and number of revolutions of the vial were adapted according to various experimental set-ups until the desired particle size was reached for concept testing of the nanosuspension as long-acting formulation: a typical milling duration was 3 days, while in-process control was performed by regular sampling of the milled suspension in order to obtain the targeted nanoparticle size. The suspensions were harvested from the roller mill with a syringe (initial small batches) or by pumping the suspension through an appropriate stainless steel filter, retaining the beads. All formulations were produced under aseptic conditions; for this purpose, TMC278 starting material was subjected to gamma-radiation, which does not affect its stability (Tibotec, data on file).

For the 800 nm nanoparticles, 60 mL of an aqueous suspension, containing 1.875 g Vit-E TPGS and 7.5 g of TMC278 in water for injection (WFI), was milled with 300 g of zirconium beads, resulting in a 125 mg/mL suspension. After removal of the beads, the nanosuspension was diluted with WFI up to 100 mg/mL of TMC278 (10%). In the case of 400 nm nanoparticles, the same amounts were used but milling time was adapted in order to obtain smaller nanoparticles. The nanosuspensions were further diluted with Vit-E TPGS (5% in WFI) to 25 mg/mL.

To obtain the targeted 200 nm particle size, milling volumes were adapted as follows: for use in dogs, 5 g of TMC278, base or HCl salt, was milled with the zirconium beads in the presence of 1.25 g poloxamer 338 and WFI qs per 60 mL; the TMC278 base nanosuspension was diluted to 25 mg/mL, the HCl salt nanosuspension to 25 mg base equivalent/mL, by diluting, respectively, 18.2 and 19.87 g of milled concentrate with 50 mL of poloxamer 338 solution (10 mg/mL in WFI).

The 200 nm nanosuspensions for use in mice were prepared as described above, but 24.83 mL of the concentrate, prepared with poloxamer 338, was diluted with 7.6 mL of a 50 mg/mL poloxamer 338 solution, and 24.4 mL, prepared with Vit-E TPGS, with 7.8 mL of 50 mg/mL Vit-E TPGS, respectively, and subsequently with WFI qs to obtain a final concentration of 25 mg/mL for injection.

2.2. Particle size determination and stability testing

Particle sizes of the nanosuspensions were measured for the in-process control by laser diffraction with a Beckman Coulter LS230 diffractometer (Beckman Coulter, Inc., Fullerton, CA, USA), using 1.57 for the product refractive index and 0.1 for the product absorption index as the optical model according to Mie theory. The average particle size was expressed by the mean volume diameter of the particles.

All nanosuspensions were inspected visually following manual shaking before injection throughout the study. Stability data for the 200 nm suspension (25 mg/mL of TMV278 and prepared with poloxamer 338 as surfactant) were generated at ICH room temperature and protected from light, after 45 days and 1 year. The 400 and 800 nm nanosuspensions were used for the *in vivo* studies following their preparation and not further tested for stability as they were not retained for further development.

The concentrations of TMC278 and aldehydes (degradation products) were checked in the test formulations during stability testing and on the day of dosing by means of HPLC. The concentration of TMC278 was determined after dissolving into dimethyl formamide, dilution in 10 mM ammonium acetate in water/acetonitrile (80/20), and equilibration for at least 1 h, before its injection into the HPLC system. HPLC was performed using a Zorbac Extend C18 column, a column temperature of 45 °C, and a gradient programme of 10 mM ammonium acetate in water and acetonitrile at a flow rate of 1.0 mL/min during a 40 min analytical run, and UV detection at 280 nm. The concentration of aldehydes were determined after reaction with 2,4-dinitrophenylhydrazine (2 mL) and 0.2 M hydrochloric acid solution (1.0 mL) added to a dilution of 1.00 g of supernatant (obtained after centrifugation for 10 min at 5000 rpm) and 3 mL water and 0.5 mL acetonitrile. Reverse phase HPLC (YMC Pro C18 column at 35 °C, injection volume 20 μL, mobile phase consisting of 45% 0.01 N HCl/55% acetonitrile, flow rate of 1.2 mL/min and run time of 8 min) was performed with UV detection at 367 nm. On the day of dosing, TMC278 concentrations had to fall within the predefined concentration range of 85–115%.

2.3. Pharmacokinetic study design

Two exploratory studies were performed in Beagle dogs to determine the release profile over 3 months following a single dosing and one study was performed in mice, evaluating the release over 3 weeks. A first study in dogs explored the intramuscular (IM) release profile of 200 nm particles as nanosuspensions, prepared with TMC278 base or HCl salt, both containing poloxamer 338 as surfactant. IM and subcutaneous (SC) administration were only directly compared for the TMC278 base nanosuspension. Six male beagle dogs, each weighing 7–8 kg, were used, two per treatment group and per route of administration, using 20 G needles for injection: 5 mg/kg of TMC278 base or its equivalent dose as HCl salt was administered over two sites (0.1 mL/kg/site) by IM injection in the *m.biceps femoris* or by SC injection in the thoracic region, using a 23G needle. Blood samples were taken for at least 3 months after dosing, to determine the plasma release profiles and relative bioavailability (F_{rel}).

As the first study suggested the best release profile after SC injection, the second study assessed the release profiles of

TMC278 after single SC administration of the 400 and 800 nm formulations with Vit-E TPGS in nine dogs weighing 7–10 kg before drug administration (three male beagle dogs per nanosuspension and per dose): 5 mg/kg (400 nm particles) or 20 mg/kg (400 and 800 nm particles) was administered per dog by SC injection (0.2 mL/kg or 0.1 mL/kg/site) in the thoracic region. Blood samples were regularly taken up to 3 months.

In order to evaluate a potential effect of the surfactant on TMC278 release from the nanosuspension, the third study assessed the pharmacokinetics of the TMC278 200 nm nanosuspension prepared with either 25 mg/mL poloxamer 338 or Vit-E TPGS: 12 mice were studied per treatment group, each receiving a single SC injection of 20 mg/kg dose in the thoracic region; they were subjected to alternate sampling (each animal sampled twice) up to 18 days after injection.

2.4. Blood and tissue sampling and plasma preparation

For each experiment, blood sampling was reduced to a minimum for ethical reasons. In dogs, blood samples (2 or 3 mL on EDTA, EDTA Vacuette Greiner, Cat. No. 454086, Greiner Labortechnik N.V.) were taken from a jugular vein: in the study of the 400 and 800 nm TMC278 nanosuspensions, samples were taken on day 0 at 0 h (= predose), 1, 3, 8 and 24 h post-dose and further once (at 8 am) on days 2, 3, 6, 8 and then weekly up to 3 months. In the study with the 200 nm nanosuspension, an additional blood sample was taken on day 0 at 20 or 30 min post-dose in order to characterize the early release phase.

In mice, 0.3 mL blood on EDTA (EDTA Microvette Sarstedt) was taken by puncture of the orbital venous plexus under light isoflurane anaesthesia. Samples were taken on day 0 at 0 h (= predose) and 1, 2, 4, 8 and 24 h post-dose and further once on days 2, 4, 7, 9, 11, 14 and 18. At each sampling time, two animals were used per treatment group, while each animal was sampled twice at different occasions, with a minimum of 48 h in between successive samplings per animal. At the terminal time point (432 h), a sample was taken from four mice.

Within 2 h of blood sampling, samples were centrifuged at room temperature at about 1900×*g* for 10 min to allow plasma separation (1500×*g* for the mice samples). Plasma was immediately transferred into a second tube and stored in the freezer within 2 h after the start of centrifugation. At all times, blood and plasma samples were protected from light.

2.5. Sample and data analysis

Plasma samples were first subjected to a selective sample clean-up and analysed individually for unchanged drug (TMC278) by means of a qualified research LC-MS/MS method, as previously described [20]. Samples were quantified against calibration curves prepared in plasma, to match the matrix and covering the concentration range of the study samples (1.00–2000 ng/mL in dogs, 2.00–4000 ng/mL in mice). The lower limit of quantification (LLOQ) was 1.00 ng/mL in dogs and 2.00 ng/mL in mice.

Release profiles and pharmacokinetic parameters obtained after administration of the different nanosuspensions were compared in a qualitative fashion. Mean plasma concentrations were calculated per administration route, per drug substance and per sampling time. The following pharmacokinetic parameters were derived by non-compartmental analysis of averaged plasma TMC278 concentration–time profiles, using WinNonlin Professional software (Version 4.0.1a, Pharsight): peak plasma concentration (C_{max}), corresponding peak time (T_{max}) and area-under-the-plasma concentration versus time curve from time zero to 92 days ($AUC_{0-92days}$) in dogs and to infinity (AUC_{0-inf}) in mice. In dogs, individual plasma concentration–time curves were constructed and pharmacokinetic parameters were calculated for each animal. In mice, generally two samples were drawn from each animal (alternate sampling) and a single ("mean") plasma concentration–time curve was constructed using the data from all mice: for each time point, except for the last sampling time ($n = 4$ mice), the mean plasma concentration was calculated based on separate samples derived from two individual mice. The plasma concentration–time plots were constructed with a total of 13 time points. From 0 to 336 h, the plasma concentration is thus the average of two independent samples, whereas the last point is the average of four values. These mean plasma levels and plasma concentration–time curve were used to calculate the pharmacokinetics parameters. To guide the decisions in selection of the nanosuspension and the route of administration for study, F_{rel} was calculated. In the studies with the 200 nm nanosuspensions in dogs, F_{rel} was calculated relative to the IM administration of the 200 nm TMC278 base nanosuspension, while in mice F_{rel} was expressed relative to the poloxamer 338 containing nanosuspension.

2.6. Ethics

The animals were housed, with free access to water all day. Food was available all day for mice, while dogs were fed in the morning. All animals were treated in accordance with the provisions for protection of vertebrates that are used for experimental and other scientific purposes and for protection of laboratory animals, as per Belgian laws and European convention (European Council Directives (1986) and European Commission's Protocol on the protection and welfare of animals used for experimental and other scientific purposes, European Commission Directives 2007 online). The study was approved by the Local Ethics Committee on animal experiments, and was performed in an AAALAC-accredited laboratory, complying with European and Belgian regulations for animal experiments.

3. Results

3.1. Preliminary characterization of the nanosuspensions

Pharmaceutical formulation with the Elan's NanoCrystal® technology proved that TMC278 could be nanosized. The particle size distributions of the targeted 200, 400 and 800 nm test-nanosuspensions of TMC278 base are given in Fig. 1. When measured with laser diffraction, the mean volume diameter was smaller than projected. For instance, in case of the 200 nm nanosuspensions with Vit-E TPGS, 10% of the particles were sized below 77 nm, 25% below 93 nm, 50% below 117 nm, 90% below 185 nm and 95% below 208 nm. The widths of the particle size distributions became

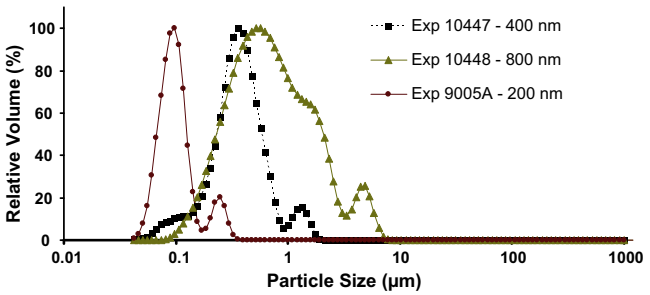

Fig. 1. Particle size distribution of the test TMC278 nanosuspensions with differently targeted particle sizes: Exp 9005A (200 nm), Exp 10477 (400 nm) and Exp 10488 (800 nm). Relative volume (%): percent of particles of a given size interval (below the size) relative to the total volume as determined by laser diffraction, using Coulter LS230.

broader, the more the targeted particle size increased. All nanosuspensions had a bimodal distribution, with a comparable small second peak. Overall, the targeted size proportionality was maintained, making the test batches suitable for proof-of-concept testing *in vivo*.

3.1.1. Impact of drug form

Use of the TMC278 base resulted in a pH of 7.0, while the use of the HCl salt of TMC278 resulted in a physiologically unacceptably low pH of 1.9.

3.1.2. Impact of surfactant

With both poloxamer and Vit-E TPGS, sufficiently stable, homogeneous and resuspendable nanosuspensions could be prepared.

3.1.3. Preliminary stability assessment

Visual inspection of the nanosuspensions obtained by wet milling showed that the formed sediments were well resuspendable on manual shaking, resulting in visually homogeneous, syringeable, injectable formulations for administration. Although not formally assessed, the sedimentation rate appeared to be very low. There was no evidence of tendency to form hard cakes. Stability testing after 45 days and 1 year of storage at room temperature is illustrated in Table 1 for the 200 nm particle size. After 1 year of storage there was minimal growth toward larger particles.

3.2. Pharmacokinetic results

3.2.1. Proof-of-concept: pharmacokinetic release profile

As shown in Figs. 2 and 3, all tested nanosuspensions resulted in maintained plasma concentrations, consistent with a sustained release profile of TMC278, albeit with differences in extent of early and overall release according to the route of administration, particle size and chemical form of TMC278.

After a single 5 mg/kg dose of the 200 nm nanosuspensions of TMC278 base in dogs, stable, sustained release of TMC278 was obtained over 3 months, with little inter-individual variability in TMC278 plasma concentrations and PK parameters among dogs (Fig. 2). Three months after administration, mean plasma concentration levels were 1.4 ng/mL for long-acting TMC278 base and 2.9 ng/mL for long-acting TMC278.HCl after IM administration, and 2.8 ng/mL for the base after SC administration. The SC administered TMC278 base nanosuspension showed the most stable sustained release, as indicated by the low $C_{max,}$ reached at 144 h (~6 days) post-dose (= T_{max}).

With both the 400 and 800 nm nanosuspensions, plasma concentrations were lower and a higher inter-individual variability in TMC278 plasma concentrations was observed per treatment group of three dogs, particularly during the first 24 h post-dose. After single SC injection of the 400 nm nanosuspension (Vit-E TPGS), 5 or 20 mg/kg, plasma concentrations rapidly increased until approximately 24 h post-dose, reaching a mean C_{max} of 19.6 or 39.4 ng/mL, respectively (Fig. 3). Subsequently, plasma concentrations fluctuated between 10 and 20 ng/mL for about 1 month after which levels slowly decreased. Three months after dosing with 5 mg/kg, plasma levels were still detectable in only two of three dogs (with plasma concentrations of approximately 2 ng/mL). The increase in plasma concentrations was not dose-proportional: 4-fold dose increase from 5 mg/kg to 20 mg/kg yielded an increase in exposure only 2- to 2.7-fold. The 800 nm nanosuspension (20 mg/kg, Vit-E TPGS) resulted in a similar release profile as the 400 nm nanosuspension. Three months after administration, mean plasma levels were 2.3, 10.1 and 5.4 ng/mL in the three treatments

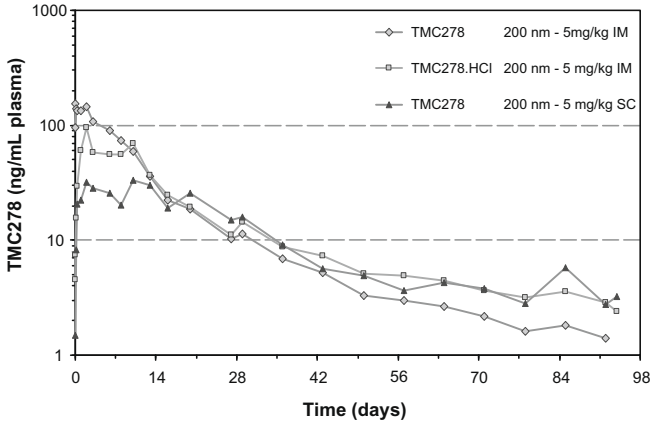

Fig. 2. Mean plasma concentration profiles (ng/mL) of TMC278 long-acting nanosuspensions in beagle dogs after SC and IM administration of 200 nm nanosuspensions at 5 mg/kg (base or HCl salt in mg equivalent).

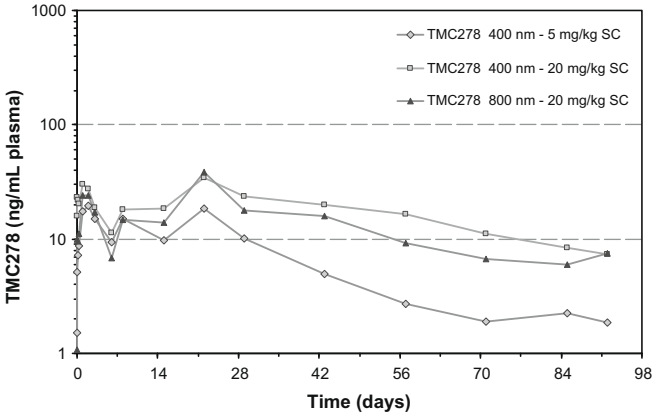

Fig. 3. Mean plasma concentration profiles (ng/mL) of TMC278 long-acting nanosuspensions in beagle dogs after SC dosing of 400 and 800 nm nanosuspensions at 5 or 20 mg/kg (TMC278 base).

Table 1
Stability of a 200 nm TMC278 suspension (25 mg/mL, using 10 mg/mL of poloxamer 338 as surfactant). Particle size distribution by Coulter LS230 after storage at room temperature (two samples).

	Sample	Diameter of cumulative percent[a] (μm)				
		10%	25%	50%	90%	99%
Day 1	1	0.065	0.077	0.094	0.179	0.294
	2	0.067	0.079	0.096	0.162	0.297
After 45 days	1	0.069	0.081	0.098	0.164	0.296
	2	0.066	0.079	0.096	0.185	0.294
After 1 year	1	0.059	0.071	0.088	0.215	0.312
	2	0.060	0.072	0.088	0.193	0.303

[a] Mean volume diameter as determined by Coulter LS230: see Section 2.

groups, representing 12%, 26% and 14% of their highest mean plasma concentrations, respectively.

3.2.2. Impact of administration route

As demonstrated by the early and late plasma release profiles of the 200 nm nanosuspension of TMC278 (Fig. 2), as well as the pharmacokinetic parameters in Table 2, IM and SC administrations resulted in different early release profiles. After the initial rapid release of TMC278 following IM dosing, leading to peak plasma concentrations of almost 100 ng/mL for the HCl salt and up to 180 ng/mL for the base, plasma concentrations progressively declined after T_{max} to less than 5% of its peak plasma with similar levels for salt and base on day 42 (~5–8 ng/mL), and an apparently slower decline from day 49 onwards until values were around 2–3 ng/mL on day 94. After SC administration of the base, plasma concentrations stabilized 8–24 h post-dose, after which they remained fairly constant until day 24 (still reaching 22 ng/mL). Thereafter, plasma levels declined slowly (see pharmacokinetic release profile, Fig. 2).

After IM administration, a rapid onset of drug availability was observed, irrespective of the nature of the drug suspension (base or salt), leading to a relatively pronounced peak in the pharmacokinetic profile. This initial peak was not observed, and release was more sustained, after the SC administration of TMC278 base (C_{max} = 31–38 ng/mL at a T_{max} of 144 h post-dose) when compared with its IM administration (C_{max} = 619 ng/mL at T_{max} of 24 h post-dose). The mean exposure over the 3-month period ($AUC_{0-92days}$) amounted to 41.4 μg h/mL after IM administration and 24.4 μg h/mL after SC administration (F_{rel} = 62% of the IM exposure), which appeared to be related to the more gradual release after SC administration. Also after SC administration of the 400 and 800 nm nanosuspensions, high initial drug exposure was not observed (Fig. 3).

3.2.3. Impact of particle size

At 5 mg/kg SC dosing, the 200 nm nanosuspension of TMC278 base (poloxamer 338) resulted in a higher mean C_{max} and $AUC_{0-92days}$ than the 400 nm nanosuspension (Vit-E TPGS) (C_{max} = 38.0 versus 19.6 ng/mL; $AUC_{0-92days}$ = 24.4 versus 14.4 ng.h/mL, respectively; see Table 2).

On increasing the particle size from 400 to 800 nm (20 mg/kg SC dosed), C_{max} values remained the same, but the mean exposure decreased from 38.2 to 29.6 μg h/mL, while $T_{max,}$ increased from 408 to 528 h (see Table 2).

3.2.4. Comparison of surfactant

On testing of 200 nm TMC278 nanosuspensions in mice, a faster release of TMC278 from the nanosuspension depots was observed than that in dogs, with C_{max} values amounting to 2160 ng/mL after SC injection with Vit-E TPGS, and 2850 ng/mL when using poloxamer 338. The AUC_{0-inf} values were 74.2 and 65.4 μg.h/mL, respectively. The F_{rel} of the nanosuspension with Vit-E TPGS relative to the nanosuspension with poloxamer 338 was 88%, essentially within the normal range of experimental variation and not suggesting a relevant impact by the change of surfactant.

3.2.5. Comparison of base and HCl salt

On comparison of base and HCl salt of TMC278, both administered IM in dogs (Fig. 2), the most rapid onset of drug release occurred with the base; after a high initial drug release over a period of 2 days (C_{max} = 173 ng/mL, T_{max} = 24 h), the plasma concentrations declined progressively to 10.2 and 1.4 ng/mL after 1 and 3 months, respectively. After IM dosing of the HCl salt, the initial rapid drug release was still apparent but less extensive, and plasma concentrations increased up to 48 h post-dose (C_{max} = 95 ng/mL; T_{max} = 48 h), after which they remained constant for 10 days (at approximately 65 ng/mL) and subsequently declined slowly to 14.5 ng/mL at 1 month and 2.9 ng/mL at 3 months. Table 2 summarizes the pharmacokinetic parameters. The values for F_{rel} indicate that the use of the salt form did not result in a biologically relevant increase in bioavailability when compared with the base form.

4. Discussion

With these studies, proof-of-concept was provided that:

1. The antiretroviral TMC278 (rilpivirine) can be nanosized by wet milling to suitable formulations for parenteral application. The wet milling with zirconium beads in the presence of a non-ionic surfactant allowed the formulation of sufficiently stable, homogeneous and resuspendable nanosuspensions, suitable for testing as long-acting injections.
2. Long-acting formulations of TMC278 can be developed, using nanotechnology by wet milling in combination with a non-ionic surfactant: TMC278 nanoparticles, ideally with a 200 nm particle size, can be administered as a single-dose long-acting injection and can achieve stable sustained plasma concentration profiles detectable up to 3 months after IM or SC administration in dogs. The formulation characteristics (narrowest particle size distribution) combined with the pharmacokinetic observations (best exposure with a sustained release profile and low inter-individual variability) supported the selection of 200 nm particle size for more in-depth study of TMC278 nanosuspensions in dogs and mice.

Wet milling in an aqueous carrier containing non-ionic surfactant under aseptic conditions resulted in sterile, syringeable and

Table 2
Pharmacokinetics of long-acting formulations of TMC278 in male dogs: single dose studies with different doses, particle sizes and routes of administration: mean values and range.[a]

Study	Drug	Surfactant	Route[b]	Dose (mg/kg)	Particle size[c] (nm)	C_{max} (ng/mL)	T_{max} (h)	$AUC_{0-92days}$ (ng h/mL)
Dogs 2 males/group	TMC278.HCl	Poloxamer 338	IM	5	200	95.0 (86–105)	48 (48–48)	33,600 (27,400–39,900)
	TMC278	Poloxamer 338	IM	5	200	173.0 (159–186)	24 (0.3–48)	39,400 (38,200–40,500)
	TMC278	Poloxamer 338	SC	5	200	38.0 (34–42)	144 (48–240)	24,400 (22,400–26,400)
Dogs 3 males/group	TMC278	Vit-E TPGS	SC	5	400	19.6 (1702–2501)	256 (48–192)	14,400 (1060–18,600)
	TMC278	Vit-E TPGS	SC	20	400	39.4 (28.1–58.8)	408 (1–528)	38,200 (33,900–43,900)
	TMC278	Vit-E TPGS	SC	20	400	38.8 (26.2–52.4)	528 (528–528)	29,600 (24,400–37,300)
Mice 12 females/group	TMC278	Poloxamer 338	SC	20	200	2160	4.0	74200[d]
	TMC278	Vit-E TPGS	SC	20	200	2850	2.0	65400[d]

[a] Mean values calculated from the individual data obtained per dog (repeated sampling per dog), or value obtained from the mean plasma concentrations obtained per time point in mice (use of alternated sampling); between brackets: range of values.
[b] IM, intramuscular; SC, subcutaneous administration.
[c] Targeted particle size, for particle size distribution see Fig. 1.
[d] In mice, the AUC_{0-inf} was calculated from the mean plasma concentrations at the different time points of plasma sampling.

injectable depot formulations, well shakeable and resuspendable for administration. The nanosuspensions were easily injectable using 20G and 30G needles for 400–800 and 200 nm suspensions, respectively. Both non-ionic surfactants tested, poloxamer 338 and Vit-E TPGS, proved to be useful. The particle size distribution was the narrowest for the targeted 200 nm nanosuspension, while the broadness of the particle size distribution increased, the more the targeted particle size increased, and this towards larger spec sizes when going from 200 to 400 nm and further to 800 nm as targeted particle size. Laser diffraction showed that the average particle size was slightly lower than the targeted diameter for all batches and also showed a small second peak towards higher spec sizes in all batches. As this second small peak was comparable across all nanosuspensions tested, and as the test samples behaved proportionally in size according to the targeted diameters, the formulations were considered to be sufficiently suitable for proof-of-concept testing. Absence of highly amorphous or ultrafine nanoparticles was confirmed *in vivo* by the observation that none of the plasma concentration profiles showed a burst or very high initial drug release due to very rapid dissolution of the ultrafine particles and release of drug in the bloodstream. Moreover, the 200 nm nanosuspension, protected from light, proved to be stable during at least 1 year at room temperature. Protection from light was chosen because earlier stability testing of TMC278 had evidenced the formation of a degradation compound when submitted to ICH light conditions.

The pharmacokinetic studies provided the first proof-of-concept that TMC278 nanosuspensions may be suitable as long-acting formulations. Following their administration, plasma levels were sustained during the 3 months of observation in dogs. Sustained release of TMC278 was already seen at a dose of 5 mg/kg. This is relevant, as HIV drugs often have to be given in substantial doses (>500 mg/day) to obtain lasting therapeutic success, while, in contrast, the average amount of drug used to treat most diseases is 10–30 mg/day [21]. Some HIV drugs have poor aqueous solubility, undergo a high first-pass effect and have low bioavailability, which may limit their potential for reformulation [22]. Long-acting parenteral formulations of HIV agents may overcome these limitations by use of nanotechnology and by avoiding the first-pass metabolism following oral intake.

The pharmacokinetic tests further showed that the particle size and route of administration, but not the chemical form of TMC278 (base or HCl) and choice of non-ionic surfactant, had an important impact on the drug release of TMC278. With respect to the particle size of the nanosuspension, 200 nm particles resulted in an improved early release (higher C_{max}) in dogs, when compared with 400 or 800 nm particles. Also the total exposure was better with 200 nm than 400 nm, as indicated by the overall AUC over 3 months following 5 mg/kg SC administration. These differences could not be attributed to the change of surfactant between formulations: decreasing particle size resulted in a much larger effect (more than 70% increase in AUC from 400 to 200 nm in dogs) than predictable from changing surfactant (13% increase from Vit-E TPGS to poloxamer 338 in mice, which falls within the expected range of variation between *in vivo* experiments). A faster release from the smaller particles, with an associated larger relative particle surface, does not come as a surprise. It cannot be excluded that with a longer follow-up in dogs, a similar overall exposure would have been observed with the nanosuspensions consisting of different sizes of particles, since well-maintained plasma levels demonstrated that the release from the depots was still ongoing at the terminal time point of 3 months.

The pilot study in dogs also indicated that the route of parenteral administration influenced the release profile of TMC278 from the nanosuspension: after IM release of the 200 nm TMC base suspension, higher initial drug release, higher C_{max} plasma levels and reduced T_{max} values were observed after IM administration than after SC administration. Also IM injection of the TMC278.HCl salt 200 nm nanosuspension resulted in initial, though smaller, drug release than after TMC278 base IM, but this nanosuspension had an unacceptably low pH 1.9; yet, injection did not appear to cause untoward effects, likely due to the low buffering effect by the low concentration of dissolved TMC278.HCl. The most stable plasma concentration profile was obtained after SC injection of the 200 nm nanosuspension of TMC278 base: this approach best attenuated C_{max} while sustaining detectable plasma levels during the 3 months of follow-up. The absence of high initial drug release following SC dosing was also confirmed by the SC experiments with the 400 and 800 nm nanosuspensions. The observations are not unexpected because on IM administration, the blood in the highly vascularised muscle, certainly when compared to skin, has a higher draining effect than is seen following SC administration.

Our findings that particle size and route of administration may profoundly affect the release kinetics of insoluble drugs, both initially and throughout the observation period, are in line with the earlier systematic studies, performed by Japanese investigators in the micron range in the 1980s [1,2]. They also showed that the release from insoluble solid particles is not necessarily dose-proportional: although only tested for 400 nm in these pilot studies, no dose-proportionality was found between 5 and 20 mg/kg.

Finally, with respect to TMC278's chemical form, one could have expected in theory that, in so far this poorly soluble drug gets dissolved in the suspension, release is more complete with the salt than with the base: yet, release was lower and slower with the HCl salt, with only small differences in exposure between both forms (F_{rel} = 86%). This can also be attributed to other factors. First, hydrolysis of TMC278.HCl salt in aqueous medium causes conversion of the salt form to the base form. This conversion may have occurred during storage in the vial prior to injection, or/and also just following injection into the muscle. Second, the test batches for this proof-of-concept study were subjected to minimal characterization of the particle size distribution by Coulter laser diffraction: the presence of some finer or fewer rough crystal particles in the base nanosuspension may have affected the dissolution kinetics in favour of the base [14].

Overall, the results strongly support clinical investigation of TMC278 nanosuspension as a novel way to improve compliance during antiretroviral therapy. Apart from enhancing compliance via reducing missed doses and decreasing pill burden, this way of administration avoids the impact of food on the bioavailability of TMC278 observed with oral administration [23]. Based on the in vitro potency of TMC278 corrected for protein binding effects [24], plasma concentrations exceeding 20 ng/mL could in theory be therapeutically effective. Although not a primary objective of the current studies, such exposures were approximated for 4 weeks from SC dosing in dogs at 5 mg/kg for the 200 nm nanosuspensions. Single-dosing of TMC278 nanosuspensions as long-acting formulation has meanwhile also been evaluated in humans [24], in whom sufficiently high TMC278 plasma levels (shown to be effective after oral treatment) were obtained. Hence, TMC278 nanosuspensions could possibly become part of future HAART and be useful in pre-exposure prophylaxis. Other potential opportunities to be investigated could be to establish minimal inhibitory plasma concentrations for longer periods of time, in the hope to reduce mutations and antiviral resistance or to prevent mother-to-child transmission. In several studies using other anti-HIV agents, trough plasma levels were found to be predictive of virologic response and rebound of the infection [25–29]. Further pharmacokinetic characterization of this formulation is thus highly desirable.

In conclusion, stable sustained plasma concentration profiles were obtained for several months in dogs after administration of single doses of nanosuspensions of TMC278. These findings open

a new opportunity for improving compliance and for development of HIV pre-exposure prophylaxis.

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