European Journal of Pharmaceutics and Biopharmaceutics 88 (2014) 252–260

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Nanosuspension for the delivery of a poorly soluble anti-cancer kinase inhibitor

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ARTICLE INFO

Article history:
Received 5 February 2014
Accepted in revised form 8 May 2014
Available online 21 May 2014

Keywords:
Nanosuspension
Micelles
Nanoparticles
Tumor targeting
Kinase inhibitor
MTKi-327
Anti-cancer drug

ABSTRACT

We hypothesized that nanosuspensions could be promising for the delivery of the poorly water soluble anti-cancer multi-targeted kinase inhibitor, MTKi-327. Hence, the aims of this work were (i) to evaluate the MTKi-327 nanosuspension for parenteral and oral administrations and (ii) to compare this nanosuspension with other nanocarriers in terms of anti-cancer efficacy and pharmacokinetics. Therefore, four formulations of MTKi-327 were studied: (i) PEGylated PLGA-based nanoparticles, (ii) self-assembling PEG_{750}-p-(CL-*co*-TMC) polymeric micelles, (iii) nanosuspensions of MTKi-327; and (iv) Captisol solution (pH = 3.5). All the nano-formulations presented a size below 200 nm. Injections of the highest possible dose of the three nano-formulations did not induce any side effects in mice. In contrast, the maximum tolerated dose of the control Captisol solution was 20-fold lower than its highest possible dose. The highest regrowth delay of A-431-tumor-bearing nude mice was obtained with MTKi-327 nanosuspension, administered intravenously, at a dose of 650 mg/kg. After intravenous and oral administration, the $AUC_{0-\infty}$ of MTKi-327 nanosuspension was 2.4-fold greater than that of the Captisol solution. Nanosuspension may be considered as an effective anti-cancer MTKi-327 delivery method due to (i) the higher MTKi-327 maximum tolerated dose, (ii) the possible intravenous injection of MTKi-327, (iii) its ability to enhance the administered dose and (iv) its higher efficacy.

1. Introduction

Poor water solubility of many anti-cancer drugs or drug candidates constitutes a major obstacle to their development and their clinical application. To overcome this problem, several strategies have been developed such as the use of cyclodextrins, salt formation of ionizable drugs and the use of co-solvents or surfactants (which are often toxic; e.g. Cremophor® EL) [1]. Alternatively, numerous nanosystems have been investigated for the colloidal dispersion of poorly water soluble drugs, including liposomes, nanoparticles, polymeric micelles, dendrimers, etc. These nanocarriers have been largely described for their ability to deliver the drug specifically to tumor, decreasing systemic side-effects. Some nanoformulations have been clinically approved such as Abraxane® and Doxil® [2]. Because of the fenestrations present in tumoral endothelium, these nanocarriers can enter the interstitium, be entrapped in the tumor and remain inside the tumoral tissue because of the poor lymphatic drainage. This "passive" targeting is based on the enhanced permeability and retention (EPR) effect, discovered by Matsumura and Maeda [3].

Recently, various nanosizing strategies have emerged. Nanosuspensions (also called nanocrystals) are nanoscopic crystals of the compound with a size below 1 μm [1]. Nanosuspensions present several advantages: (i) Nanosuspensions allow the colloidal dispersion of poorly water soluble drugs [4]. (ii) These particles possess a very high drug loading (pure drug coated with surfactant

Abbreviations: AUC, area under the curve; CL, caprolactone; DMEM, Dubelcco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; EMA, European Medicine Agency; EPR, enhanced permeability and retention effect; FDA, Food and Drug Administration; HPLC, high performance liquid chromatography; IV, intravenous; LOQ, limit of quantification; MTD, maximum tolerated dose; NMR, nuclear magnetic resonance; PCL, polycaprolactone; PDI, polydispersity index; PEG, polyethylene glycol; PK, pharmacokinetic; PLGA, poly(lactide-co-glycolide); PTX, paclitaxel; PVDF, polyvinylidene fluoride; RES, reticulo-endothelial system; SEC, size exclusion chromatography; SEM, standard error of the mean; TMC, trimethylene carbonate; VEGFR, vascular endothelial growth factor receptor.

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http://dx.doi.org/10.1016/j.ejpb.2014.05.014
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or polymer as stabilizer) in contrast to matrix nanoparticles [5]. (iii) The scaling-up is easy and the nanosuspension technology constitutes a cost effective simple alternative [5]. (iv) Nanosuspensions allow physical and chemical stability of drugs [1]. (v) Nanosuspensions may show passive targeting (EPR effect) similar to colloidal drug carriers after intravenous injection [6]. (vi) Finally, nanosuspensions are one of the most important strategies to enhance the oral bioavailability of hydrophobic drugs but also for their intravenous route [5].

More than 10,000 patent applications for kinase inhibitors have been field since 2001 in the United States [7]. This massive investment has been filed by the realization that kinases are intimately involved cancer cell growth [7]. MTKi-327 (JNJ-26483327) is an anti-cancer multi-targeted kinase inhibitor developed by Johnson and Johnson (Fig. 1). Kinase inhibitors have revolutionized the treatment of a selected group of diseases such as chronic myeloid leukemia and gastrointestinal stromal tumors, which are driven by a single oncogenic kinase. However, most of cancers involve multiple kinases [7]. In this context, the development of multi-targeted kinase inhibitor has emerged. MTKi-327 has been demonstrated *in vitro* as a potent inhibitor of various kinases, including (i) EGFR, Her-2, Her-4 and Ret (playing a role in proliferation and migration); (ii) VEGFR-3 and Yes (decreasing lymphangiogenesis and angiogenesis); and (iii) Src, VEGFR and Raf (limiting the metastasic process) [8]. *In vivo*, MTKi-327 (orally administered) presented an anti-tumor activity on various subcutaneous tumors (such as A-431 and A-549), orthotopic tumors (such as DU-145), intracranial tumors (A-431) and bone tumors (MDA-MB-231). MTKi-327 decreased the tumor growth more efficiently than lapatinib or erltinib [9]. Phase I studies in patients with advanced solid tumors demonstrated that MTKi-327 was well tolerated and showed predictable pharmacokinetics profiles [9]. This compound is practically insoluble in water (<0.001 mg/ml) at pH = 7.4. Therefore, the current challenge is to formulate this drug for intravenous and oral routes in a non-toxic formulation. No formulation convenient for the parenteral route is currently available.

We hypothesized that nanosuspensions could be promising for the delivery of MTKi-327. Hence, the aims of this work were (i) to evaluate the MTKi-327 nanosuspension for parenteral and oral administrations and (ii) to compare this nanosuspension with other nanocarriers in terms of anti-cancer efficacy and pharmacokinetics.

Therefore, different formulations of MTKi-327 were studied: (i) PEGylated PLGA-based nanoparticles. The poly(lactide-co-glycolide) (PLGA) polymer (approved by the FDA and EMA in parenteral products) was chosen for its biocompatibility and its biodegradability properties. Poly(ε-caprolactone-co-ethyleneglycol) (PCL–PEG) was added to take advantage of PEG repulsive properties [10]. These nanoparticles have been previously shown suitable for the encapsulation of poorly water soluble drugs such as paclitaxel (PTX) or a cyclin dependent kinase inhibitor, JNJ-7706621 [11,12];

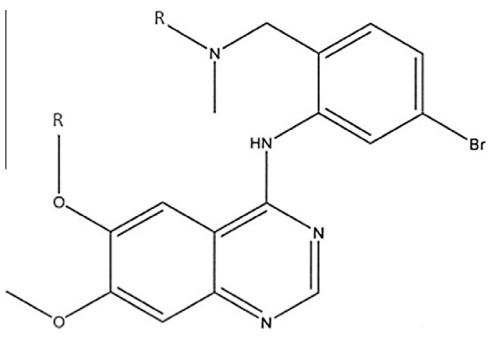

Fig. 1. Chemical structure of the tyrosine kinase inhibitor pharmacophore.

(ii) self-assembling diblock copolymers made up of ε-caprolactone (CL) and trimethylene carbonate (TMC) and mmePEG$_{750}$ (mmePEG$_{750}$-p-(CL-*co*-TMC)) have been shown to form micelles spontaneously upon gentle mixing with water. These copolymers are biocompatible, non-cytotoxic and non-hemolytic. They increase the solubility of poorly soluble-water drug (such as PTX) by at least two orders of magnitude. These polymeric micelles significantly reduced the toxicity of paclitaxel allowing the dose enhancement for better therapeutic response [12,13]; (iii) a nanosuspension of MTKi-327; and (iv) a Captisol solution (pH = 3.5). This solution was designed for the oral delivery of MTKi-327 (pK_a of MTKi-327 = 3.9) but was not adapted for repeated intravenous administrations because of its acidity. Orally administered to mice, MTKi-327 had a maximum tolerated dose of 100 mg/kg/day. For the present study, the Captisol solution was thus used as control.

These four formulations were characterized and the concentration of MTKi-327 was determined. Maximum tolerated doses were determined. The *in vivo* anti-tumor activity was performed in A-431-tumor bearing nude mice. To demonstrate the potential of nanosuspension, MTKi-327 nanosuspension was investigated more precisely in comparison with the Captisol solution: the effect of the dose, the route of administration and the number of administered doses were evaluated. Pharmacokinetics studies were also performed.

2. Material and methods

2.1. Materials

PLGA, PCL-*b*-PEG, PLGA-*b*-PEG polymers were synthesized by ring opening polymerization and were kindly provided by Pr. Ch. Jérôme (CERM, ULg). Molecular weights were determined by size exclusion chromatography (SEC) and NMR as described previously [14,15]. The PEG-p(CL-*co*-TMC) diblock copolymers were synthesized by the Johnson and Johnson Advanced technologies and Regenerative Medicine (ATRM) (Somerville, NJ, USA). Characteristics of polymers are summarized in Table 1. MTKi-327 was provided by Johnson and Johnson, Pharmaceutical Research and Development, Division of Janssen Pharmaceutica (Beerse, Belgium) [9]. A-431 (Human Epidermoid Carcinoma) cells were acquired from ATCC (American Type Culture Collection, Manassas, VA, USA). Dubelcco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin–EDTA, penicillin–streptomycin mixtures and L-glutamine were purchased from Invitrogen (Merelbeke, Belgium). Ultra-purified water was used throughout and all other chemicals were of analytical grade. NMRI and athymic nude mice were purchased from Janvier, Genest-St-Isle, France.

2.2. Formulations

2.2.1. Nanoparticles

35 mg of PLGA, 7.5 mg of PLGA–PEG, 7.5 mg PCL–PEG and 5 mg of MTKi-327 were dissolved in 600 μl of dichloromethane. 2 ml of sodium cholate (1%) was added to the polymers solution and sonicated two times 15 s (60 W). This emulsion was added with a glass syringe to 100 ml of sodium cholate (0.3%) under magnetic stirring 2 h to allow the evaporation of dichloromethane. Nanoparticles were ultra-centrifuged 1 h at 4 °C at 22,000*g* and washed three times in water. Pellets were finally suspended in 3 ml ultra-purified water [11].

2.2.2. Polymeric micelles

500 mg of PEG-p(CL-co-TMC) 50:50 was added to an excess of MTKi-327 (12.5 mg) in a formulation flask and mixed overnight at 37 °C. 4.5 ml ultra-purified water was added to the polymer

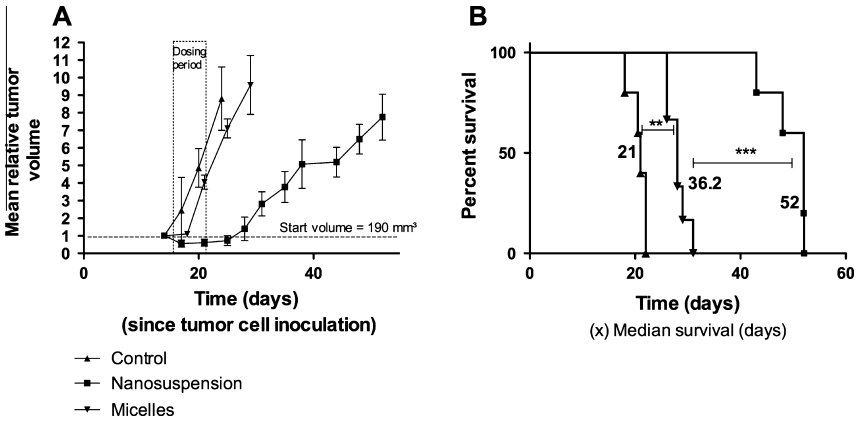

Fig. 2. Anti-tumor effect on A-431-tumor-bearing mice treated with different formulation of MTKi-327. (A) Evolution of the A431 tumor volume in nude mice after intravenous injections for 5 consecutive days of MTKi-327 nanosuspension (650 mg/kg/day) and intravenous injections for 5 consecutive days of polymeric micelles (6 mg/mg/day). Each point represents the mean ± SEM. (B) Kaplan Meyer survival curve and mean survival times. $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 1
Chemical description of the polymers included in the formulations.

Nanoparticle polymers	Mw (g/mol)[a]	Mn (SEC)[b] (g/mol)	Mn (NMR)[a] (g/mol)	PDI[c]
PLGA	39,600	22,000	–	1.8
PLGA-FITC	37,800	23,600	–	1.6
PLGA-b-PEG	49,800	29,300	16,500–4600	1.7
PCL-b-PEG	25,800	22,400	15,200–4600	1.15
Micelle polymers	Mw (g/mol)[d]		CL/TMC ratio in final polymer[e]	PDI[d]
PEG-p(CL-*co*-TMC)	5300		49.3 ± 0.4/50.1 ± 0.8	1.9

[a] Determined by SEC and NMR by the following formula: $(I_{4.7}/2)/(I_{5.2} + I_{4.7}/2)) \times 100$, where $I_{4.7}$ is the signal intensity of the glycolide unit at 4.7 ppm ($CH_2OC{=}O$) and $I_{5.2}$ is the signal intensity of the lactide unit at 5.2 ppm ($CH(CH_3)OC{=}O$).
[b] Polystyrene calibration.
[c] PDI = Mw/Mn, determined by SEC using polystyrene standard.
[d] The molecular weight and the polydispersity of the diblock polymer were determined by gel permeation chromatography and the monomer ratio in the polymer by NMR spectroscopy.
[e] The monomer composition was evaluated by NMR.

mixture drop wise and mixed for 1 h. The excess drug was removed by filtration through 0.45 μm PVDF filters (Acrodisc, Pall, USA) [12,16].

2.2.3. Nanosuspension

MTKi-327 nanosuspension was prepared by the roller milling method described previously [1,4]. The MTKi-327 powder was dispersed into an aqueous solution containing various stabilizers and ionic agents (Pluronic® F108 (25 mg/ml), Lipoid S75 (3 mg/ml) and glucose (40 mg/ml)). The obtained pre-mixture was milled for 66 h at room temperature with ZrO_2 of 0.5 mm. The nanosuspension was harvested with a syringe with a fine bore needle microlance G23.

2.2.4. Captisol solution

The Captisol solution contained 10 mg/ml of MTKi-327, sulfobutyl-*b*-cyclodextrin (150 mg/ml) (Captisol®, La Jolla, USA), citric acid (2 mg/ml), Tween 20 (1 mg/ml), HCl (5 μl/ml), NaOH (q.s. ad pH 3.5) and water for injection.

2.3. Physico-chemical characterization

The drug concentration was determined by HPLC (Agilent 1100 series, Agilent Technologies, Diegem, Belgium). The samples were eluted with a linear gradient of ammonium acetate 0.2% in water (A) and acetonitrile (B), from 70% (A) (0 min) to 0% (12 min). The reverse phase column was Hypersil BDS RP 18 (10 cm × 4.6 mm). The column temperature was maintained at 30 °C. The flow rate was set at 1.2 ml/min and the detection wavelength was 252 nm. Sample solution was injected at the volume of 10 μl. The HPLC was calibrated with standard solutions of 10–200 μg/ml dissolved in HCl 0.3 N and acetonitrile (correlation coefficient of $R^2 = 0.998$, coefficient of variation <5.1%, LOQ = 10 μg/ml). The Captisol solution, the nanosuspension and polymeric micelles were diluted in acetonitrile. Nanoparticles were also dissolved in acetonitrile and vigorously vortexed to get a clear solution.

The average size of particles and size polydispersity was determined by photon correlation spectroscopy in water, using a Malvern NanoZS (Malvern Instruments, UK).

The zeta potential (ζ) was determined in KCl 1 mM by Laser Doppler Electrophoresis, using a Malvern NanoZS (Malvern Instruments, UK). The measurements were performed in triplicate.

2.4. Maximum tolerated doses (MTD)

The MTD is the dose that induces a median body weight loss of less than 15% of the control and causes neither death due to toxic effects nor remarkable changes in general signs within 1 week after administration [16]. The MTD was studied to determine the highest possible dose administrable without inducing side effects. The 4 formulations were intravenously injected every day for 5 days in the tail vein of NMRI mice. The volume injected was 150 μl, corresponding to the highest injectable volume to mice: Group 1: MTKi-327 nanosuspension at the highest possible dose (16.35 mg/injection); Group 2: the Captisol solution at the highest possible dose (1.6 mg/injection); Group 3: MTKi-327-loaded polymeric micelles at the highest possible dose (0.14 mg/injection) and Group 4: MTKi-327-loaded nanoparticles at the highest

possible dose (0.09 mg/injection). The Captisol solution was also tested at different dilutions (in water). Mice were weighted every day and potential side effects were observed for 10 days post-treatments.

2.5. In vivo anti-tumoral activity

All experiments were performed in compliance with guidelines set by national regulations and were approved by the Ethical Committee for animal care of the Université Catholique de Louvain (Permit number: UCL/MD/2008/025). All tumor implantation was performed under anesthesia and all efforts were made to minimize suffering.

A-431 tumor cells (human epidermoid carcinoma) were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml of penicillin G sodium, 100 μg/ml of streptomycin sulfate and L-glutamine (1%). Cells were maintained in an incubator supplied with 5% CO_2/95% humidified atmosphere at 37 °C. At the beginning of the experiment, 4–5 weeks-old female nude athymic mice weighed approximately 25 g. A solid tumor was established upon subcutaneous injection of 100 μl of A-431 human cancer cells suspension (1×10^7 cells per ml) to the right flank of the mice. Treatments were started when tumor reached a volume of 190 mm^3 (measurement with an electronic caliper).

2.5.1. Formulations at their highest possible dose (intravenous route)

The aim of the first growth delay study was to compare the efficacy of various nanoformulations delivered by the intravenous route. The Captisol solution administered intravenously was not tested because of its low MTD. Because the poor drug loading in nanoparticles, the *in vivo* efficacy of the nanoparticles was not tested in this experiment. The mice were thus divided in 3 groups: Group 1: Untreated (negative control) ($n = 5$); Group 2: MTKi-327 nanosuspension at the highest possible dose (16.35 mg/injection = 650 mg/kg), injected in the tail vein every day for 5 consecutive days ($n = 5$) and Group 3: MTKi-327 loaded-polymeric micelles of at the highest possible dose (0.14 mg/injection = 0.6 mg/kg), injected in the tail vein every day for 5 consecutive days ($n = 5$). The volume of the tumors was measured twice per week with an electronic caliper. The data are expressed as tumor volume normalized to the initial volume.

2.5.2. Influence of the dose, the route of administration and the number of the dose

The aim of the next study was to better assess the effect of MTKi-327 nanosuspension on A431 tumor growth. The influence of (i) the dose (650–150 mg/kg), (ii) the route of administration (intravenous *versus* oral) and (iii) the number of doses (one administration *versus* 5 consecutive administrations) were evaluated. The Captisol solution administered orally (150 mg/kg) was chosen as control group.

(i) The mice were divided in 3 groups: Group 1: untreated ($n = 5$); Group 2: MTKi-327 nanosuspension (650 mg/kg), injected in the tail vein every day for 5 consecutive days ($n = 5$); Group 3: MTKi-327 nanosuspension (150 mg/kg) injected in the tail vein every day for 5 consecutive days ($n = 5$).

(ii) The mice were divided in 3 groups: Group 1: untreated ($n = 5$); Group 2: the Captisol solution (150 mg/kg) administered *per os* for 5 consecutive days ($n = 5$); Group 3: MTKi-327 nanosuspension (150 mg/kg) injected in the tail vein every day for 5 consecutive days ($n = 5$); Group 4: MTKi-327 nanosuspension (150 mg/kg) administered *per os* for 5 consecutive days.

(iii) The mice were divided in 3 groups: Group 1: untreated ($n = 5$); Group 2: MTKi-327 nanosuspension (130 mg/kg), injected in the tail vein every day for 5 consecutive days (cumulative dose = 650 mg/kg) ($n = 5$); Group 3: MTKi-327 nanosuspension (650 mg/kg) injected in the tail vein once ($n = 5$); The volume of the tumors was measured twice per week with an electronic caliper. The data are expressed as tumor volume normalized to the initial volume.

2.6. Pharmacokinetics

The aim of the study was to determine the pharmacokinetics (PK) of MTKi-327 delivered as a nanosuspension or in the Captisol formulation, after intravenous and oral administrations. Treatments were administered on 4–5 weeks-old female nude athymic mice: Group 1: MTKi-327 nanosuspension (3 mg/kg), intravenously, ($n = 5$); Group 2: MTKi-327 nanosuspension (10 mg/kg), *per os*, ($n = 5$); Group 3: Captisol solution, intravenously, MTKi-327 (at the MTD = 3 mg/kg) ($n = 5$); Group 4: Captisol solution, *per os*; MTKi-327 (10 mg/kg) ($n = 5$). At designated times (5, 15, 30 min, 1, 2, 6, 24 h), blood samples were drawn by orbital puncture. No more than three blood samples were drawn per mouse. 300 μl of blood was collected for every time point (yielding a bit more than 100 μl plasma) in Multivette 600K3E, No./REF 15.1671 tubes with EDTA as anticoagulant. Within 2 h, the tubes were centrifuged at 1500*g* at ambient temperature for 10 min and the plasma was transferred into an amber colored container and stored at −20 °C.

Plasma concentrations of MTKi-327 were determined by a validated liquid chromatography–mass spectrometry bioanalytical method (Johnson and Johnson) [9]. In brief, plasma samples were de-proteinized with acetonitrile and the supernatant was analyzed by LC/MS/MS. The HPLC column was a C18-ODS3 column (2 mm × 50 mm, 3 μm particles) (Torrance, CA) maintained at 60 °C with a flow rate of 0.5 ml/min. The mobile phase consisted of 5 mM ammonium acetate pH 3.75 (A) and acetonitrile (B). The initial mobile phase composition was 87.5% A/12.5% B. After sample injection, the mobile phase was changed to 37.5% A/62.5% B over 2 min, and was held at that composition for 1.5 min. The HPLC was interfaced to a Finnigan LCQ Advantage (Thermo Electron Corp, San Jose, CA, USA) ion-trap mass spectrometer operated in the positive ion electrospray and full MS/MS mode. The standard curve ranged from 0.5 to 5000 ng/ml and was fitted with a quadratic regression weighed by reciprocal concentration (1/×). The limit of quantitation (LOQ) for the purposes of this assay was 0.5 ng/ml [17]. The PK parameters gathered were area under the plasma concentration – time curve from time zero and extrapolated to infinity ($AUC_{0-\infty}$) after the dose and calculated over the 24 h dose interval at steady state (AUC_{0-24h}), maximum observed plasma concentrations (C_{max}), time to reach C_{max} (T_{max}) and apparent terminal half-life ($t_{1/2}$).

2.7. Statistics

Results are expressed as mean ± standard error of the mean (SEM). The Kaplan–Meier survival rate and the median survivals were performed using the software Graph Pad Prism 5 for Windows to demonstrate statistical differences. For Figs. 3–5, because the tumor volume measurements were not performed at the same time in the different groups, a "time to reach" graph was chosen for statistical analysis (One-way ANOVA). The pharmacokinetic analysis was performed using PKAA 2.00 (developed by Johnson and Johnson) [9].

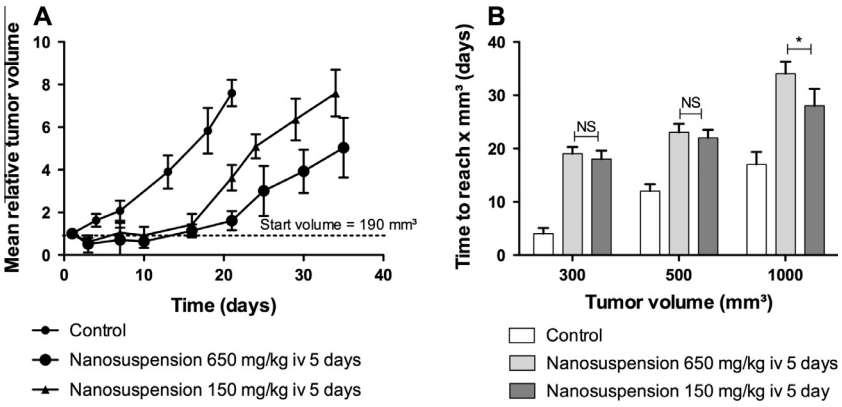

Fig. 3. Anti-tumor effect on A-431-tumor-bearing mice treated with different formulation of MTKi-327. (A) Evolution of the A431 tumor volume in nude mice after intravenous injections for 5 consecutive days of MTKi-327 nanosuspension (650 mg/kg/day) and MTKi-327 nanosuspension (150 mg/kg/day). Each point represents the mean ± SEM. (B) Comparison of time for A-431 tumor to reach 300, 500 or 1000 mm^3 of MTKi-327 formulations. $^{*}p < 0.05$, ns: not significant $p > 0.05$.

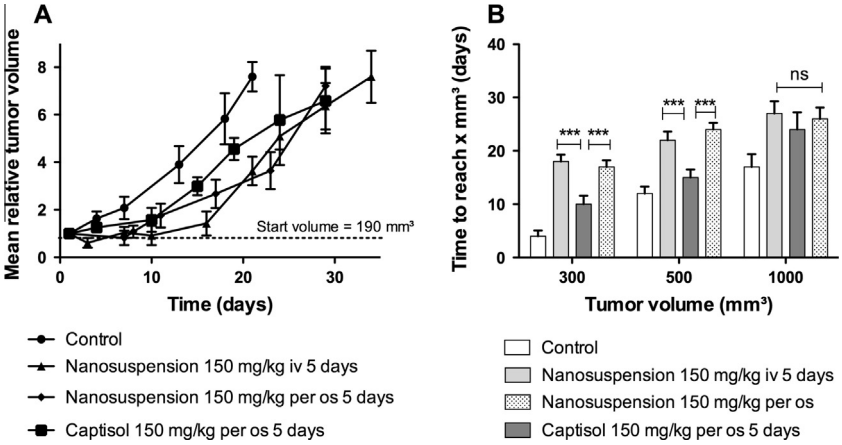

Fig. 4. Anti-tumor effect on A-431-tumor-bearing mice treated with different formulation of MTKi-327. (A) Evolution of the A431 tumor volume in nude mice after intravenous injections for 5 consecutive days of MTKi-327 nanosuspension (150 mg/kg/day), gavage for 5 consecutive days of MTKi-327 nanosuspension (150 mg/kg/day) and gavage for 5 consecutive days of Captisol solution (150 mg/kg/day). Each point represents the mean ± SEM. (B) Comparison of time for A-431 tumor to reach 300, 500 or 1000 mm^3 of MTKi-327 formulations. $^{***}p < 0.001$, ns: not significant $p > 0.05$.

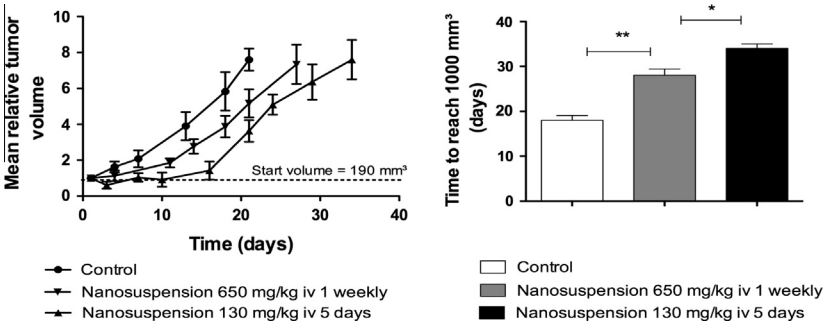

Fig. 5. Anti-tumor effect on A-431-tumor-bearing mice treated with different formulations of MTKi-327. (A) Evolution of the A431 tumor volume in nude mice after intravenous injections for 5 consecutive days of MTKi-327 nanosuspension (130 mg/kg/day) and intravenous injection of MTKi-327 nanosuspension (650 mg/kg/day) in one unique administration. Each point represents the mean ± SEM. (B) Comparison of time for A-431 tumor to reach 1000 mm^3 of nanosuspension of MTKi-327. $^{*}p < 0.05$.

3. Results

3.1. Physico-chemical characterization

Results of the drug concentration, size and ζ potential are summarized in Table 2. All the nanoformulations presented a size below 200 nm, which is adapted for the EPR effect [18]. All the PDI (polydispersity index) were below 0.2, showing a narrow size distribution. The achievable drug concentration varied from 109 mg/ml for the nanosuspension to 0.6 mg/ml for nanoparticles as compared to 10.6 mg/ml for the Captisol solution.

3.2. Maximum tolerated doses (MTD)

The four formulations were injected to mice at their highest possible dose. To determine the MTD of each formulation, treated

Table 2
Physico-chemical characterization of the 4 different formulations of MTKi-327 ($n = 3$).

	Nanosuspension	Polymeric micelles	Nanoparticles	Captisol solution
MTKi-327 amount (mg/ml)	109 ± 6.8	0.92 ± 0.15	0.615 ± 0.8	10.6 ± 2.1
Size (nm)	195 ± 5.6	22.4 ± 0.4	153 ± 6.7	NA
PDI	0.1 ± 0.001	0.036 ± 0.012	0.216 ± 0.05	NA
ζ potential (mV)	−19.6 ± 2.1	4.5 ± 0.35	−36.9 ± 0.55	NA
ζ deviation (mV)	6.1 ± 1.3	18.4 ± 7.1	6.1 ± 5.3	NA

NA: not applicable.

mice were evaluated for 10 days post-treatment. The injections of the highest possible dose of the nanosuspension (16.35 mg/injection), polymeric micelles (0.14 mg/injection) and nanoparticles (0.09 mg/injection) did not induce any side effects or body weight change in NMRI mice. However, the injection of the highest possible dose of the Captisol solution (1.59 mg/injection) induced a severe necrosis of the tail 2 days after the injection. Dilutions 1/3 and 1/10 of the highest possible dose induced a necrosis of tail after 1 and 2 weeks, respectively. Injection of a dilution 1/20 of the highest possible dose (0.08 mg/injection) did not induce any side effects or body weight change (Table 3).

3.3. In vivo anti-tumoral activity

3.3.1. Formulations at their highest possible dose

To compare the efficacy of different formulations of MTKi-327, the *in vivo* anti-tumor efficacy of the nanosuspension and polymeric micelles was evaluated in A-431-tumor-bearing mice (Fig. 2). As the maximum dose which could be injected was too low due to low MTD for the Captisol solution or poor drug loading for the nanoparticles, their *in vivo* anti-tumoral efficacy was not tested. All treatments were administered for 5 consecutive days, intravenously, at their highest possible dose. All treatments were statistically different as compared to untreated mice. Nanosuspension delayed the tumor growth the most efficiently as compared to polymeric micelles ($p < 0.001$). The survival rate (corresponding to the sacrifice of mice when tumors reached a mean relative volume of 10) was significantly higher for mice treated with the nanosuspension (52 days) ($p < 0.001$). Interestingly, micelles, administered with a dose of only 6 mg/kg, presented a higher survival rate (36 days) than untreated mice (21 days) ($p < 0.01$).

3.3.2. Influence of the dose, the route of administration and the number of the dose

Based on the physico-chemical characterization and the *in vivo* anti-tumoral activity, the most effective nanoformulation is the nanosuspension. Hence, we further evaluated this formulation in comparison with the Captisol solution (control) by studying the effect of: (i) the dose, (ii) the route of administration and (iii) the number of doses, on A-431 tumor bearing mice.

Firstly, we compared the dose of MTKi-327 (650 mg/kg *versus* 150 mg/kg) administered intravenously. As expected, the higher dose of MTKi-327 (650 mg/kg) delayed more the growth delay than the lower dose (150 mg/kg). Indeed, the time to reach 1000 mm^3 was longer for MTKi-327 nanosuspension (650 mg/kg) than MTKi-327 nanosuspension (150 mg/kg) ($p < 0.05$) (Fig. 3A and B).

Secondly, we compared the intravenous route *versus* the oral route. Hence, we evaluated the MTKi-327 nanosuspension and the Captisol solution at the same dose (150 mg/kg) intravenously and *per os*, respectively. When compared to the Captisol solution (*per os*), at the same dose (150 mg/kg), nanosuspension (intravenous) delayed more the growth delay within 15 days after treatment. Indeed, the time to reach 300 and 500 mm^3 was longer for nanosuspension than Captisol solution ($p < 0.001$), while at the end of the experiment we did not observe any difference between these two treatments ($p > 0.05$) (Fig. 4A and B).

Finally, a comparison between two schedules of administration (one administration each day for 5 consecutive days *versus* one unique administration) was performed at the same dose (130 mg/kg * 5 *versus* 650 mg/kg). As illustrated in Fig. 5, the administration of MTKi-327 nanosuspension for 5 days was more effective than when administered once (Fig. 5A). Indeed, the time to reach 1000 mm^3 was significantly different between these two groups ($p < 0.05$) (Fig. 5B).

3.4. Pharmacokinetics

The PK profiles of the Captisol solution and the nanosuspension of MTKi-327 were evaluated both intravenously (IV) and orally at the MTKi-327 doses of 3 and 10 mg/kg, respectively. The plasma concentration–time profiles are shown in Fig. 6. Main pharmacokinetics parameters are summarized in Table 4. Higher initial plasma concentrations were observed for mice treated intravenously when compared with orally treated mice. Indeed, the peak plasma concentrations (C_{max}) achieved after oral administration (77.6 and 381 ng/ml for Captisol and nanosuspension, respectively) were significantly lower than those observed after IV administration (940 and 5076 ng/ml for Captisol solution and nanosuspension, respectively). Similarly, the plasma $AUC_{0-\infty}$ was higher after IV administration and the highest value was found for the nanosuspension (1785 h ng/ml). After oral administration, maximum plasma concentrations were observed at 0.3 and 0.8 h (T_{max}) for the nanosuspension and Captisol, respectively. After intravenous administration, plasma levels gradually declined with $t_{1/2}$ of 5.98 and 5.28 h for Captisol and the nanosuspension, respectively. *Per os*, $t_{1/2}$ was increased up to 6.88 and more interestingly 11.76 h for Captisol solution and nanosuspension. The absolute oral bioavailability in the female nude athymic mouse was 6.9% for the Captisol and 7.1% for the nanosuspension.

Table 3
Administrable doses of MTKi-327 of the different formulations and MTD.

	Nanosuspension	Polymeric micelles	Nanoparticles	Captisol
Highest possible dose (mg/ml)	109	0.92	0.6	10.6
Highest possible dose administrable/injection (mg/150 μl)	16.35	0.14	0.09	1.59
MTD (mg/150 μl)	16.35	0.14	0.09	0.08

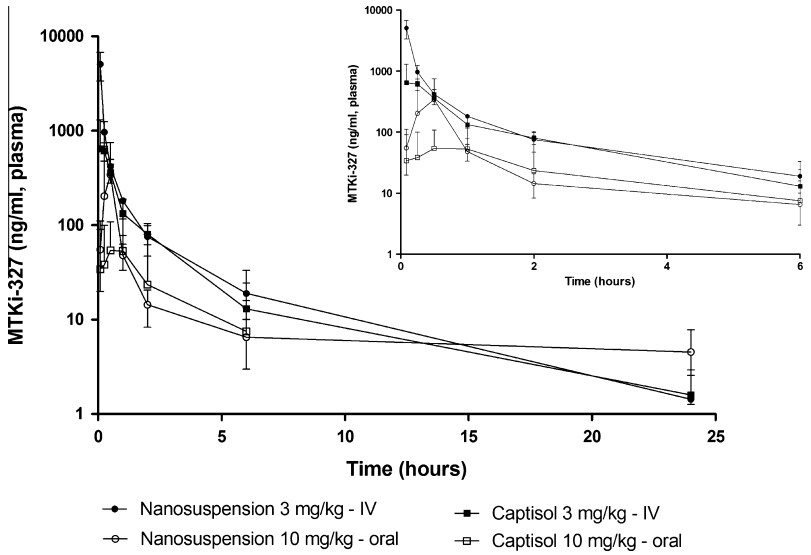

Fig. 6. Plasma profile of MTKi-327 (Captisol solution or nanosuspension of MTKi-327) after oral (MTKi-327 dose 10 mg/kg) or intravenous (MTKi-327 dose 3 mg/kg) administration in female athymic nude mice ($n = 5$).

Table 4
Pharmacokinetics parameters of Captisol solution and the nanosuspension of MTKi-327) after oral (MTKi-327 dose 10 mg/kg) or intravenous (MTKi-327 dose 3 mg/kg) administration in female athymic nude mice ($n = 5$).

Drug	Oral administration (10 mg/kg)		Intravenous administration (3 mg/kg)	
	Captisol	Nanosuspension	Captisol	Nanosuspension
T_{max} (h)	0.8 ± 0.27	0.32 ± 0.18	0.18 ± 0.09	0.08 ± 0.00
C_{max} (ng/ml)	77.6 ± 21.6	381 ± 438	940 ± 393	5076 ± 1709
$t_{1/2,6-24h}$ (h)	6.88 ± 0.52	11.76 ± 2.63	5.98 ± 1.84	5.28 ± 2.02
AUC_{0-24h} (ng h/ml)	170 ± 22.9	323 ± 215	745 ± 193	1771 ± 338
$AUC_{0-\infty}$ (ng h/ml)	175 ± 23.5	428 ± 289	760 ± 190	1785 ± 323
F_{abs} (%)	6.8	7.1		

4. Discussion

We hypothesized that nanosuspensions could be promising for the delivery of poorly soluble anti-cancer drugs multi-targeted kinase inhibitor, MTKi-327, based on (i) easy scaling up of nanosizing techniques and stabilization with GRAS excipients (ii) high drug loading compared to other nanocarriers whose drug loading can be as low as 1% (iii) 200 nm size appropriate for passive targeting in tumor by EPR effect [19]. Hence, we investigated if polymeric biodegradable nanocarriers (micelles and nanoparticles) and nanosuspension could overcome the poor water solubility and enhance the therapeutic index of MTKi-327. The major findings as well as the rationale of the studied formulations for each experiment are summarized in Table 5.

As size reduction is an efficient method to improve the performance of poorly water soluble drugs (the increased surface area of smaller particles enhances dissolution rate and bioavailability based on the Noyes–Whitney equation), Roller Milling was used to obtain a nanosuspsension of MTKi-327 [20]. This procedure is known as an efficient method to prepare nanoparticles with easy scale-up and limited batch-to-batch variability when the method is optimized [1]. Pluronic® F108 and Lipoid S75 were selected as stabilizers because the polymers have already been successfully used to stabilize nanosuspensions [19]. Additionally, Pluronic® is known to have cytotoxicity-promoting properties as it can interact with multi-drug resistance cancer tumors, resulting in drastic sensitization of tumors to cytostatic drugs [21].

To our knowledge, no previous report describes encapsulation of multi-targeted kinase inhibitors in nanocarriers. We demonstrated that MTKi-327 can be efficiently formulated in polymeric micelles, nanoparticles or as nanosuspension. These nanocarriers, with a size below 200 nm – convenient for the EPR effect, can be intravenously injected and can deliver therapeutic doses MTKi-327. Nanosuspension allowed an enhancement of the drug concentration of two orders of magnitude because of its high drug loading when compared to the polymeric micelles or nanoparticles formulations and of one order of magnitude when compared to the control Captisol solution. The low drug loading in the nanoparticles and micelles as well as the targeted dose needed for anti-tumor activity limit their potential use for MTKi-127 delivery.

To determine the concentrations of MTKi-327 for the *in vivo* anti-tumor efficacy, formulations were injected to mice at their highest possible dose, aiming at determination of the MTD of each formulation. For the three nano-formulations, injections of the highest possible dose did not induce any side effects or body weight change in NMRI mice for 10 days. Hence, we concluded that the MTD of each formulation was equal to their highest possible dose. In contrast, for the control Captisol solution (pH 3.5), there was a need to dilute the solution 20-fold to not induce any sign of toxicity, showing that this formulation is not adapted for the intravenous route and confirming the challenge to develop new formulation able to be administered intravenously.

The *in vivo* anti-tumor efficacy was performed on A-431-tumor-bearing nude athymic mice. At their highest possible dose, the nanosuspension delayed the tumor growth the most efficiently when compared to polymeric micelles, probably linked to a dose effect (Fig. 2). Then we compared the dose of MTKi-327 (650 mg/kg *versus* 150 mg/kg) administered intravenously. As expected,

Table 5
Major findings of the study and the rationale of the studied formulations for each experiment.

	Nanoparticles	Micelles	Captisol solution	Nanosuspension
Formulation & Physico-chemical characterization (Table 2)	153 nm -36,9 mV 0,615 mg/ml	22,4 nm 4,5 mV 0,92 mg/ml	NA NA 10,6 mg/ml	195 nm -19,6mV 109 mg/ml
Maximum Tolerated Dose (Table 3)	0,09 mg/150µl (IV)	0,14 mg/150µl (IV)	0,08 mg/150µl (IV)	16,35 mg/150µl (IV)
Anti-tumor efficacy Mean survival rate (Figure 2)		36 days		52 days
Anti-tumor efficacy - Influence of the dose (Figure 3) - IV *versus* oral (Figure 4) - Number of the doses (Figure 5)			NA Oral (150 mg/kg) NA	650 mg/kg IV (150 mg/kg) 5 days
Pharmacokinetics (Figure 6, Table 4)			--	++

the higher dose of MTKi-327 delayed more the growth delay than the lower dose. Nevertheless, this regrowth delay was not drastically enhanced, probably because of a saturation of the response (Fig. 3A and B). Secondly, we compared the intravenous route *versus* the oral route. Hence, we evaluated the MTKi-327 nanosuspension and the Captisol solution at the same dose (150 mg/kg) intravenously and *per os*, respectively. Whatever the formulation or the route of administration, at the dose of 150 mg/kg, no difference was observed (Fig. 4A and B). Finally, because it is possible to administer a higher dose of MTKi-327 with the nanosuspension, we evaluated the influence of the time schedule (administration for 5 consecutive days *versus* one unique administration). As expected, the administration of the nanosuspension for 5 consecutive days was more effective than administered once. Nevertheless, this regrowth delay was not drastically enhanced when compared with the unique administration, also probably because of a saturation of the response (Fig. 5). All these data clearly demonstrate that intravenous and oral delivery of MTKi-127 lead to a significant delay in A431 tumor growth.

The PK parameters indicate that the $AUC_{0-\infty}$ of the nanosuspension was 2.4-fold higher than the $AUC_{0-\infty}$ of the Capstisol solution both after intravenous and oral delivery. After intravenous administration, the nanosuspension PK profile exhibited a 5.4-fold higher C_{max} for the MTKi-327 nanosuspension compared with the Captisol solution. Nevertheless, the $t_{1/2}$ was similar. The *in vivo* properties of a nanosuspension formulation strongly depend on the nanoparticle size, dissolution rate and nature and density of the coating. Particles that dissolve rapidly in the blood would be expected to exhibit similar pharmacokinetic behavior to a drug in solution form [22]. However, surprisingly, the C_{max} for the MTKi-327 nanosuspension was higher than for the Captisol solution. This might be explained by a putative precipitation of the MTKi-327 occurring directly after the intravenous injection because of the dilution and the higher pH of the blood (pH = 7.4) [23]. Biodistribution studies will be needed to check if the nanosupension accumulates in the tumor by EPR effect and if a rapid uptake of rather slowly dissolving nanoparticles by the reticulo-endothelial system (RES) can be avoided. Previous studies have shown that slow dissolving nanocrystals were taken up by the phagocytic cells of the RES, mainly the Kupffer cells in the liver, spleen and lung [24–26]. After oral administration, the C_{max} and $AUC_{0-\infty}$ of MTKi-327 nanosuspension was 4.9 and 2.4-fold greater than those of the Captisol solution. Additionally, the $t_{1/2}$ was also enhanced (1.7-fold). For oral administration, the ability to increase the rate and extent of dissolution in gastric/intestinal fluid is the main driver for the enhanced bioavailability of compounds formulated as nanosuspensions. The increased surface area which elicits rapid dissolution irrespective of the presence or absence of food, can eliminate and/or greatly reduce variations in bioavailability typically encountered with more conventional non-nanosized formulations of poorly water soluble compounds [23]. The higher C_{max} and $t_{1/2}$ of the nanosuspension should also be explained by the fact that small nanometer sized particles can embed themselves within the vast microvillus borders of the epithelium [23]. The nanosuspension maintained thus higher plasma concentration for a longer period of time, suggesting that the nanosuspension is also convenient for oral administration of MTKi-327.

5. Conclusion

The multi-targeted kinase inhibitor MTKi-327 was incorporated in three nano-formulations: polymeric micelles, nanoparticles and nanosuspension. These nanocarriers, with a size below 200 nm – adapted for the EPR effect, can be intravenously injected and can deliver therapeutic doses MTKI-327. It was demonstrated that the highest regrowth delay of A-431-tumor-bearing nude mice was obtained with the MTKi-327 nanosuspension administered intravenously at a dose of 650 mg/kg. Nanosuspension may thus be considered as an effective anti-cancer MTKi-327 delivery system due to (i) the higher MTKi-327 MTD, (ii) the possible intravenous injection of MTKi-327 for potential targeting by EPR effect, (iii) its ability to enhance the administered dose and (iv) its higher efficacy.

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