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Safety assessment in rats and dogs of Acoustic Cluster Therapy, a novel concept for ultrasound mediated, targeted drug delivery

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Abstract

Acoustic Cluster Therapy (ACT) represents a novel concept for targeted drug delivery. Ultrasound is applied to activate intravenously administered free-flowing clusters of microbubbles and microdroplets within the target pathology, depositing 20–30 μm large bubbles in the microvasculature for 5–10 min. Further application of ultrasound induces biomechanical effects which increase vascular permeability and enhance localized extravasation of coadministered drugs. Herein we report investigations done to assess the preclinical safety of ACT, using doses up to 1 mL/kg (3 μL perfluoromethyl-cyclopentane/kg). In dogs, half the animals were exposed to ultrasound activation in the heart for 1 min, no ultrasound was applied in the other half. Posttreatment observation time was 24 h. Clinical signs, ophthalmoscopy, clinical pathology, macro-, and microscopy were used as endpoints. No differences between groups with and without ultrasound activation were observed. Short-lasting leukopenia and thrombocytopenia, possibly secondary to a slight and short-lasting increase in plasma histamine and complement split products, were the only effects noted. In rats ACT was activated in the liver for 5 min. Histopathology and clinical chemistry parameters remained unchanged. Lastly, rats were treated with ACT activated in the heart and thereafter placed on a rotarod for evaluation of motor coordination. No differences were observed between animals treated with ACT and controls. In conclusion, ACT appeared safe at dose-levels up to 1 mL/kg and with activation either in the heart or the liver. These results, together with positive efficacy data upon coinjection with cytotoxic compounds encourage further preclinical safety studies with the objective of entering subsequent clinical trials.

Abbreviations

ACT, Acoustic Cluster Therapy; APTT, activated partial thromboplastin time; DSPC, distearoylphosphateidyl-choline; GC-MS, gas chromatography-mass spectrometry; GLP, Good Laboratory Practice; HEPS–Na, hydrogenated egg phosphatidylserine–sodium; PDAC, pancreatic ductal adeno-carcinoma; PFB, perfluorobutane; PF-MCP, perfluoromethyl-cyclopentane; PT, prothrombin time; TT, thrombin time.

Introduction and Background

A prerequisite for successful therapy with a medicinal drug is that the active substance reaches its target pathology and that toxicity to healthy tissue and nontargeted organs is limited. This fundamental necessity limits the effective use of a large number of drug molecules with low therapeutic index, and has led to a massive hunt for concepts that enable localized release or enhanced uptake of active ingredients *within* the target pathology.

A wide variety of approaches for localized drug delivery or enhanced uptake have been explored over the last couple of decades (Devarajan and Jain 2015), but transition to drug products and clinical practice has been very limited. In truth, the idea of targeted drug delivery currently remains essentially unresolved in medical practice. In recent years, several concepts for Ultrasound (US) mediated drug delivery have been investigated, some with quite encouraging results (Tsusui et al. 2004; Martin and Dayton 2013; Unga and Hashida 2014). Most of the these concepts explore the use of regular US contrast microbubbles such as Sonovue™ or Optison™, either loaded with or coinjected with various active ingredients. US insonation of the target pathology containing microbubbles in vascular compartments leads to a variety of biomechanical effects that enhance extravasation, distribution to and uptake of drug molecules in target tissue (Kooiman et al. 2014; Lentacker et al. 2014). Coinjection of Gemcitabine and Sonovue, with localized US insonation for enhanced drug uptake and therapeutic effect during treatment of pancreatic ductal adenocarcinoma is currently being explored in clinical trials (Kotopoulis et al. 2013). A similar approach is being investigated for treatment of Glioblastoma in humans (Assistance Publique - Hôpitaux de Paris, 2014; InSightec, 2014). Whereas various drug delivery approaches exploring the use of contrast microbubbles have shown quite some promise, their effectiveness is hampered by several issues. Being just a few microns in diameter, the level of biomechanical work contrast microbubbles can induce is limited. In addition, they are free flowing and hence display limited contact with the endothelial wall, reducing the level and range of the biomechanical effects. Furthermore, contrast microbubbles are typically cleared from the vascular compartment in 2–3 min, limiting the effective time window and finally, these approaches typically utilize Mechanical Indices MI) that induce inertial cavitation, with ensuing potential safety aspects.

Recently, a novel approach for US mediated, targeted drug delivery; Acoustic Cluster Therapy (ACT), has been suggested (Sontum et al. 2015; Healey et al. 2016; Van Wamel et al. 2016; Van et al. 2016). Acoustic Cluster Therapy explores similar mechanism as with regular microbubbles, but addresses important shortcomings of the latter. Details and attributes of the ACT formulation concept are described in (Sontum et al. 2015). In brief, the approach comprises coadministration of a drug together with a dispersion of microbubble/microdroplet clusters, followed by a two-step, local US activation and enhancement procedure. US activation induces a liquid-to-gas phase shift of the microdroplet component and the formation of a large (~25 μm) ACT bubble that transiently (5–10 min) lodge in the targeted microvasculature, occluding blood flow. The subsequent US enhancement step induces controlled volume oscillations of the activated bubbles, which in turn induce biomechanical effects that enhance the local permeability of the vasculature, allowing for improved extravasation and distribution of drug into the tumor tissue extracellular matrix. The nature of these biomechanical effects is not completely understood. However, the oscillating bubbles exert direct forces on the endothelial cells, creating deformations, increasing or creating fenestrations, and potentially affect the morphology and fluid dynamics in the interstitium. In addition, strong shear force fields are created which increase the convection of fluid in the vascular compartment, and may lead to enhancement of various transcellular uptake mechanisms. The ACT concept represents an unprecedented approach to targeted drug delivery that may improve significantly the efficacy of, for example, current chemotherapy regimen.

In previous papers, we have described the basics of the ACT formulation and concept (Sontum et al. 2015) and provided proof of principle for targeted, tumor-specific uptake (Van Wamel et al. 2016). We have also demonstrated proof of concept for this new treatment strategy by demonstrating a very strong increase in the therapeutic efficacy of paclitaxel and nab-paclitaxel (Abraxane®), when combining these with ACT for treatment of human prostate adenocarcinoma in mice (Van et al. 2016). Furthermore, we have studied the size, number, and dynamics of the activated/deposited bubbles in a dog myocardial model. In (Healey et al. 2016), we have demonstrated that the volume median diameter of the activated bubbles in vivo is ~20 to 22 μm with 91% being between 10 and 25 μm. At a dose of 100 μL ACT/kg bodyweight, the number of deposited bubbles was estimated to ~40.000 per mL myocardial tissue. In (Van Wamel et al. 2016) the typical residence time of the activated bubbles in a prostate adenocarcinoma tumor has been demonstrated to be ~5 to 10 min.

Acoustic Cluster Therapy has very clear theranostic attributes; the activated bubbles display copious US backscatter in regular B-mode imaging and are easily detected as very bright, stationary echoes in the image. They can be clearly differentiated from regular contrast

microbubbles, which in B-mode appears as rather weak, flickering echoes, and are depleted completely within 1–2 min from injection (Healey et al. 2016; Van Wamel et al. 2016). US imaging has hence been used to confirm activation and deposition of large activated bubbles during the studies presented.

In many respects, ACT represents a novel class of drugs with a strictly mechanical mechanism of action; it contains no active ingredient in the classical definition of the term. With exception of a minute amount of stearylamine, the constituents are perfluorated compounds that should be completely biologically inert, stabilized with endogenous phospholipids. The strictly *chemical* toxicity potential of the ACT formulation should hence be quite low. However, the formulation is presented as a dispersion of particles which upon intravenous administration potentially could cause biological effects irrespective of their chemical composition. Circulating particles are generally cleared from the blood stream by opsonization followed by phagocytosis by cells being part of the mononuclear phagocyte system, also known as the reticuloendothelial system, and this has been shown to be the case for several gas-filled microbubbles used as ultrasound contrast agents (Yanagisawa et al. 2007). This process may activate several biological systems and release mediators that could have implications for the safety of using a product containing particulate matters. In addition, in its very nature, ACT induces a local and transient occlusion of a fraction of the microcapillaries, and it exerts mechanical stress on surrounding tissue, both attributes which could potentially cause further unintended effects.

The objective of the investigations reported in this paper was to assess the general acute toxicity of the constituents in the ACT formulation. In addition, experiments were designed to detect potential effects related to the in situ generation of large bubbles, transiently occluding blood flow at the microcapillary level, in the presence of an applied US field.

Materials and Methods

Test and control items

The ACT test item was prepared by reconstituting a lyophilized composition of negatively charged microbubbles with an emulsion of positively charged microdroplets. The microbubble component explored in this paper consisted of Sonazoid™, a US contrast agent manufactured by GE Healthcare (Sontum 2008). Sonazoid comprises perfluorobutane (PFB) (F2 Chemicals Ltd., Lea Town, UK) microbubbles stabilized with a hydrogenated egg phosphatidylserine–sodium (HEPS–Na) (NOF Corp., Tokyo, Japan) phospholipid membrane, embedded in a lyophilized sucrose matrix. After reconstitution, Sonazoid contains ~8 μL PFB or 11×10^8 microbubbles/mL, with a number weighted mean diameter of ~2.0 μm. The microbubbles in Sonazoid display a negatively charged surface, with a zeta potential of ~−70 mV. The microdroplet composition consisted of perfluoromethyl-cyclopentane (PF-MCP) (F2 Chemicals Ltd.) microdroplets stabilized with a distearoylphosphateidyl-choline (DSPC) (Lipoid AG, Steinhausen, Switzerland) phospholipid membrane added 3% (mol/mol) stearylamine (SA) (Sigma-Aldrich, St. Louis, MO, USA), a positively charged surfactant. The emulsion component contains ~3 μL PF-MCP or 6×10^8 microdroplets/mL with a number weighted mean diameter of ~1.8 μm. The microdroplets display a positively charged surface, with a zeta potential of ~+30 mV.

Upon reconstitution of Sonazoid™ with the emulsion component, small microbubble/microdroplet conjugates ("clusters") are formed. The final ACT test item contains $\sim 1 \times 10^8$ clusters/mL with a median diameter of ~5 μm, in addition to a significant fraction of non-clustered Sonazoid™ microbubbles. The concentrations of the chemical constituents are ~8 μL PFB/ml, 3 μL PF-MCP/mL, 150 μg DSPC/mL, 70 μg HEPS-Na/mL, and 1.5 μg SA/mL.

The control item used was either 0.9% physiological saline (Sodium chloride 9 mg/mL), supplied by Fresenius Kabi, or mannitol (50 mg/mL with 5 mmol/L Tris-buffer), supplied by Amersham Health (Amersham, UK).

General safety assessment in the dog with ultrasound exposure to the heart

This investigation was done to obtain general safety data, including clinical pathology and histopathology, for ACT with and without ultrasound activation in the heart using a nonrodent animal species. Activation in the left ventricle assured maximal activation of the administered dose and that the large activated bubbles were fed into the aorta and hence causing exposure of the entire systemic circulation. The study was performed in compliance with the Good Laboratory Practice (GLP) regulations and the use and care of the animals had been approved by the institutional animal ethics committee prior to the start of the experiments.

Thirty-two male Beagle dogs from Harlan Winkelmann GmbH, Germany were used. Only males were included since no sex differences were expected. A pretreatment period of 19–22 days, including an acclimatization period of 5 days, was allowed during which the animals were observed daily in order to reject animals in poor condition. At the start of the acclimatization period the animals were ~8 months old and in the weight range 9.8–16.3 kg.

On the day of arrival, the animals were allocated to eight groups of four animals per group, using a randomization scheme. Prior to commencement of treatment, the animals were reallocated in order to distribute litter-mates evenly between groups and in order to reduce intergroup mean body weight differences. Each animal was identified from the supplier by an individual number tattooed in the pinna of one ear. In addition, they carried an individual number plate on a neck collar chain. Each pen was identified by a color coded card marked with study number, group number, and animal number.

The animals were housed individually in floor pens (1.2 m^2) with sawdust "Lignocel 3–4" from J. Rettenmaier & Sohne GmbH, Rosenberg, Germany as bedding, in rooms with filtered air at a temperature of 21°C ± 3°C and a relative humidity of 55 ± 15%. Room air was changed 10 times per hour and the room was illuminated to give a cycle of 12 h light and 12 h darkness. Light was on from 6 AM to 6 PM.

A commercially available diet (Teklad 2021 from Harlan CPB, Postbus 167, NL-3700 AD Zeist, The Netherlands) was offered once daily in an amount of 450 g per meal. The animals had ad libitum access to domestic quality drinking water in beakers. The water was renewed twice daily.

Each day the animals were allowed to exercise in indoor pens.

Dosing and ultrasound exposure

The dose was given intravenously by injection in the left foreleg and the dose and dose volume were calculated according to the most recent body weights. The injections were performed using Venflon (20 G, BOC Ohmeda AB Helsingborg, Sweden) at a nominal speed of 0.2 mL/sec. After injection of the test article the Venflon was flushed with 1.5 mL physiological saline. The treatment in each of the dose groups is specified in Table 1.

Ultrasound exposure of the heart was performed in groups 5 to 8 by a Vivid 3 GE Medical ultrasound machine (GE Healthcare, Milwaukee, WI,USA) equipped with a 2.2–5.0 MHz transducer. The scanner was operated in fundamental B-mode at 3.3 MHz with mechanical index (MI) at ~1 and a frame rate of ~25 MHz. In order to maximize activation of the ACT clusters, a high, short axis, left-sided parasternal view was used, with focus in the middle of the left ventricle. The image depth was adjusted to the smallest value that still covered the whole heart. The exposure and the presence of deposited ACT bubbles in the myocardium after activation were documented by ultrasound imaging.

For each animal in groups 5 to 8 the imaging procedure was started shortly before injection of the ACT product. The animal was shaved on the left side of the body at the heart region and acoustic coupling gel was applied to facilitate the imaging procedure. The heart was imaged continuously before dosing, during dosing and for 1 min after completion of dosing. After a time interval (~3 min) to allow the free Sonazoid™ microbubbles to be washed from the blood pool, ultrasound was reapplied to the heart to assess deposit and retention of activated ACT bubbles in the myocardium.

Study observations and measurements

All animals were weighed on arrival, on Day -12, 1 week before start of dosing (Day -7), on the day of reallocation,

Table 1. Overview of experiments and design of the Acoustic Cluster Therapy (ACT) safety studies in dogs and rats.

Study type	Group no.	No. of animals (sex)	Test item	Dose (mL/kg)	Ultrasound activation
Toxicity in dogs, ACT activation in the heart	1	4 (m)	Saline	1.0	No
	2	4 (m)	ACT	0.1	No
	3	4 (m)	ACT	0.3	No
	4	4 (m)	ACT	1.0	No
	5	4 (m)	Saline	1.0	Yes
	6	4 (m)	ACT	0.1	Yes
	7	4 (m)	ACT	0.3	Yes
	8	4 (m)	ACT	1.0	Yes
Toxicity in rats, ACT activation in the liver	1	4 (m)	Saline	1.0	Yes
	2	4 (m)	ACT	0.1	Yes
	3	4 (m)	ACT	0.3	Yes
	4	4 (m)	ACT	1.0	Yes
Motor coordination test in rats, ACT activation in the heart	1	10 (m)	Mannitol	1.0	Yes
	2	10 (m)	ACT	0.1	Yes
	3	10 (m)	ACT	0.3	Yes
	4	10 (m)	Chlorpromazine	5 mg/kg	No

on the day of treatment (Day 1) and on the day of necropsy (Day 2). From Day -7, food consumption was estimated daily for each animal by weighing unconsumed diet.

All visible signs of ill health and any behavioral changes were recorded daily. Any deviation from normal was recorded with respect to time of onset, duration, and intensity. The first 3 h after dosing the animals were closely watched for signs of altered behavior.

An ophthalmoscopic examination was performed predose, ~10 min after treatment (to check for circulation disturbances) and just before necropsy.

Blood samples were taken into Vacutainers for clinical pathology analyses on Day 1 (the day of treatment) at the following time points: predose, ~5, 40, and 120 min after end of dosing and shortly before necropsy on Day 2. Blood samples were drawn from the jugular vein. Three mL EDTA-stabilized blood was taken for hematology and 2 mL citrate stabilized blood for coagulation tests. Twenty milliliter blood was taken with micropipette with dry Na-heparin for the blood glucose analysis. Three ml samples were taken into plain glass tubes to prepare serum for clinical chemistry analysis. Samples for hematology, coagulation, and glucose analysis were analyzed fresh while the samples for clinical chemistry were frozen before analysis due to the large number of samples. The following clinical chemical parameters were analyzed in serum by a Hitachi 902 clinical chemical analyzer: Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, total cholesterol, triglycerides, blood urea nitrogen, creatinine, sodium, potassium, calcium, inorganic phosphorus, chloride, total protein, albumin, and albumin/globulin ratio. Glucose was analyzed in whole blood by a Cobas Mira analyzer.

The hematology parameters were analyzed by an ABX Pentra 120 analyzer and included hemoglobin, red blood cell count, hematocrit, mean cell volume, mean cell hemoglobin, white blood cell count, differential white blood cell count (neutrophils, lymphocytes, eosinophils, basophils, monocytes), and platelet count. Coagulation parameters included activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT) and were analyzed by ILTest™/ACL™ (Instrumentation Laboratories).

A minimum of 2 mL blood was collected into EDTA Vacutainer tubes on Day 1 predose and at 2, 5, and 10 min after end of dosing for measurement of histamine. Another sample of minimum 2 mL blood was collected into EDTA Vacutainer tubes on Day 1 predose and at 10 and 120 min after end of dosing for measurement of complement split products. Only plastic equipment was used after blood collection for both histamine and complement split products. Gloves were worn during sampling handling to avoid contamination. Hemolysis during sample collection and processing was avoided. The blood samples were kept at +2°C to +8°C until centrifugation, which took place within 20 min after the blood sampling. The blood samples were centrifuged at 1500***g*** for 10 to 15 min at +2°C to +8°C. The plasma was carefully collected without approaching the white cell layer and transferred to prechilled, appropriately labeled plastic storage cryo-tubes. Each tube contained ~500 μL plasma. The samples were frozen at ~−80°C immediately after collection and until analysis. Histamine was analyzed by enzyme immunoassay and complement split products by electrophoresis and immunoblotting.

Blood samples were collected in 3 mL Venoject tubes containing heparin at predose and 2, 5, 10, 20, 40, 60, and 180 min after end of dosing, and at necropsy on Day 2, for determination of pharmacokinetic parameters for PFB and PF-MCP. At each time point, duplicate aliquots of ~1.0 mL heparinized blood were transferred from the Venojects to tared, capped 20 mL headspace vials containing 6.0 mL 1.5N KOH in 30% ethanol. Before use, the vials were stored at 2–8°C. The vials were weighed to determine the weight of blood dispensed to each vial. Samples were stored in the refrigerator. Samples were analyzed for determination of concentrations of PFB and PF-MCP using a gas chromatography-mass spectrometry (GC-MS) assay (Hvattum et al. 2001). This assay was originally developed for PFB only but is also applicable for determination of PF-MCP concentrations. A minimum of five samples per animal were analyzed. The maximum observed plasma concentration (C_{max}), the area under the concentration-versus-time curve from zero to the last time-point where the compound was detected (AUC, last) estimated by the trapezoidal rule and elimination half-lives calculated by the formula $t/2 = \ln 2/k$ (k is the estimated slope of the terminal decline of the log drug concentration versus linear time curve, assessed by linear regression) were characterized for each dose using a validated software program, Pharm-NCA (Innaphase, Philadelphia, PA, USA).

On Day 2, that is, 24 h after administration and ultrasound exposure, the animals were weighed, examined externally and anesthetized by an intravenous injection of Mebumal® containing lidokain as a local anesthetic. The injection was not performed at the site where the animals were treated with the test article as this site was sampled for microscopic analysis. The animals were killed by exsanguination during anesthesia and were not fasted prior to necropsy. A macroscopic examination was performed on all animals after opening the cranial, thoracic, and abdominal cavities and by observing the appearance of the organs and tissues in situ. Any macroscopic change was recorded with details of the location, color, shape, and size.

The following organs were excised and weighed: Adrenals, brain, heart with aortic arch, kidneys, liver, pituitary, prostate, spleen, testes, thymus, and thyroids including parathyroids. These organs and in addition the following organs were initially fixed in phosphate-buffered neutral 4% formaldehyde with the exception of the eyes (Davidson'S fixative) and testes (Bouin's fixative): Thoracic aorta, femur including bone marrow, the knee joint with bone, epididymides, eyes with lens and optic nerve, gall bladder, tongue, sublingual and submandibular salivary gland, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, larynx, trachea, cranial and caudal lobes of both sides of the lungs, right mandibular and mesenteric lymph nodes, mammary gland, pancreas, right parotid, sciatic nerve, skeletal muscle, thoracic and lumbar spinal cord, sternum (for collection of bone marrow), and urinary bladder. The injection site and any lesion observed during necropsy were also excised and fixed. Seven serial sections of the brain were prepared.

The fixative for long-term preservation of all tissues was phosphate-buffered, neutral 4% formaldehyde. The lungs were infused with fixative at necropsy. Either whole organs or selected samples of the indicated organs and tissues were subjected to these procedures. Paired organs were weighed together. The relative organ weights, that is, the organ weight as a percentage of the body weight, were calculated for each animal.

Samples from all animals were trimmed and representative specimens were taken for histological processing. The specimens were embedded in paraffin and cut at a nominal thickness of 5 μm, stained with hematoxylin and eosin, and examined by light microscopy. Histological alterations were graded on a five grade system: Grade 1 – Minimal/Very few/Very small; Grade 2 -Slight/Few/Small; Grade 3 -Moderate/Moderate number/Moderate size; Grade 4 -Marked/Many/Large; Grade 5 -Massive/Extensive number/Extensive size. If appropriate, a finding could also be scored as Present - Finding present/Severity not scored.

All organs and tissues from control (groups 1 and 5) and high dose (groups 4 and 8) animals were examined microscopically. In addition, brain (seven serial slices), eyes, heart, liver, ileum, colon, cecum, kidneys, and lungs of the low and mid-dose groups (groups 2, 3, 6, and 7) were examined histologically.

Data analyses and statistics

Data were processed to give group mean values and standard deviations where appropriate. Thereafter, each continuous variable was tested for homogeneity of variance with Bartlett's test. As significant differences were detected, possible intergroup differences were assessed with Dunnett's test. If the variance was heterogeneous, each variable was tested for normality by the Shapiro–Wilk method. In case of normal distribution, possible intergroup differences were identified with Student's t-test. Otherwise the possible intergroup differences were assessed by Kruskal–Wallis's test. If any significant intergroup differences were detected, the subsequent identification of the groups was carried out with Wilcoxon Rank-Sum test. For all tests, the level of significance was defined as $P < 0.05$.

The statistical analysis on body weight, hematology, clinical chemistry, and organ weight data was performed by comparing group 1 (control) with groups 2, 3, and 4 (all groups without ultrasound activation) and by comparing group 5 (control) with groups 6, 7, and 8 (all groups with ultrasound activation).

Furthermore, a statistical analysis on white blood cell count and platelet count was performed where the effect of the ultrasound activation was assessed *(i.e.,* comparison of group 1, predose, with group 5, predose, etc.).

The above statistical analyses were done with the SAS software (SAS Institute Inc., Cary, NC) and StatXact procedures described in StatXact 5 for Windows (Cytel Software Corporation, Cambridge, MA).

Sigmastat v. 2.0 (Jandel Corporation, San Rafael, CA, USA) was used to perform a Student's *t*-test to reveal any statistical differences in pharmacokinetic parameters between groups with ultrasound activation and those without.

Studies in the rat

Two investigations were performed in rats in order to study safety aspects different to those addressed in the dog. One studied the safety profile of ACT following activation in the liver while the other addressed effects on motor coordination after activation of ACT in the blood pool of the heart, a situation where enlarged microbubbles potentially could be transported by arterial blood to the brain. All care and use of the animals were in compliance with the institutional regulations for animal ethics.

The strain of rats used in these investigations were Sprague–Dawley (Hsd:SD) obtained from Harlan in the Netherlands or the UK. Upon arrival the animals were randomly allocated to cages and a numbered ear-tag was fixed in one ear to uniquely identify each individual animal. They were kept in polycarbonate cages type IV with Beekay laboratory bedding from B&K Universal AS, Nittedal, Norway. The cages with bedding were changed every other day but on the day of treatment the animals were placed in new cages with fresh bedding. The animals were allowed minimum 5 days of acclimatization prior to study start. On the day before treatment commenced the animals in each cage were weighed and the cages assigned

to groups so that the average animal weight was approximately the same in all groups. Animals within one cage belonged to the same group and received the same treatment.

Throughout the experiments the rats were kept in rooms with temperature of 21 ± 2°C, humidity 55 ± 10%, and 12 h dark/light cycle in phase with natural daylight. Ventilation provided ~20 air-changes per hour.

An Acuson Sequoia 512 ultrasound machine was used for exposing the animals to ultrasound in order to activate the microbubbles, and for imaging purposes. The machine was equipped with a 15L8 linear array probe, operated in B-mode with mechanical index (MI) at ~1, lowest frequency 8 MHz, frame rate ~25 MHz (maximum obtainable), dynamic range 40–60 dB and depth and focus at ~20 and 10 mm, respectively. A water-based acoustic contact gel was used between the transducer and the skin of the rats.

Safety assessment in the rat with ultrasound exposure to the liver

Twenty male Sprague–Dawley rats (Hsd:SD) were purchased. Upon arrival the animals weighed between 350 and 400 g, when treatment started the bodyweights were between 378 and 430 g. They were fed a 4012.02 reference diet from Hopes farm, Woerden, The Netherlands, containing 5% cellulose and 10% starch. Municipally tap water was supplied ad libitum.

Prior to dosing the animals were weighed and then anesthetized with isoflurane using the FluoSafe® Induction chamber and the abdomen clipped free of hair and shaved. A single injection of either saline or ACT was administered as a bolus (0.1 mL/sec) into a lateral tail vein according to the details given in Table 1. Ultrasound exposure was done for about 5 min postdosing by moving the probe back and forth to cover the entire liver.

The rats were euthanized 24 h after administration of the control and test items by an overdose of thiopental given intravenously.

Study observations and measurement

Animals were observed daily during the acclimatization period; very closely the first 2–4 h after dosing and ultrasound exposure, and any signs of morbidity or altered behavior were recorded. The condition of the rats was also assessed prior to euthanasia on Day 2.

Body weights were recorded during pretreatment, on the day of dosing and again prior to euthanasia on Day 2. Due to the short posttreatment period, food consumption was not recorded.

A 0.5 mL blood sample was collected from a tail vein predose and just prior to necropsy at Day 2. Serum was prepared after clotting and the following clinical chemical parameters were analyzed on a Beckman Synchron CX5 clinical chemical analyzer (Beckman Coulter Inc., Brea, CA, USA) at 37°C: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatine kinase, 5'nucleotidase, glutamate dehydrogenase, total bilirubin, triglycerides, cholesterol, urea, inorganic phosphate, calcium, total protein, and albumin.

At necropsy on Day 2, that is, 24-h after dosing and ultrasound exposure, the animals were opened by a midventral incision of the abdomen and chest wall. The liver was weighed and the liver, ileum, cecum, and colon were examined thoroughly for grossly visible changes and then taken out and fixed in 10% neutral-buffered formalin. After fixation the liver samples were processed into paraffin wax, sectioned at nominal thickness of 5 μm, stained with hematoxylin and eosin and examined by light microscopy.

Data analysis and statistics

Statistical analysis, using the LabCat system, compared the animals given ACT and exposed to ultrasound with the animals given saline and exposed to ultrasound. Dunnett's test with ANOVA was applied and level of significance was defined as $P < 0.05$.

Assessment of effects of ACT on motor coordination in the rat after ultrasound exposure to the heart

The assessment was done by placing the animals on a rotarod treadmill device designed to test locomotor coordination in rats.

A total of 80 male Sprague–Dawley rats (Hsd:SD) were purchased from Harlan, UK. Upon arrival, a 4012.02 reference diet from Hopes farm, Woerden, The Netherlands, containing 5% cellulose and 10% starch, and municipally tap water were supplied ad libitum.

When treatment started the rats weighed 170–219 g. The animals were to be tested on a rotarod treadmill (San Diego Instruments Inc., San Diego, CA, USA) after injection of the control or test item and exposure to ultrasound (Table 1). Therefore, within 5 days before testing, the animals were familiarized with the rotarod by doing three training sessions separated by 2–3 h. The rotarod was set to accelerate from 0 to 30 rounds per minute (rpm) during 2 min and the time spent on the rotarod was recorded. The time for each animal was limited to 180 sec. Rats performing poorly were excluded from the test and the test continued until 40 suitable rats were identified. These animals were randomly allocated to

the four test groups with 10 animals in each group, in a way securing that the mean bodyweights were equal in all groups. On the day before dosing, the ventral thorax of the animals in groups 1–3 was clipped free of hair and shaved to allow ultrasound exposure to the heart.

On the day of testing the animals were placed in the test room at least 1 h before the test commenced. Mannitol was given to group 1 as vehicle control and ACT to groups 2 (0.1 mL/kg) and three (0.3 mL/kg) by intravenous bolus injections, and all these animals were exposed to ultrasound to the heart during and for 60 sec after the injection. Activation in the heart, including the left ventricle, was applied to create a situation with a potential for large, activated bubbles to move with the blood stream to the brain. Immediately after completion of ultrasound the rats were placed on the rotarod for the first testing period of 180 sec. During the test the rotarod was set to accelerate from 0 to 30 rpm during 2 min and the time spent on the rotarod was recorded for each animal. After a time interval of ~3 min after the first rotarod test, ultrasound was reapplied to the heart to assess retention of microbubbles in the myocardium. The rats were retested on the rotarod 60 min after dosing.

Chlorpromazine, a commonly used positive control substance in rotarod experiments due to its sedative effects (Jones and Roberts 1968), was given subcutaneously at a dose of 5 mg/kg. The rats in the chlorpromazine group (group 4) were not exposed to ultrasound. They were placed on the rotarod 10 min after injection.

Due to the short period the animals were being tested in this study, no other specific parameters were recorded. After the end of the second rotarod test, performed 60 min postdosing, the animals were euthanized by an overdose of pentothal-Na solution.

Data analysis and statistics

Statistical analyses were done by SigmaStat v. 2.0. First, the distribution of each dataset was assessed for homogeneity of variance using the Levene Median test, and for normality using the Kolmogorov–Smirnov Test with Lilliefors correction. The variance test indicated homogeneity ($P \geq 0.05$), and a one-way analysis of variance (ANOVA) was performed. A post hoc Dunnett's test was used to identify any differences between reference and each treatment group.

Results

General safety assessment in the dog with ultrasound exposure to the heart

All dogs were considered healthy during the pretreatment period and upon entering the experimental phase of the study.

Ultrasound imaging of the heart was performed in all animals in groups 5 to 8. In all treated groups (groups 6 to 8) the ACT test item was easily visible as a white cloud reaching the heart within seconds after start of injection. In the high-dose group (group 8) the cloud was so intensive that the image became unclear. At the second ultrasound imaging performed ~3 min after treatment the presence of deposited, activated bubbles in the myocardium was demonstrated (Fig. 1).

No clinical signs were recorded that could be ascribed to the treatment with the test item or the procedures used. Body weights and body weight gain were unremarkable for all dogs throughout the pretreatment and treatment periods. Food consumption was normal.

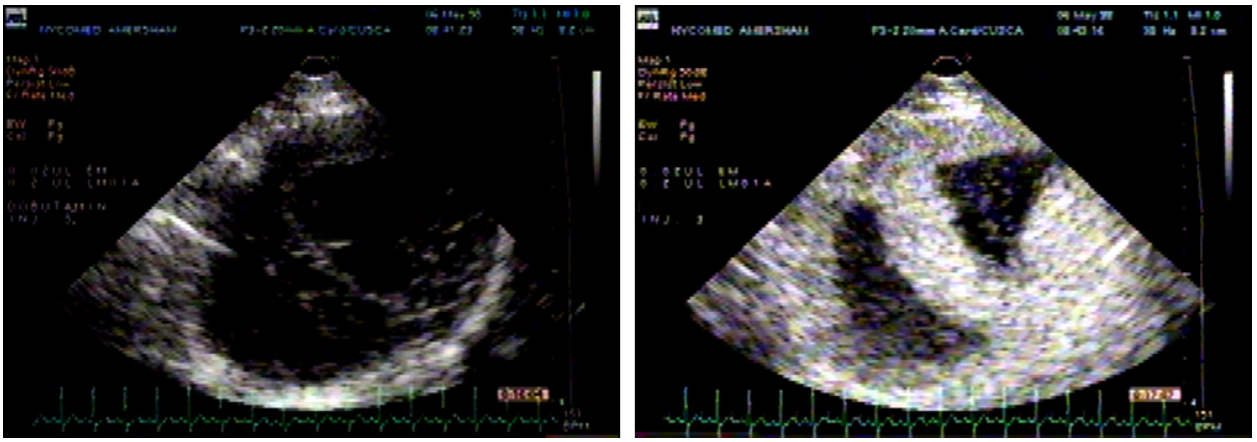

Figure 1. US imaging of dog heart. Baseline B-mode image (left) and ~3 min after administration and US activation of Acoustic Cluster Therapy (ACT) compound. Note that the ventricular volumes are almost completely void of contrast (i.e., free-flowing microbubbles are depleted), whereas the myocardial tissue display copious backscatter from deposited ACT bubbles.

Occasionally, the animals did not eat their entire daily food ration but this was not considered to be related to the treatment procedures.

The ophthalmoscopic examinations performed before treatment, before necropsy and 10 min after treatment were all unremarkable. Although ophthalmoscopic findings were noted on a few of the animals, these findings were present both before and/or after treatment. No signs of circulatory disturbances were noted in any of the animals 10 min after injection and ultrasound exposure.

In the hematology analyses, no significant differences were observed in any of the erythroid parameters (i.e., hemoglobin, red blood cell count, hematocrit, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration), neither were any of the coagulation parameters (APTT, TT, and PT) significantly changed. A treatment-related decrease in white blood cells was observed 5 and 40 min after treatment (Table 2). The decrease was statistically significant for groups 3 and 4 (no ultrasound activation) and groups 6, 7, and 8 (ultrasound activation) both 5 and 40 min after treatment. In both cases the decrease was caused by a general (and in some cases statistically significant) decrease in the number of all of the white blood cell subsets. The decrease was less pronounced 40 min after treatment and was no longer present 120 min after treatment.

A treatment-related decrease in the platelet count was seen 5 and 40 min after treatment (Table 2). The decrease was statistically significant for groups 3 and 4 (no ultrasound activation) and groups 7 and 8 (ultrasound activation) 5 min after treatment and in group 8 40 min after treatment. Although no longer statistically significant, the tendency toward a treatment-related decrease in the platelet count persisted 120 min and 24 h after treatment.

The statistical analysis referred to above was performed by comparing groups 2, 3, and 4 (treated with ACT) with group 1 (saline control group), and by comparing groups 6, 7, and 8 (treated with ACT and activated with ultrasound) with group 5 (given saline and activated with ultrasound).

When performing a statistical analysis on the white blood cell and platelet count between groups treated with the same dose of the test item, but with or without ultrasound activation, no effects of ultrasound activation of ACT was found.

No treatment-related effects were observed in the clinical chemistry parameters between the treated and the control groups whether activated with ultrasound or not.

The results of the histamine and complement split product analyses are given in Table 3. Histamine was present in all dogs both pre and postdosing, but levels ≤1 ng/mL are considered within the normal range. In 12 out of 24 dogs treated with ACT, histamine levels above 1 ng/mL

Table 2. White blood cell and platelet values in the dogs at predose and at 5, 40, and 120 min and 24 h postinjection.

			WBC (10^9/L)					Platelets (10^9/L)				
Group	Dose (ml/kg)	US activation	Predose	5 min	40 min	120 min	24 h	Predose	5 min	40 min	2 h	24 h
1	0	No	10.25 ± 1.04	9.40 ± 1.35	10.40 ± 0.48	9.20 ± 0.77	10.58 ± 0.99	389 ± 81	342 ± 58	330 ± 48	365 ± 82	350 ± 78
2	0.1	No	11.95 ± 1.99	10.25 ± 1.11	9.93 ± 0.91	10.05 ± 1.09	11.63 ± 2.50	338 ± 116	266 ± 70	322 ± 112	318 ± 93	316 ± 101
3	0.3	No	11.03 ± 1.41	4.83 ± 0.62†	7.30 ± 0.71†	9.43 ± 0.91	10.53 ± 2.57	344 ± 109	169 ± 80†	284 ± 74	311 ± 81	295 ± 88
4	1.0	No	12.00 ± 2.02	3.05 ± 1.96†	8.83 ± 0.43*	10.13 ± 0.81	10.18 ± 1.18	249 ± 72	53 ± 21†	191 ± 57	213 ± 57	207 ± 58
5	0	Yes	12.55 ± 0.93	12.05 ± 1.98	12.50 ± 2.32	12.03 ± 2.08	11.53 ± 2.49	343 ± 45	355 ± 42	349 ± 43	341 ± 55	355 ± 54
6	0.1	Yes	11.38 ± 1.52	8.95 ± 0.86*	6.98 ± 1.35*	9.83 ± 2.10	11.03 ± 0.35	362 ± 90	342 ± 112	340 ± 84	344 ± 97	344 ± 88
7	0.3	Yes	11.35 ± 1.47	5.65 ± 2.19†	8.70 ± 1.22*	9.28 ± 1.78	11.35 ± 0.65	282 ± 51	174 ± 69†	262 ± 48	242 ± 90	287 ± 63
8	1.0	Yes	10.95 ± 2.85	2.25 ± 0.79†	8.90 ± 1.84*	9.78 ± 1.60	9.55 ± 1.91	295 ± 46	94 ± 29†	242 ± 38*	270 ± 44	267 ± 42

*$P < 0.05$: Group 2, 3, and 4 versus group 1 or group 6, 7, and 8 versus group 5.
†$P < 0.01$: Group 2, 3, and 4 versus group 1 or group 6, 7, and 8 versus group 5.

Table 3. Histamine and complement split products as measured in the eight groups of dogs. Histamine values ≤1 ng/mL is considered to be within the normal range for dogs. For complement split products, + means "slight signs observed", ++ means "visible signs observed".

				Histamine (ng/mL)				Complement split products		
Group no.	Test item	Dose (mL/kg)	Ultrasound activation	Pre	2 min	5 min	10 min	Pre	10 min	120 min
1	Saline	1.0	No	≤1 (n = 4)	≤1 (n = 4)	≤1 (n = 4)	≤1 (n = 4)	0/4	0/4	0/4
2	ACT	0.1	No	≤1 (n = 4)	≤1 (n = 2) 2.5 1.5	≤1 (n = 4)	≤1 (n = 4)	0/4	0/4	0/4
3	ACT	0.3	No	≤1 (n = 4)	≤1 (n = 2) 4.1 7.3	≤1 (n = 4)	≤1 (n = 4)	0/4	3/4 +	0/4
4	ACT	1.0	No	≤1 (n = 4)	≤1 (n = 2) 38.6 15.1	≤1 (n = 2) 14.4 3.2	≤1 (n = 3) 3.9	0/4	4/4 ++	1/4 ++
5	Saline	1.0	Yes	≤1 (n = 4)	≤1 (n = 4)	≤1 (n = 4)	≤1 (n = 4)	0/4	0/4	0/4
6	ACT	0.1	Yes	≤1 (n = 4)	≤1 (n = 3) 3.5	≤1 (n = 4)	≤1 (n = 4)	0/4	0/4	0/4
7	ACT	0.3	Yes	≤1 (n = 4)	≤1 (n = 3) 17.9	≤1 (n = 3) 1.9	≤1 (n = 4)	0/4	1/4 +	0/4
8	ACT	1.0	Yes	≤1 (n = 4)	6.3 7.1 1.7 1.3	≤1 (n = 2) 1.2 1.1	≤1 (n = 4)	0/4	4/4 + +	0/4

ACT, Acoustic Cluster Therapy.

were measured 2 min after dosing. In some dogs the levels of histamine were relatively high 2 min after dosing. In five dogs histamine levels above 1 ng/mL were still present 5 min postdosing and in one dog a histamine concentration of 3.9 ng/mL was measured 10 min postdose. The higher levels and prolonged presence of histamine in the animals given the highest doses of ACT indicate that histamine release was dose-dependent. There is no apparent difference in the number of animals and/or the histamine values between animals treated with ultrasound exposure and those without.

Complement split products were present in 4/8 dogs dosed with 0.3 mL/kg and 8/8 dogs dosed with 1.0 mL/kg. There was no apparent difference in the number of animals and/or severity of complement activation between animals treated with and without ultrasound exposure. Complement activation might be considered a normal physiological response to injection of particles, due to opsonization processes in which the complement system is involved.

Blood samples were analyzed for both PFB and PF-MCP and the data were used to calculate pharmacokinetic parameters for these two analytes, which are summarized in Table 4. The calculated pharmacokinetic parameters for both PFB and PF-MCP in blood were similar in the animal exposed to ultrasound or and those not exposed.

At necropsy a few findings were reported in animals from control and treated groups but all were judged to be incidental and of no toxicological importance. Slight to marked hemorrhage was observed at the injection sites of all dogs but there was no trend of relationship with treatment. There were no significant differences in organ weights between treated and control groups.

The histopathological examination revealed no treatment-related findings in the tissues examined. All findings reported by the pathologist were considered to be within the background incidence of findings reported in this age of laboratory maintained Beagle dogs and as such to be of no toxicological importance. In line with the macroscopic findings, perivascular and/or subcutaneous hemorrhage occasionally associated with polymorphs, necrosis, and scab were observed at the injection sites in all animals. These changes did not show any intergroup variations which could be treatment-related and it was concluded that they were associated with the treatment procedures rather than the test compound. No hemorrhagic lesion was reported in the cecum or colon.

Safety assessment in the rat with ultrasound exposure to the liver

The ultrasound images were analyzed during dosing and the 5-minute ultrasound exposure period and showed

Table 4. Pharmacokinetic parameters for perfluorobutane (PFB) and perfluoromethylcyclopentane (PF-MCP) measured in whole blood from the dogs. Values given are mean ± SD. No statistical differences between animals exposed or not exposed to ultrasound were detected.

				PFB			PF-MCP		
Group	Test item	Dose (mL/kg)	US activation	C_{max} (ng/g)	AUClast (min × ng/g)	Half-life (min)	Cmax (ng/g)	AUClast (min × ng/g)	Half-life (min)
1	Saline	1.0	No	N/A	N/A	N/A	N/A	N/A	N/A
2	ACT	0.1	No	27.8 ± NC	259 ± 50.3	4.8 ± 1.5	1894 ± NC	22826 ± 2929	115 ± 5.6
3	ACT	0.3	No	69.1 ± 16.9	647 ± 128	22.1 ± 1.7	4059 ± 1143	59179 ± 15752	93.2 ± 32.1
4	ACT	1.0	No	165 ± 73.6	1660 ± 550	20.1 ± 2.4	9034 ± 4111	135960 ± 30405	54.8 ± 7.8
5	Saline	1.0	Yes	N/A	N/A	N/A	N/A	N/A	N/A
6	ACT	0.1	Yes	27.8 ± 3.2	295 ± 62.8	6.3 ± 4.0	2086 ± 354	34477 ± 13014	110 ± 30.4
7	ACT	0.3	Yes	53.0 ± NC	518 ± 117	21.2 ± 1.4	3868 ± NC	49019 ± 10378	69.8 ± 9.6
8	ACT	1.0	Yes	160 ± 45.8	1946 ± 413	18.9 ± 4.0	9607 ± 3937	162475 ± 34426	58.1 ± 8.4

NC: not calculable since some animals were excluded from calculations due to T_{max} > 2 min. ACT, Acoustic Cluster Therapy.

that the microbubbles entered the liver, and in the mid- and high-dose groups the liver was darkened by the shadowing effect. Due to the fact that Sonazoid™ microbubbles accumulate in the liver, the US signal did not deplete sufficiently for demonstration of activation and deposition of large ACT bubbles. However, based on the observations from activation in the heart and from previous studies (Sontum et al. 2015; Healey et al. 2016; Van Wamel et al. 2016) it is highly unlikely that the activation was not successful.

There were no mortalities and none of the animals included in the study showed any clinical signs of untoward effects or reduced well-being. Bodyweights were not affected in any of the treated groups when compared with the control group. Neither were there any effects on the liver weights when comparing the treated groups with the controls. All clinical chemical parameters in the ACT groups were unaffected as compared to the control animals.

During necropsy, no adverse observations were observed. The microscopic evaluation of the liver showed normal tissue in all animals in all groups.

Assessment of effects of ACT on motor coordination in the rat after ultrasound exposure to the heart

The mean group performance on the rotarod is tabulated in Table 5. There was no statistical significant difference in the performance between the control group and the groups treated with ACT and ultrasound activation. However, the group given chlorpromazine performed significantly poorer than all other groups and proved the validity of the test. Hence, activated ACT was shown to have no untoward effects in the brain leading to reduced ability to coordinate motoric movements.

Table 5. Performance on the rotarod by rats where the Acoustic Cluster Therapy (ACT) was activated in the heart. Chlorpromazine, acting as a positive control item (Group 4), was administered intraperitoneally at a dose of 5 mg/kg 10 min prior to the first rotarod test.

			Rotarod performance (sec)	
Group	Dose (mL/kg)	ACT activation	Immediately posttreatment	60 min posttreatment
1	0	Yes	67.5 ± 8.2	63.1 ± 6.1
2	0.1	Yes	63.3 ± 7.1	79.8 ± 5.8
3	0.3	Yes	71.9 ± 7.0	76.6 ± 9.4
4	N/A	No	33.3 ± 4.7	17.3 ± 2.6

The animals were closely observed during the entire experimental period, including the periods between the rotarod testing. No clinical signs of adverse reactions to the treatment were noted.

Discussion

ACT represents a novel concept for targeted drug delivery, in which ultrasound is applied to activate free-flowing clusters of negatively charged microbubbles and positively charged microdroplets. The clusters are activated within the target pathology by diagnostic ultrasound, undergo an ensuing liquid-to-gas phase shift and transiently deposit 20–30 mm large bubbles in the microvasculature, occluding blood flow for about 5–10 min. Further application of US induces biomechanical effects that increase the vascular permeability, leading to a locally enhanced extravasation of components from the vascular compartment (e.g., coadministered drugs) (Sontum et al. 2015; Healey et al. 2016; Van Wamel et al. 2016). These effects are a prerequisite for the functionality of the concept, but could also create untoward effects in

the target tissue. In addition, the temporary occlusion of small vessels could be suspected to induce ischemic or other local effects in the tissue, therefore a series of investigations was undertaken to assess the safety of the ACT concept in laboratory animals. The investigations reported in the present article demonstrated that an intravenous injection of up to 1 mL/kg of the ACT to rats and dogs was well tolerated, also when the product was activated by ultrasound in the heart or in the liver. This highest dose tested is assumed to represent 5–10 times the dose needed in humans for targeting drugs to a certain tissue and demonstrates a reasonable safety margin as a basis for moving into future clinical trials within oncology.

The parameters analyzed for safety assessment included general observations of the behavior of the animals during and after ultrasound activation, ophthalmoscopy, clinical chemistry including histamine and complement split product analyses, hematology, coagulation, and macro- and microscopic pathology of relevant tissues. A special experiment to assess effects on motor coordination, which could be affected if the microbubbles occluded either large or several small blood vessels in the brain, was part of the safety assessment.

The heart may be considered among the most sensitive organs in a setting where the microvasculature in the tissue is deliberately occluded. Furthermore, activation in the left ventricle will assure a maximum systemic exposure to the large, activated bubbles. The ultrasound imaging done of the heart of the dogs in which ACT was activated by insonation when the product was in the blood pool, demonstrated retention of the bubbles in the myocardium a few minutes after activation. However, no lesions or abnormalities were observed in the myocardial tissue by the histopathological examination, which indicates that the 5–10 min the bubbles occlude the small vessels is too short to create ischemia or other lasting damage to the tissue.

Ophthalmoscopy provides the only means for direct inspection of blood vessels in a living individual and is used to observe, among other types of pathology, changes in the circulation in small vessels in the fundus of the eye. The procedure is considered a sensitive and clinically useful technique for this purpose (Schneiderman 1990). In the present investigations, the examination of the fundus of the dogs was done both before injection of ACT, shortly after and as long as 24 h after. No abnormalities were observed at any of the time-points, indicating no lasting occlusion of the small vessels in the fundus that affected the blood circulation in this tissue.

A significant occlusion of blood vessels of the brain would be expected to result in immediate effects, in particular on complicated motoric tasks like coordination and movement. The rotarod test for rats is considered an appropriate tool to detect disturbances in the ability of rodents to maintain motor coordination well enough to run at an accelerating speed for a certain time (Cartmell et al. 1991). When testing rats given up to 0.3 mL/kg of ACT, followed by ultrasound activation in the heart, no differences were seen between a control group given saline and the ACT groups. Knowing that retention of bubbles was observed in the myocardium in the ACT-treated animals, it is encouraging that no apparent affection of the coordination ability of the animals could be observed, indicating no significant occlusion of circulation in the central nervous system.

Although the investigations described here included activation of the ACT by ultrasound, making a comparison of the safety between activated and nonactivated product possible, one additional objective was to study the general, acute safety of the chemical constituents of the formulation. Although the observation period was only 24 h from treatment to euthanasia, thorough examinations were undertaken in order to detect any acute reaction to treatment with the ACT product. The only significant adverse effects observed in the dogs were a short-lasting reduction in the number of circulating leukocytes, sometimes affecting all subtypes of this cell type, and a reduction in platelets. In this context, it is also of note that an apparent dose-dependent increase in the concentration of histamine was measured in plasma, and complement split products were occasionally detected, also in an apparent dose-dependent manner. The latter was only observed at the 10-minute time-point, and might indicate that the complement system is involved in the opsonization of the microbubbles prior to phagocytosis by the mononuclear phagocytic cells. No untoward clinical signs were noted in any of the dogs and this supports the suggestion that the slight increase in the level of split products was a result of complement factors being used in microbubble opsonization and not coming from the classical complement activation cascade.

The number of leukocytes was normalized at 120 min after treatment and this short-lasting effect may indicate that the reason for the leukopenia was not a direct toxic effect on the cells but more likely caused by leukocyte extravasation, possibly as a secondary effect to the histamine release which was observed during the first minutes after injection and activation of the ACT.

The reduction in platelets was statistically significant only up to 40 min after treatment but the trend of reduction was seen up to 24 h. The etiology of this temporary thrombocytopenia was not investigated further and is therefore unknown. The coagulation tests performed simultaneously with this platelet reduction did not indicate that the bleeding time was affected.

The experiment in rats with activation of ACT in the liver was quite uneventful as no adverse findings were

noted. Serum levels of enzymes indicative of liver toxicity were normal, and the microscopic examination of this organ did not reveal any abnormalities. A weakness in this evaluation of liver effects is the relatively short post-treatment observation period of 24 h, as toxic effects in the liver often takes longer to develop. This will, however, be addressed in future safety studies with ACT and certainly prior to any clinical trials in which ACT activation in the liver is planned.

Whether ACT was activated or not did not influence any of the parameters measured in the investigations. This was also the case for the pharmacokinetics of the two components of the formulations that were analyzed in dog blood, PFB, and PF-MCP. No significant differences were seen in the exposure parameters, maximum concentration (C_{max}) and the area under the blood concentrations-versus-time curves (AUC).

In a clinical setting, ACT would represent an image guided, localized therapy; the activated bubble produce copious US backscatter in regular B-mode imaging, giving the operator a tool for confirmation of the spatial distribution and level of bubble deposition. By its nature, ACT would not be indicated toward systemic or strongly metastatic conditions, but toward diseases where it is clinically meaningful to treat known, solid tumors with medicinal therapy (e.g., chemotherapy). A number of relevant clinical scenarios exists; most cases where chemotherapy is used as a neoadjuvant or adjuvant before/after surgical resection, and several diseases where a known, solid and nonresectable tumor is the primary reason for morbidity and mortality. A particularly interesting indication for ACT could be treatment of nonresectable, localized pancreatic ductal adeno-carcinoma (PDAC).

In conclusion, a single intravenous injection of the ACT product to rats and dogs at a dose-level up to 1 mL/kg and a posttreatment observation period of 24 h caused no significant toxicity to the animals. Activation of the product by ultrasound, either in the heart or in the liver, had no impact on this conclusion. This finding, together with promising animal efficacy data when combining ACT with cytotoxic drugs (Van et al. 2016), encourages our further development of the ACT concept and will form the basis for future preclinical safety studies with repeated dosing and longer periods of observation. Provided acceptable safety is confirmed in these studies, subsequent studies in humans are planned to fully explore the potential of this concept for improved targeted drug delivery.

Disclosures

Dr Sontum has a patent WO2015047103 pending and is cofounder and coowner of Phoenix Solutions AS, currently developing ACT in a commercial setting. Other authors have nothing to disclose.

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