Fluorescently labelled monoclonal antibodies for real-time molecular imaging

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CHAPTER 3

Development, preclinical safety, formulation, and stability of clinical grade bevacizumab-800cw, a new near infrared fluorescent imaging agent for first in human use

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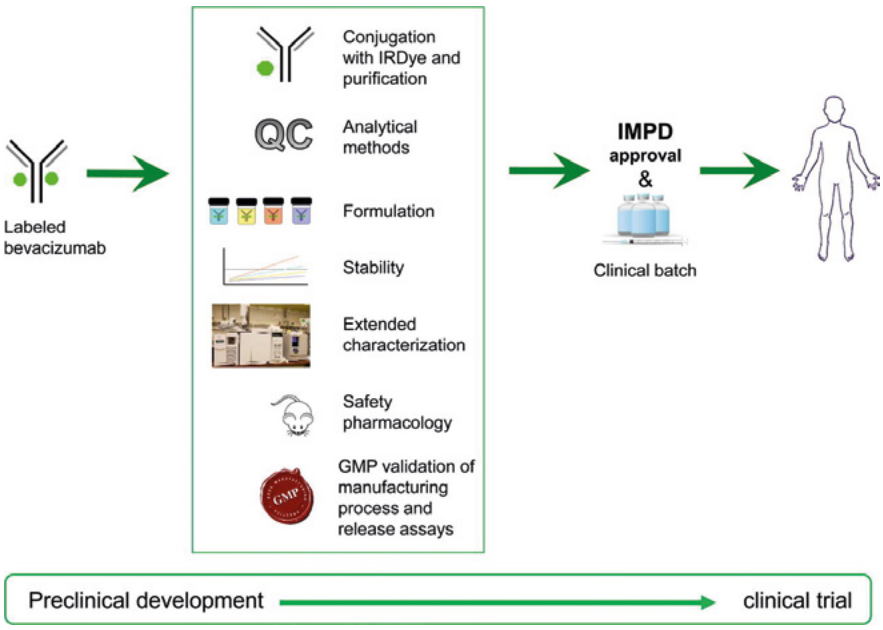
ABSTRACT

There is a dire need for better visualization of cancer and analysis of specific targets *in vivo*. Molecular imaging with fluorescence is gaining more and more attention, as it allows detection of these targets and has advantages over radioactivity, such as no radiation dose, and lower costs. A key challenge in optical imaging however, is translation of the newly developed tracers from pre-clinical phase to clinical application. We describe the development and safety testing of clinical grade bevacizumab-800CW, an antibody-based targeted agent for non-invasive imaging of vascular endothelial growth factor A (VEGF-A).

Development included implementing the manufacturing process and analytical methods according to current Good Manufacturing Practice (cGMP), formulation studies, extended characterization and stability testing. For safety pharmacology an extended single dose toxicity study in mice was performed.

Bevacizumab-800CW was formulated in isotonic phosphate buffered sodium chloride solution at pH 7. The production was robust and showed a reproducible labelling efficiency, and no impurities. The binding affinity to VEGF-A remained intact. The optimized product meets all release specifications, is stable up to at least 3 months and its characteristics did not significantly differ from the unlabelled bevacizumab. Toxicity testing in mice showed no remarkable findings.

In conclusion, sterile bevacizumab-800CW (6 mg = 6 ml) can be produced in stock according to current Good Manufacturing Practice. It is ready for first-in-human use.



INTRODUCTION

In the past decade there has been a growing interest in clinical translation of optical and fluorescence imaging in surgery. In surgical oncology, fluorescence imaging can provide the surgeon with reliable and real-time intraoperative feedback to identify surgical targets, including positive tumour margins. (1) Besides a camera system sensitive to light in the 700–800 nm spectrum, the fluorescent dye or contrast agent is of importance. Different types of optical contrast agent can be used. For example, non-targeted agents can be used because of the enhanced permeability and retention effect of tumours. However, the main research focus is on exploiting the intrinsic properties of tumours by labelling a fluorescent moiety to an antibody targeting such a specific property. When developing fluorescent contrast agents the translation from laboratory to clinic is often the bottleneck.

The implementation process of contrast agents for intraoperative imaging is not straightforward. Like other substances for clinical use, attention must be paid to efficacy, accuracy, quality control (QC) and stability according to current Good Manufacturing Practices (cGMP). In this manuscript we will use bevacizumab (Avastin®), an anti-vascular endothelial growth factor (VEGF-A) agent used in the clinic for several indications, as an example to discuss the different aspects necessary for clinical development. We will describe the production (including QC), formulation, stability, extended characterization and pre-clinical safety of a fluorescent imaging agent suitable for first-in-human application. Molecular imaging with PET using radiolabelled zirconium-89 (89Zr)-bevacizumab already indicated that VEGF-A is a valid target for imaging purposes in different tumour types (2–4), and a concise radiolabelling protocol has been published. (5) Moreover, the feasibility of 89Zr-bevacizumab has been shown in cancer patients with several tumour types using a microdose; a single dose less than 30 nmol (~4.5 mg antibody). (6) We labelled bevacizumab with fluorescent IRDye 800CW which can be bound to primary amines in lysine residues of antibodies when used in its N-hydroxysuccinimide (NHS) ester form. The dye is produced under GMP conditions and no toxicity was found in mice after one single intravenous dose of IRDye 800CW. (6) Intraoperative imaging with bevacizumab-800CW was very sensitive in mice bearing human A2780 ovarian xenografts, detecting even sub-millimetre lesions (2) using a real-time intraoperative fluorescence imaging system as described earlier. (7)

The aim of this paper was to describe the implementation process including production and QC, formulation, stability, extended characterization and pre-clinical safety of a fluorescent imaging agent suitable for first-in human application (Clinical Trial identifiers: NCT02113202; NCT01972373; NCT02129933).

MATERIALS AND METHODS

Two bevacizumab-800CW products are described in the paper. The initial formulation as developed for administration in the first clinical study is described first and thereafter, in sections "optimization of bevacizumab-800CW formulation" and "Stability of the optimized formulation" a second, optimized formulation is described. This product is used in ongoing clinical studies.

Production of bevacizumab–800CW initial formulation

The development of bevacizumab-800CW and its good laboratory practice (GLP) production for animal experiments has been described previously. (2) GMP production of the initial bevacizumab-800CW product consisted of conjugation, purification and sterile filtration. For conjugation, bevacizumab (25 mg/ml, Roche AG, Basel Switzerland) was diluted in phosphate buffered saline (PBS) and adjusted to a pH of 8.5–9.0 with 2 M sodium bicarbonate (Na_2CO_3, 2 M, produced in house). The IRDye 800CW NHS ester (LICOR Biosciences Lincoln, NE, USA) was added in a ratio of 4 molecules dye per antibody (4:1 ratio) and incubated at room temperature for 2 h. After incubation, and before purification, in process control (IPC) samples are taken to determine the efficiency of the conjugation and the dye/mAb ratio. The bevacizumab-800CW mixture was then added to a pre-equilibrated PD-10 column (GE Healthcare, Buckinghamshire, UK) and eluted with 0.9% NaCl. IPC samples are also taken hereafter to determine the concentration. The collected bevacizumab-800CW was diluted with 0.9% NaCl to a concentration of 1 mg/ml and was sterile filtered over a bacteria-retaining filter (0.22 µm pore size, Millex GV, Millipore, Darmstadt, Germany) immediately before filling into sterile type I tubular glass vials (Aluglas, Uithoorn, Netherlands). After aseptic filling, the vials are immediately closed with a rubber stopper (Omniflexplus 20 mm, Pont Packaging, Almere, Netherlands) and aluminium closure with a polymer flip-off cap (CTO closure white 20 mm, Pont Packaging, Almere, Netherlands). Samples are taken for release QC and the final drug product was stored at 2–8 °C for 7 days, see Fig. 1 for an overview of the manufacturing process. Qualified personnel in a grade A LAF-hood with grade C background performed the production. The manufacturing process was validated by performing three consecutive runs and full release testing.

Quality control

Quality control strategy consists of IPC and final release testing. Release specifications are described in Table 1. All analytical methods are validated according to ICH guidelines and justification of specifications is described in the investigational medicinal product dossier such as appearance, pH, osmolality, extractable volume, bioburden, endotoxins, residual solvents and filter integrity and is determined according to the European Pharmacopoeia (Ph. Eur.).

An HPLC analysis consisting of a 5110 chromaster pump; 5210 chromaster injector; and a DAD 5430 chromaster detector (VWR/Hitachi, Tokio, Japan); eluens: PBS pH 7.4; column: Biosep SEC S3000 (Phenomenex, Torrance, CA, USA) 300 x 7.8 mm; flow 1 ml/min; detection by Diode-array from 200 to 900 nm was used to determine identity, purity and concentration of final product. Determination of purity was focused on amount of aggregates, antibody integrity, and amount of free dye.

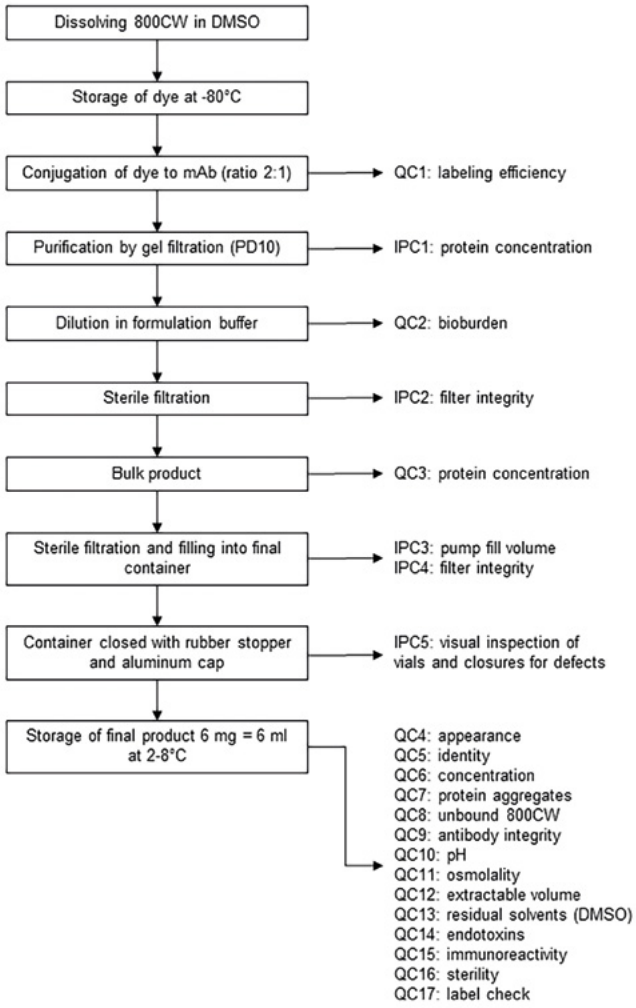


Figure 1: Flowchart of the production process of bevacizumab-800CW.

Table 1: Product criteria for release and end-of-shelf life

Specification	Test	Criterium at release	Criterium at end of shelf life
Identity API	SE-HPLC retention time	Matches with standard	Matches with standard
Content of API	SE-HPLC	0.95 -1.05 mg/ml	0.90-1.05 mg/ml
Content aggregates	SE-HPLC	<15%*	<15 %
Content free dye	SE-HPLC	<5%	<10%
Percentage protein-bound dye	SE-HPLC	>95%	>90%
Integrity antibody	SE-HPLC	No secondary peaks	No secondary peaks
Labelling efficiency	SE-HPLC	≥ 75% efficiency	NA
Dye:antibody ratio	SE-HPLC	≥ 1.5:1	NA
Amount aggregates before purification	SE-HPLC	For information only	NA
Appearance	Visual inspection	Clear or slightly opalescent solution, green to bluegreen	Clear or slightly opalescent solution, green to bluegreen
Visual particles	Visual inspection against a black and white background	No visible particles	No visible particles
pH	pH-electrode	6.8-7.2	6.8-7.2
Osmolality	Osmol measurement	270-300 mOsm	NA
Extractable volume	Test on extractable volume Ph. Eur.	>4.5 ml	NA
Residual solvents: DMSO	GC** according to Ph. Eur	<5000 ppm	NA
Endotoxins	LAL-test	<5 EU/ml	NA
Bioburden	According to Ph. Eur.	<1CFU/ml	NA
Filter integrity	Bubble point test	<2 ml air at 10 ml	NA
Immunoreactivity	Immunoassay	S-shape, upper plateau, max. 5% binding	NA

*This criterion was adjusted to <10% based on acquired data.
**GC = gas chromatography

The specification for the percentage of aggregates in the product is based on the Ph. Eur. Monograph for Normal Immunoglobulin for Intravenous Use. This states that a product may not contain more than 3% aggregates. However, all registered immunoglobulin products have IgG concentrations of 50 mg/ml or higher, thus a concentration of 1.5 mg/ml of aggregates is accepted for commercially available products. Since our protein concentration is far lower (1 mg/ml) we will not reach this concentration of aggregates in regular production. And also the total protein dose in the clinical study is only 4.5 mg, so the absolute amount of aggregates even for the maximum of 15% is low. We therefore consider it acceptable to set the limit for aggregates at 15%

An immunoreactivity assay was performed to determine whether labelling with IRDye 800CW impairs binding to VEGF. This assay was set up as a competition immunoassay. The assay was performed according to the design as described earlier. (2) Briefly, wells are coated with recombinant human vascular endothelial growth factor (rhVEGF165, R&D systems, Minneapolis, MN, USA) and left to incubate overnight at 4 °C. After coating, the plate was washed with 0.5% Tween 80 (Sigma Aldrich, Steinheim, Germany) in PBS and blocked with 1% HSA in PBS. The block was incubated for 1 h at room temperature on a plate shaker. A series of dilutions of unlabelled bevacizumab was made in PBS, to which are then added a series of fixed concentration bevacizumab-800CW. These sample dilutions are added to the wells after washing. The sample solution was incubated for 2 h at room temperature on a plate shaker. After sample incubation the plate was washed and dried on a tissue. The intensity of the fluorescence was determined by Odyssey scanner (Licor Biotechnology, Lincoln, NE, USA). The fluorescence intensity was plotted against the logarithm of the inhibitor concentration and the resulting graph was assessed for (a) the presence of an inverse S-shaped curve formed by the data points, (b) an upper plateau reached at the lowest concentration of competitor (unlabeled bevacizumab), and (c) a maximum of no more than 5% binding is reached at the highest concentration of competitor (unlabeled bevacizumab). Specific binding of bevacizumab-800CW to its target VEGF-A is confirmed by the results of the competition assay if all three criteria are met.

The dye:protein ratio is calculated based on the amount of antibody-bound and free IRDye 800CW present in the product before purification (as determined by SE-HPLC-DAD). The results from 29 batches produced for stability testing between February 2014 and February 2015 demonstrated that the labelling efficiency is 79.5% (±2%), and dye:protein ratio is on average 1.59 (±0.04). This resulted in QC requirements of an efficiency of at least 75% and a dye:protein ratio of at least 1.5. These requirements apply to final (optimized) formulation, not the initial formulation as is added to Table 1. Further data on these batches can be found in Supplementary Table 1.

Extended characterization

Additional extended characterization of bevacizumab-800CW in its initial formulation was performed. Three independent batches of bevacizumab-800CW were sent to Roche for extended measurements. At all analyses (except for label ratio) bevacizumab was used as reference standard (RS) and measured in parallel.

For the analysis of dye/mAb ratio any not-bonded dye in bevacizumab-800CW samples was removed by ultrafiltration. Label ratio was determined by calculating the ratio of the UV absorption at 280 nm (Protein) and 774 nm (Label) using a Nano UV/VIS-photometer (IMPLEN).

In order to confirm the amino acid sequence of bevacizumab-800CW, tryptic digests of denatured and reduced samples were analyzed by LCMS peptide mapping (Thermo Fisher Scientific, Orbitrap Velos). Microheterogeneities of the light chain (LC) and the heavy chain (HC) were investigated by evaluation of specific ion chromatograms (SIC). The data were evaluated with respect to the appearance of oxidation of methionine, deamidation of asparagine, formation of succinimides and the heterogeneities of the N- and C-terminus.

The determination of the molecular masses of the non-glycosylated light chain (LC_NG), the non-glycosylated heavy chain (HC_NG) and their fluorescence labeled variants (1FL and 2FL) was investigated by mass spectrometry. All bevacizumab-800CW samples were deglycosylated, denatured and reduced. After buffer exchange the measurements were performed offline with an electrospray mass spectrometer (Waters, Synapt G2). The experimentally determined molecular masses were calculated from different charge states of the heavy and light chain and compared to the theoretical molecular masses.

The determination of the N-linked oligosaccharides was investigated by 2-amino-benzamide (2-AB) labelling. All bevacizumab-800CW samples were deglycosylated by enzymatic cleavage with N-glycosidase F and the released glycans were labeled with 2-AB. The analysis was performed with hydrophilic interaction chromatography (HILIC) using an ultra-high performance liquid chromatography with a fluorescence detector (Waters, Acquity UPLC). The antiproliferative activity of bevacizumab-800CW was determined by a bioassay measuring cell growth inhibition due to neutralization of VEGF. The assay is based on the ability to inhibit recombinant human (rh)VEGF-induced proliferation of endothelial cells (HUVEC). rhVEGF binds to the HUVEC and stimulates their proliferation. Bevacizumab inhibits this process by blocking rhVEGF binding to its receptors. In this assay, varying concentrations of bevacizumab are mixed with rhVEGF followed by the addition of HUVEC suspension (10,000 cells/well). After an incubation period of four days, the relative number of viable cells is quantitated indirectly using the fluorescent redox dye, alamarBlueTM. The results, expressed in relative fluorescence units (RFU), are plotted against the bevacizumab concentrations and the inhibitory activity of bevacizumab samples is determined relative to the RS of unlabeled bevacizumab.

Animal toxicity study

An extended single dose toxicity study (microdose approach I) was conducted according to the guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) M3 (R2): guidance on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. (8) This guideline states that a maximum dose of 1000-fold the clinical dose can be used on an mg/kg basis for intravenous administration and mg/m2 for oral administration. We aimed for an intravenous administration of 4.5 mg in the clinical study.

This dose was derived from the FDA guideline Exploratory IND studies stating that the maximum dose for protein products in microdosing studies is ≤30 nmol (i.e. 4.5 mg bevacizumab-800CW). Based on an average body weight of 75 kg this is a dose of 0.06 mg/kg. We therefore used doses of 6 and 60 mg/kg body weight for the toxicity study. The study was performed at the GLP facilities of WIL Research in The Netherlands.

The nature and purpose of this toxicity study was to assess the toxic potential of bevacizumab-800CW when administered as a single intravenous dose to CD-1 mice, and to study the reversibility of possible effects. It has to be noted that there is no crossreactivity between bevacizumab and mouse-VEGF. This study only evaluates the toxic potential of bevacizumab-800CW, since the safety profile of the fluorescent dye and of bevacizumab itself has already been investigated. (9,10) The mice (CRL:CD-1(Icr), outbred, SPF-Quality) were obtained from Charles River (Sulzfeld, Germany). Females were nulliparous and non-pregnant. This type of protocol was reviewed and agreed by the Animal Welfare Officer and the Ethical Committee of WIL Research as required by the Dutch Act on Animal Experimentation. All the animals were carefully monitored during the testing period.

The study animals were assigned to groups as shown in Table 2. Randomization of animals was performed by computer-generated random algorithm according to body weight, with all animals within ±20% of the sex mean. Groups consisted of 10 animals (main groups) and 5 recovery animals/sex (control and high dose group), which received a single intravenous bolus injection. Main animals were sacrificed on day 2; recovery animals were observed during a 2 week follow-up phase and sacrificed on day 15. Bevacizumab-800CW was administered at a dose level of 6 and 60 mg/kg body weight. The control animals received the vehicle (0.9% NaCl solution) alone. At time of tracer injection, the animals were approximately 6 weeks old. They received intravenous injection via the tail vein and were observed at least twice daily. Duration of recovery phase was from day 2 to day 15.

Table 2: Toxicity study groups

Group	Subgroup	Dose (mg/kg)	Injection volume (ml/kg)	Number of animals	
				Males	Females
1	Main	0	14	10	10
1	Recovery	0	14	5	5
2	Main	6*	6	10	10
3	Main	60**	14	10	10
3	Recovery	60**	14	5	5

*6 mg/kg is 100 times the intended human iv microdose of ≤30 nmol (i.e. 4.5 mg bevacizumab-800CW)
**60 mg/kg is 1000 times the intended human iv microdose of ≤30 nmol (i.e. 4.5 mg bevacizumab-800CW)

Blood samples were taken just prior to scheduled termination. While under anaesthesia, the samples were collected from the retro-orbital sinus of the mice; subsequently they were exsanguinated and transferred to termination area. Blood was collected for clinical biochemistry, haematology and electrolyte analysis. Tissues and organs were processed from all animals euthanized during the safety study and evaluated pathologically.

Blood samples were collected in EDTA containing tubes for haematological parameters (0.35 ml) and tubes treated with Li-heparin (at least 0.25 ml) for clinical biochemistry parameters.

Plasma samples were stored at ≤-75 °C prior to analysis on the Olympus AU400 (Sysmex Etten-Leur, The Netherlands). Blood was stored in a refrigerator prior to analysis. Haematology parameters were determined with the ADVIA120 (Siemens Medical Solution Diagnostics). Biochemistry parameters were determined in plasma samples.

Samples, tissues, and organs were collected from all animals at necropsy and fixed in 10% buffered formalin (neutral phosphate buffered 4% formaldehyde solution, Klinipath, Duiven, The Netherlands). Organs and tissue samples, were processed, embedded and cut in 2–4 µm slices and stained with hematoxylin and eosin.

All organs and tissues collected from all animals of groups 1 (controls) and 3 (high dose group) were examined and all gross lesions of all main and recovery animals (all dose groups).

Optimization of bevacizumab–800CW formulation

Ten formulation buffers with variations in buffer components and additives were designed and tested for 28 days. Buffer composition was either based on the composition of the commercially available bevacizumab, or on a 0.9% NaCl solution. Variations between buffer solutions were designed to investigate the effect of pH on protein concentration and aggregation. All tested solutions were kept at a predetermined pH using a 50 mM phosphate buffer. Storing the product in the dark at 2–8 °C minimized the effects of light, temperature and freeze–thaw cycles. Treating the buffers with nitrogen gas before filling and sterilization of the optimized buffer container minimized the effect of oxidation. To further minimize the effect of oxidation on the product, after filling of bevacizumab-800CW, the headspaces of the product vials were filled with nitrogen gas as well. For the full list of buffers used and their components, see Table 3.

Conjugation of bevacizumab to IRDye 800CW and purification of the conjugated antibody was generally performed as described before under ‘‘production of bevacizumab-800CW initial formulation”. However, a dye/mAb ratio of 2:1 instead of 4:1 was used and PD-10 elution was performed with the assigned buffer solution. With each buffer 6 vials were manufactured. One vial contained 6 ml, and was used for visual inspections. The other 5 vials contained 1 ml and were used as sample vials for HPLC analyses. On days 0, 7, 14 and 28 the product was

assessed for protein concentration, percentage of aggregates, and percentage of free dye by SE-HPLC as described under "Quality control". After 28 days the 6 ml vial was visually inspected for particles according to Ph. Eur. 2.9.20; as well as color, turbidity or opalescence, and precipitation of the product.

Stability of the optimized formulation

The two formulations from the previous section that showed the best results based on protein concentration, absence of aggregates, and appearance of the solution after 28 days, were further investigated over a period of 3 months in a real-time stability study. For this purpose, a large scale production was performed, resulting in approximately 100 product vials for each of the buffers. After production, vials were randomly distributed over 2 groups; real-time and accelerated stability test groups, with the real-time group containing 3 times as many vials as the accelerated group. The real-time group was stored in the refrigerator (2–8 °C, Fridge) and the accelerated group was stored at room temperature (15–25 °C, Room). Temperature was monitored and logged with validated temperature probes (174H, Testo, Lenzkirch, Germany). The stability of the product in the real-time groups was assessed up to 12 months after production, and was measured at 0, 1, 2, 3, 6, 9, and 12 months. The stability of the accelerated stability batches was assessed up to 3 months after production and was measured at 0, 1, 2, and 3 months. At 0, 3, and 12 months, both groups of both formulations were tested for the full specification of the product where applicable, with the addition of sterility testing. Container closure was tested directly after production at stability starting point. At 1, 2, 6, and 9 months after production, a lean stability test was performed. The lean test omits the test for extractable volume, test for osmolality, and the test for bacterial endotoxins. The specifications of the product and the methods used for analysis are displayed in Table 1. SE-HPLC analyses were performed on 5 independent samples from each of the groups from both formulations. The vials to be assessed were picked at random at each time point. Non-destructive tests or tests requiring a small sample volume, such as measurement of pH and osmolality, were performed on the same vials as SE-HPLC. Separate vials were used for microbiological tests and container closure test. Appearance of the solution was determined visually, and it was inspected for particles according to Ph. Eur. 2.9.20; as well as color, turbidity or opalescence, and precipitation of the product. Data were collected and interpreted in an intermediate analysis 3 months after production.

Statistical analysis

Statistical analyses were performed on the data from the animal toxicity study. If the variables could be assumed to follow a normal distribution, the Dunnet t-test (many-to-one t-test) based on a pooled variance estimate was applied for the comparison of the treated groups and the control groups for each sex. The Steel-test (many-to-one rank test) was applied instead of the Dunnet t-test if the data

could not be assumed to follow a normal distribution. The Fisher-exact test was applied to frequency data. All tests are two-sided and in all cases $p < 0.05$ will be accepted as the lowest level of significance. Mean values and 95% confidence intervals were calculated for each of the SE-HPLC analyses. For other release criteria, means were calculated.

RESULTS

Production of bevacizumab–800CW initial formulation

The product is a clear to slightly opalescent, bluish-green, sterile solution, containing 6 mg = 6 ml bevacizumab-800CW in saline with a shelf life of 7 days stored at 2–8 °C. The product meets its predefined specifications such as sterility, endotoxins, and dimethyl sulfoxide (DMSO) concentration. The percentage of protein aggregates is <10% and percentage of unbound IRDye 800CW <5%, see sample graph in Fig. 2. The product shows an intact binding affinity for the target, meeting the 3 criteria of an inverse S-shaped curve, an upper plateau at the lowest concentration unlabelled bevacizumab, and a no more than 5% binding at the highest concentration unlabelled bevacizumab. A representative example of an immunoreactivity graph is depicted in Supplementary Fig. 1.

Extended characterization

By UV measurement label ratios of 2.0 and 1.9 (mol IRDye 800CW/mol bevacizumab) were determined in the three batches.

The LCMS peptide map analysis confirmed the amino acid sequence by 100% sequence coverage for the bevacizumab-800CW and the RS. No significant differences between bevacizumab-800CW and the RS were detected with regard to microheterogeneities of the light and heavy chain. The determination of the intact mass of heavy and light chain revealed that within the limits of precision of the method all molecular masses of bevacizumab-800CW samples and the RS fit to the calculated mass.

Also the relative ratios of the different N-linked glycan species showed no significant difference between bevacizumab-800CW and the RS. The result of the potency assay was a 94% antiproliferative activity of bevacizumab-800CW compared to a non-labelled RS. 3.3.

Animal toxicity study

No changes in body weight and body weight gain were noted in any of the mice, and the mean absolute and relative food consumption were normal for all groups.

Females at the 60 mg/kg bevacizumab-800CW dose group showed a slight decrease in body weight gain during the last half of the second week when compared to the control group animals. However, the mean body weight of these mice was normal during the 14-day follow-up period. Therefore, these findings were considered to be of no toxicological significance.

In addition to monitoring weight and food intake, mice were monitored for other physiological responses. No clinical signs of toxicity were noted during the observation period, and none of the animals died.

Clinical Laboratory Investigations

No toxicologically relevant findings were noted at the hematological examinations, see Supplemental Tables 2 and 3.

Mean haematocrit values and mean corpuscular volume (MCV) were decreased in males at 6 mg/kg, and mean corpuscular haemoglobin concentration (MCHC) was increased in this group. No changes were noted in the 60 mg/kg-dosed males or at the end of the recovery period. In the females' 6 mg/kg group, white blood cell counts (WBC) were lower, but in the 60 mg/kg group values were comparable to the control level. At the end of the recovery period WBC of females at 60 mg/kg was increased, and a higher haemoglobin concentration was noted as well.

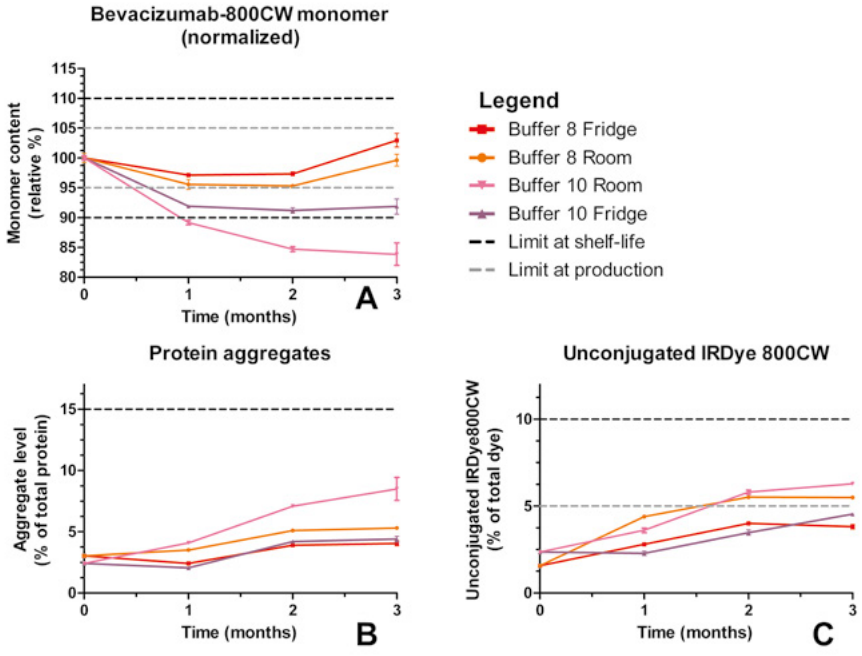


Figure 2: Stability data on two formulations (8 and 10), measuring three parameters over three months. Limits for release and for shelf-life are plotted as a horizontal line. Normalized percentage of bevacizumab monomers over time (A), percentage of aggregates measured over time (B), amount of free IRDye present over time (C). 'Fridge' samples were stored at 2–8 °C; 'Room' samples were stored at 15–25 °C

Changes in corroborative haematological parameters were absent, and no dose relationship was apparent. Further, in the absence of histopathological findings, these changes were considered not to be toxicologically relevant. Clinical biochemistry parameters of treated mice were considered not to be affected by treatment. Total bilirubin was increased in males at the 60 mg/kg dose, both on days 2 and 15. In females total bilirubin was increased in a dose related way at 6 mg/kg and 60 mg/kg, but levels returned to normal values at the end of recovery (day 15). Since there were no concurrent microscopic changes, these variations were considered to be without toxicological relevance.

Lower triglyceride and inorganic phosphate level in males at 6 and/or 60 mg/kg on day 2 were considered to be without toxicological relevance in the absence of a dose-relationship and in the absence of any changes at the end of recovery. Further, lower urea and phospholipid concentration and higher chloride concentration in females at 6 mg/kg, and higher sodium concentration in females at 6 and 60 mg/kg were considered to be without toxicological relevance since no changes were noted at the highest dose level, no dose-relationship was noted and/or no changes were observed at the end of the recovery phase. Thus, no relevance was attached to this finding.

Pathology

Autopsy did not reveal any toxicologically relevant alterations. Incidental findings among control and/or treated animals included reduced size of testes and epididymides, a nodule on the liver, cysts on the ovaries and uterus containing fluid. The incidence of these findings was within the background range of findings that are encountered among mice of this age and strain, and did not show a dose-related incidence trend. These findings were therefore considered to be of no toxicological significance.

No significant changes were noted in organ weights and organ to body weight ratios. In males at 6 mg/kg, absolute and relative liver weight was decreased and relative brain weight was increased. No changes in liver weight were noted in males at 60 mg/kg at day 2 or day 15. In females a lower absolute and relative liver weight was observed for the 60 mg/kg dose group on day 15 (not at the end of treatment). These findings were considered not to be a sign of toxicity in the absence of any corroborative findings at the microscopic examination that were indicative of organ dysfunction. Other organ weights and organ to body weight ratios among the dose groups were similar to control levels.

There were no microscopic findings recorded which could be attributed to treatment with the test substance.

Optimization of Formulation

In the initial formulation bevacizumab was labelled with IRDye-800CW at a dye/mAb ratio of 4:1 and was formulated at a concentration of 1 mg/ml in 0.9% NaCl. A 7-day shelf life was assigned to this product. As a result manufacturing

and quality control for this product when produced for clinical use in patient studies were performed frequently on a per-patient basis. This was very time consuming, therefore effort was put in optimization of a new formulation with the aim to extend its shelf life to at least three months, and longer if possible. The formulation results are presented in Table 3. In all formulations the concentration remained constant. For formulations 4 and 5 it was not possible to determine the concentration due to interference of albumin on the HPLC system. Any aggregates found remained below the limit of 15% of total peak area as was determined in the release criteria. The concentration of free IRDye 800CW remained below the limit of 5% of total peak area as determined in the release criteria.

Formulations 5, 8, and 10 performed best after 1 month in terms of visual inspection, concentration bevacizumab, percentage of aggregates, and percentage of free dye (Fig. 2 and Table 3). HPLC analysis of formulation 5 was difficult as albumin interfered with the retention time of bevacizumab, therefore formulations 8 and 10 were used for further stability testing.

Stability of the optimized formulation

Results of the stability study are presented in Fig. 2, and additional test criteria are shown in Table 1. Mean values and 95% confidence intervals of bevacizumab-800CW monomer concentration were plotted in the graph as percentage relative to the first measured value. Mean values and confidence intervals of protein aggregates and free dye were plotted similarly to the monomer concentration. The amounts of protein aggregates and unconjugated IRDye 800CW were within the set limits at all time points for both groups of both tested formulations. However, the tests show a trend toward a more rapid increase of impurities in the accelerated stability batch. The accelerated batches eventually show a rise above the 5% free dye level and consequently, these batches show a protein-bound dye level of <95% (data not shown). The monomer concentration of bevacizumab-800CW is within limits for all tested solution at most time points. After production, both the accelerated and real-time batches show a marked decrease in concentration, which continues up until 3 months. The graph in Fig. 2A accentuates this, showing the accelerated stability batch of buffer 10 dropped below 90% of the original concentration after only one month.

All tested solutions passed the tests for pH, osmolality, identity of the protein, integrity of the protein, status of the container, extractable volume, residual solvents: DMSO, and container closure. No turbidity, opalescence or sedimentation was seen in any of the vials of the tested solutions. Some vials contained a particle, however not more than 2 visible particles were found in a single vial.

The accelerated batch of buffer 10 showed a decrease in immunoreactivity at 2 and 3 months after production. No such effect was seen in the other test solutions. Samples of 4 vials from both formulations and both groups were tested for sterility immediately after preparation and 3 months after production. All vials were found to be sterile.

Based on these findings, we decided to use buffer 8 for our optimized formulation.

Table 3: Overview of formulation studies

	Components	Visual inspection after 1 month	Concentration bevacizumab	% Aggregates of total mAb	% Free IRDye of total dye
1	Trehalose dihydrate Sodium phosphate Polysorbate 20 pH6	Clear or slight opalescent solution, green – bluegreen, visible particles	0.95 -1.05 mg/ml	< 15%	< 5%
2	Sodium phosphate Polysorbate 20 pH 6	Clear or slight opalescent solution, green – bluegreen, visible particles	0.95 -1.05 mg/ml	< 15%	< 5%
3	isotonic phosphate buffered sodium chloride solution pH 6	Clear or slight opalescent solution, green – bluegreen, visible particles	0.95 -1.05 mg/ml	< 15%	< 5%
4	isotonic phosphate buffered sodium chloride solution Albumin pH 6	Clear or slight opalescent solution, green – bluegreen, visible particles	n.a.*	< 15%	< 5%
5	Trehalose dihydrate Sodium phosphate Polysorbate 20 Albumin pH 6	Clear or slight opalescent solution, green – bluegreen, **no visible particles**	n.a.*	< 15%	< 5%
6	isotonic phosphate buffered sodium chloride solution EDTA pH 6	Clear or slight opalescent solution, green – bluegreen, visible particles	0.95 -1.05 mg/ml	< 15%	< 5%
7	isotonic phosphate buffered sodium chloride solution pH 5	Clear or slight opalescent solution, green – bluegreen, visible particles	0.95 -1.05 mg/ml	< 15%	< 5%
8	isotonic phosphate buffered sodium chloride solution pH 7	Clear or slight opalescent solution, green – bluegreen, **no visible particles**	0.95 -1.05 mg/ml	< 15%	< 5%
9	Trehalose dihydrate Sodium phosphate Polysorbate 20 pH 5	Clear or slight opalescent solution, green – bluegreen, visible particles	0.95 -1.05 mg/ml	< 15%	< 5%
10	Trehalose dihydrate Sodium phosphate Polysorbate 20 pH 7	Clear or slight opalescent solution, green – bluegreen, **no visible particles**	0.95 -1.05 mg/ml	< 15%	< 5%

* The concentration of bevacizumab could not be analysed in formulation 4 and 5 due to interference of albumin

DISCUSSION

We produced bevacizumab-800CW according to cGMP, suitable for clinical use. This is to our knowledge the first report about the pharmaceutical quality and pre-clinical safety of a NIR fluorescent molecular imaging agent for first-in-man clinical use. This is of particular interest as the use of fluorescent dyes in patient imaging is still in its infancy. However, optical imaging is currently taking flight (1), as an important feature in intraoperative image-guided surgery that improves the quality of solid tumour resection. (11) In this paper we describe a route to develop and produce a fluorescent protein-based imaging agent for clinical use.

One of the major hurdles is a well-defined and efficient translation from pre-clinical to clinical use; especially proteins are notoriously difficult to translate. (12) One of the examples is a clinical study testing cetuximab-IRDye 800CW in 14 patients, where no pre-formulated product was used but instead was made fresh for every single patient study. (13) The reason for a reduced shelf-life of our initial formulation was due to aggregation, a well-known phenomenon for antibodies in solution. Several mechanisms contribute to aggregate formation: it can be influenced by pH, total protein concentration, light, shear stress, temperature, freeze–thaw cycles and oxidations. (12,14–17) We also suspected that increasing the dye/mAb ratio might result in aggregation. This may be due to changes in isoelectric point, but it was also mentioned IRDye 800CW apparently induces – via intramolecular tension – a conformation change of the mAb molecule. (18) Even though the ratio of 4 IRDye 800CW molecules per antibody was established in previous preclinical experiments and proved to be stable enough for that application (2), we lowered the dye/mAb ratio from 4 to 2 IRDye molecules per bevacizumab molecule in order to optimize the stability of the product. The lower ratio was incorporated in the formulation research. Apparently, labelling less dye results in less aggregation (see Supplementary Table 1), but it does not reduce the fluorescent signal. When labelling more than 2 IRDye 800CW molecules per antibody, it is likely that the dye molecules quench each other's fluorescence. (19,20) This self-quenching of the fluorescence occurs when two excited fluorophores of the same molecule are close enough (<10 nm) to enable them to absorb energy from each other. Besides self-quenching, self-aggregation of the dye also plays a role (21), therefore we used a start dye/mAb ratio of 2:1 to lower the chance of aggregation and quenching. Previous research has demonstrated that small differences between mAbs can induce significant differences in stability. (22) As a result of the more stable product, a relatively simple formulation, with only a buffer added to the previous saline solution, proved to be sufficient for long-term storage. The stability study shows that the new formulation using 50 mM phosphate buffer at pH 7.0 provides a superior product. The concentration of the bevacizumab-800CW monomer stays within the limits set for both release and shelf-life up to 3 months after production when stored at 2–8 °C. Real-time stability results are supported

by the accelerated stability tests, which show a similar pattern of degradation compared to their refrigerated counterparts. However, the speed of degradation is increased for both formulations when stored at room temperature (Fig. 2B and C). Therefore, when combining the information gained from the real-time and accelerated studies, buffer 8 containing isotonic phosphate buffered sodium chloride solution set at pH 7 is clearly the better option for clinical production of bevacizumab-800CW, even though some measurements resulted in protein content values over 100%. This is due to the fact that our method of analysis was still in development. Any irregularities are solved for later analyses. The test for immunoreactivity is difficult to qualify, most likely due to variation in detection of the fluorescent signal. We do however apply the following qualitative specifications to the test: (a) an inverse S-shaped curve is formed by the data points, (b) an upper plateau is reached at the lowest concentration of competitor (unlabeled bevacizumab), and (c) a maximum of no more than 5% binding is reached at the highest concentration of competitor (unlabeled bevacizumab). Specific binding of bevacizumab-800CW to its target VEGF-A is confirmed by the results of the competition assay if all three criteria are met.

The specific binding of bevacizumab-800CW to its target VEGF is confirmed by the results of the competition assay if these three criteria are met. Currently, no generally accepted guidelines exist for accelerated stability testing for proteins. We chose to use room temperature for several reasons. First, we expected the increased temperature to induce degradation and cause formation of expected degradation products in a short amount of time, allowing for control of these degradation products in the real-time study. Second, after production, a product vial or syringe containing tracer might be stored at room temperature for short amounts of time. Testing at room temperature would also give an indication of the stability during these periods. It is too early to conclude whether the accelerated stability is an accurate predictor of formulation stability. This accuracy can be evaluated at the end of the study.

Testing for immunoreactivity indicated an intact affinity for the target and levels of aggregates and free dye remain low as well. No major deviations were found in any of the other criteria that were investigated. The prolonged stability study will provide more information on the long-term stability of the product in due time. Based on the findings of the prolonged stability study and routine production of bevacizumab-800CW for clinical use, we will adjust release criteria as appropriate. For instance, the requirement set for maximum amount of aggregates has been reduced from 15% of total protein to 10% by the time of publication of this paper. The data gained from the extended characterization showed that the labelling procedure does not influence the structural integrity and post translational modifications of bevacizumab. Also the mode of action is not affected by the IRDye 800CW.

Another significant hurdle in the translation process is safety testing of a newly developed product. As imaging agents are usually administered in low doses and do not have a therapeutic intent, we considered it appropriate to test our tracer according to microdose regulations; the FDA 'Guidance for Industry, Investigators, and Reviewers – Exploratory IND Studies' (23) states that the single administration of a small dose, for proteins below 30 nmol, has very limited risks to human subjects and that information to support the clinical trials can be obtained from limited preclinical safety studies. Guidelines on clinical microdose trials can be found in ICH M3 (R2) section 7.1 and in the FDA guideline on exploratory IND studies. (8,23) In the latter guideline it is also mentioned that an extended single-dose toxicity study is sufficient to support single-dose studies in humans. For microdose studies, a single mammalian species (both sexes) can be used if justified by in vitro metabolism data and by comparative data on in vitro pharmacodynamic effects. With this in mind and the fact that the bulk of pre-clinical imaging data with antibodies has been performed in mice, we chose mice as our animal of interest. Our toxicity study indicated that labelling bevacizumab with IRDye 800CW has no negative consequences; there was no observed toxicity either in the clinical laboratory investigations and pathology, even though the mice received relative doses that were 100 and 1000 times the intended human microdose. Bevacizumab-800CW is currently being used in several clinical trials including in adenomatous poliposis coli (identifier: NCT02113202); rectal cancer (identifier: NCT01972373); and in esophageal cancer (identifier: NCT02129933).

CONCLUSION

This study proves that it is possible to develop and produce cGMP compliant bevacizumab-800CW for first-in-man use in a non-commercial setting, and provide a suitable formulation for long-term storage.

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REFERENCES

1. de Boer E, Harlaar NJ, Taruttis A, Nagengast WB, Rosenthal EL, Ntziachristos V, et al. Optical innovations in surgery. Br J Surg. 2015 Jan 27;102(2):e56–72.
2. Terwisscha van Scheltinga AGT, van Dam GM, Nagengast WB, Ntziachristos V, Hollema H, Herek JL, et al. Intraoperative Near-Infrared Fluorescence Tumor Imaging with Vascular Endothelial Growth Factor and Human Epidermal Growth Factor Receptor 2 Targeting Antibodies. J Nucl Med. 2011 Nov 1;52(11):1778–85.
3. van Asselt SJ, Oosting SF, Brouwers AH, Bongaerts AHH, de Jong JR, Lub-de Hooge MN, et al. Everolimus Reduces 89 Zr-Bevacizumab Tumor Uptake in Patients with Neuroendocrine Tumors. J Nucl Med. 2014 Jul;55(7):1087–92.
4. Oosting SF, Brouwers AH, van Es SC, Nagengast WB, Oude Munnink TH, Lub-de Hooge MN, et al. 89 Zr-Bevacizumab PET Visualizes Heterogeneous Tracer Accumulation in Tumor Lesions of Renal Cell Carcinoma Patients and Differential Effects of Antiangiogenic Treatment. J Nucl Med. 2015 Jan;56(1):63–9.
5. Cohen R, Vugts DJ, Stigter-van Walsum M, Visser GWM, van Dongen GAMS. Inert coupling of IRDye800CW and zirconium-89 to monoclonal antibodies for single- or dual-mode fluorescence and PET imaging. Nat Protoc. 2013 May 25;8(5):1010–8.
6. Gaykema SBM, Brouwers AH, Lub-de Hooge MN, Pleijhuis RG, Timmer-Bosscha H, Pot L, et al. 89 Zr-Bevacizumab PET Imaging in Primary Breast Cancer. J Nucl Med. 2013 Jul;54(7):1014–8.
7. Themelis G, Yoo JS, Soh K-S, Schulz R, Ntziachristos V. Real-time intraoperative fluorescence imaging system using light-absorption correction. J Biomed Opt. 2009;14(6):064012.
8. European Medicines Agency. ICH Topic M3 (R2), Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals.
9. Marshall M V, Draney D, Sevick-Muraca EM, Olive DM. Single-dose intravenous toxicity study of IRDye 800CW in sprague-dawley Rats. Mol Imaging Biol. 2010;12(6):583–94.
10. European Medicines Agency. Product information Bevacizumab.
11. Atallah I, Milet C, Henry M, Josserand V, Reyt E, Coll J-L, et al. Near-infrared fluorescence imaging-guided surgery improves recurrence-free survival rate in novel orthotopic animal model of head and neck squamous cell carcinoma. Head Neck. 2016 Apr;38(S1):E246–55.
12. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of Protein Pharmaceuticals: An Update. Pharm Res. 2010 Apr 9;27(4):544–75.
13. Rosenthal EL, Warram JM, De Boer E, Chung TK, Korb ML, Brandwein-Gensler M, et al. Safety and tumor specificity of cetuximab-IRDye800 for surgical navigation in head and neck cancer. Clin Cancer Res. 2015;21(16):3658–66.
14. den Engelsman J, Garidel P, Smulders R, Koll H, Smith B, Bassarab S, et al. Strategies for the Assessment of Protein Aggregates in Pharmaceutical Biotech Product Development. Pharm Res. 2011 Apr 23;28(4):920–33.
15. Kahook MY, Liu L, Ruzycki P, Mandava N, Carpenter JF, Petrash JM, et al. HIGH-MOLECULAR-WEIGHT AGGREGATES IN REPACKAGED BEVACIZUMAB. Retina. 2010 Jun;30(6):887–92.
16. Karow AR, Bahrenburg S, Garidel P. Buffer capacity of biologics-from buffer salts to buffering by antibodies. Biotechnol Prog. 2013 Mar;29(2):480–92.
17. Kerwin BA. Polysorbates 20 and 80 Used in the Formulation of Protein Biotherapeutics: Structure and Degradation Pathways. J Pharm Sci. 2008 Aug;97(8):2924–35.
18. Cohen R, Stammes MA, de Roos IH, Stigter-van Walsum M, Visser GW, van Dongen GA. Inert coupling of IRDye800CW to monoclonal antibodies for clinical optical imaging of tumor targets. EJNMMI Res. 2011 Dec 1;1(1):31.

19. Zhegalova NG, He S, Zhou H, Kim DM, Berezin MY. Minimization of self-quenching fluorescence on dyes conjugated to biomolecules with multiple labeling sites via asymmetrically charged NIR fluorophores. Contrast Media Mol Imaging. 2014 Sep;9(5):355–62.

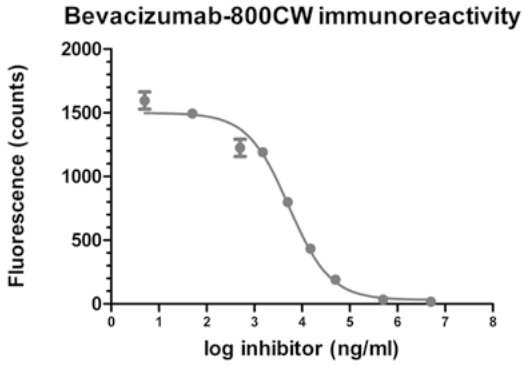
20. Rijpkema M, Bos DL, Cornelissen AS, Franssen GM, Goldenberg DM, Oyen WJ, et al. Optimization of Dual-Labeled Antibodies for Targeted Intraoperative Imaging of Tumors. Mol Imaging. 2015 Jul;14(7):348–55.

21. Mujumdar SR, Mujumdar RB, Grant CM, Waggoner AS. Cyanine-Labeling Reagents: Sulfobenzindocyanine Succinimidyl Esters. Bioconjug Chem. 1996 Jan 1;7(3):356–62.

22. Neergaard MS, Nielsen AD, Parshad H, De Weert M Van. Stability of Monoclonal Antibodies at High-Concentration: Head-to-Head Comparison of the IgG1 and IgG4 Subclass. J Pharm Sci. 2014 Jan;103(1):115–27.

23. U.S. Department of Health and Human services, Food and Drug administration, Center for Drug Evaluation and Research (CDER). Guidance for Industry, Investigators and Reviewers, Exploratory IND Studies. 2006.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1: Example of a graph obtained from an immunoreactivity assay of bevacizumab-800CW.

Supplementary Table 1: Overview of 3 key indicators regarding production of bevacizumab-800CW from February 2014 to February 2015 (n = 29)

Production Date	Concentration IPC1 (mg/mL)	Concentration Final (mg/mL)	Immuno-reactivity (%)*	Aggregates (%)	Free dye (%)
2/10/2015	5.10	1.02	173.4	12.7	0.8
1/27/2015	5.66	0.95	161.7	10.9	0.7
1/15/2015	5.35	0.94	148.3	12.5	0.5
1/5/2015	5.67	0.92		10.5	1.0
11/25/2014	5.74	0.95	162.6	11.9	0.9
11/18/2014	5.63	0.92	167.2	11.6	1.0
11/4/2014	3.64	1.07	189.7	12.1	2.8
10/21/2014	5.20	0.89	176.3	10.6	0.8
10/14/2014	5.36	0.94	169.4	11.3	0.7
9/30/2014	5.38	0.93	149.9	12.2	0.8
9/16/2014	5.60	0.94	160.3	12.0	0.6
9/11/2014	5.24	0.97	159.8	11.3	0.6
8/26/2014	5.61	0.96	159.6	9.97	0.7
7/28/2014	5.58	0.88	168.8	13.1	0.8
7/19/2014	6.15	0.93	161.4	10.5	0.9
7/15/2014	5.32	0.97	168.2	8.6	1.2
7/1/2014	5.57	0.96	133.6	8.8	0.7
6/24/2014	5.74	0.96	140.4	3.6	0.7
6/10/2014	5.90	0.91	127.0	10.6	1.0
6/3/2014	4.73	0.91	147.3	8.6	0.9
5/27/2014	5.17	0.94	144.5	10.5	0.8
4/29/2014	5.44	0.99	167.1	5.2	0.9
4/22/2014	5.15	0.98	165.8	10.4	0.7
4/8/2014	5.41	0.96	150.3	10.2	0.7
4/1/2014	5.53	0.98	163.4	7.1	0.8
3/25/2014	5.44	1.01	165.8	4.3	1.4
3/18/2014	5.48	0.92	111.3	9.4	0.7
3/4/2014	5.54	0.96	146.1	11.9	0.6
2/25/2014	4.21	1.31	174.4	<15	0.6
Mean	5.36	0.96	157.63	10.08	0.87
Standard Deviation	0.49	0.08	16.41	2.45	0.42

*n = 28

Supplementary Table 2: Clinical parameters of male mice.

Group				1				2		3			
Subgroup		**Normal**	**SD**	**Main**	**SD**	**Recovery**	**SD**	**Main**	**SD**	**Main**	**SD**	**Recovery**	**SD**
Weight													
Body	g	30.5*	2.6*	28.6	1.9	32.1	2.6	27	0.9	27.6	1.7	31.7	2.7
Heart	g	0.175*	0.023*	0.154	0.02	0.163	0.006	0.165	0.026	0.158	0.014	0.166	0.008
Lungs	g	0.177*	0.016*	0.219	0.035	0.194	0.019	0.2	0.023	0.202	0.02	0.206	0.024
Liver	g	1.878*	0.248*	2.07	0.23	2.12	0.24	1.76	0.14	1.91	0.22	2.1	0.29
Kidneys	g	0.441*	0.062*	0.489	0.038	0.533	0.038	0.462	0.047	0.459	0.042	0.539	0.074
Haematology													
White blood cells	10^9/L	8.2	1.86	5.6	1.0	6.1	2.4	7.2	1.9	6.7	1.4	5.9	1.0
Red blood cells	10^{12}/L	8.80	0.533	8.51	0.32	9.52	0.74	8.59	0.31	8.59	0.39	9.55	0.37
Hemoglobin	mmol/L	8.9	0.42	8.6	0.2	9.2	0.4	8.5	0.3	8.6	0.2	9.4	0.2
Platelet count	10^9/L	1353	199.0	1687	295	1382	94	1616	314	1783	280	1336	106
Biochemistry													
ALAT	U/L	48.9	17.96	39.3	10.2	44.7	8.7	42	16.3	40.4	10	43.5	9.9
Albumin	g/L	26.0	17.94	24.7	1.8	25.1	1.6	25.5	1.7	25.3	0.9	25.2	1.2
Total globulin	g/L	23.9	1.54	24.5	1.0	23.3	1.0	24.3	1.1	24.9	1.0	23.8	0.9
Total bilirubin	µmol/L	3.8	1.19	3.1	0.9	3.4	0.5	3.2	0.4	5.5	0.4	4.2	0.3
Glucose	mmol/L	14.08	2.470	16.22	2.88	16.3	3.85	15.31	1.7	14.59	1.73	14.09	1.87
Cholesterol	mmol/L	3.56	0.822	3.05	0.3	3.02	0.48	3.03	0.4	2.9	0.43	2.86	0.32
Urea	mmol/L	8.7	1.56	7.1	1.3	10.2	1.0	6.6	0.9	6.4	1.4	9.4	0.7
Creatinine	µmol/L	26.6	2.02	25.9	2.3	25.4	1.4	26.8	1	27.2	1.7	25.1	1.5
Total protein	g/L	50.0	2.00	49.3	1.7	48.5	1.3	49.7	2.4	50.2	1.8	49	2
Sodium	mmol/L	147.1	3.80	146.2	4.8	148.6	1.7	145.8	3.4	145.5	2.2	150	1.7
Potassium	mmol/L	4.33	0.726	3.76	0.25	3.63	0.5	3.82	0.49	3.75	0.47	3.66	0.76
Chloride	mmol/L	111	3.6	107	2	112	2	108	3	107	3	113	2
Calcium	mmol/L	2.37	0.126	2.55	0.1	2.37	0.15	2.51	0.11	2.5	0.07	2.36	0.1

*Data on CD-1 mice obtained from Charles River

Supplementary Table 3: Clinical parameters of female mice

Group				1				2		3			
Subgroup		**Normal**	**SD**	**Main**	**SD**	**Recovery**	**SD**	**Main**	**SD**	**Main**	**SD**	**Recovery**	**SD**
Weight													
Body	g	26.5*	1.5*	21.7	0.9	24.5	0.5	21.2	0.9	21.2	1.4	24.6	1.9
Heart	g	0.153*	0.024*	0.126	0.014	0.14	0.012	0.129	0.012	0.127	0.015	0.136	0.017
Lungs	g	0.167*	0.021*	0.165	0.024	0.183	0.018	0.162	0.016	0.161	0.014	0.189	0.027
Liver	g	1.213*	0.108*	1.26	0.12	1.44	0.11	1.18	0.1	1.2	0.1	1.23	0.13
Kidneys	g	0.28*	0.023*	0.311	0.025	0.341	0.031	0.313	0.036	0.307	0.042	0.339	0.029
Haematology													
White blood cells	10^9/L	4.2	0.9	6.9	1.3	5.9	1.1	4.8	1.1	7.5	2.3	8.8	1.0
Red blood cells	10^{12}/L			9.13	0.44	9.58	0.25	9	0.34	9.19	0.67	10.02	0.35
Hemoglobin	mmol/L	8.5	0.2	9.2	0.5	9	0.3	9.1	0.5	9.1	0.4	9.5	0.1
Platelet count	10^9/L	936	225	1200	326	1280	271	1186	238	1183	123	1055	156
Biochemistry													
ALAT	U/L	34	8	45.5	19.4	39.5	9.0	41.1	11.0	44.1	8.8	40.4	8.5
Albumin				26.1	2.6	25	2.4	26.5	2	26.8	1.2	25.5	1.9
Total globulin				22.8	1.8	20.1	0.9	21.7	1.3	21.8	1.0	20.6	1.3
Total bilirubin	µmol/L	<10		2.7	0.5	4.5	1.2	3.5	0.8	5.2	0.3	4.4	1.0
Glucose	mmol/L	8.6	0.8	14.81	2.79	14.49	2.72	13.91	1.53	15.45	1.75	15.34	3.12
Cholesterol	mmol/L	2.8	0.5	2.26	0.46	2.16	0.34	1.9	0.27	2.12	0.23	2.22	0.49
Urea	mmol/L	7.1	1.6	7.3	0.8	7.6	1.0	6.1	1.1	6.7	0.8	5.7	1.0
Creatinine	µmol/L	30	2	25.5	1.8	23.8	0.7	24.6	1.2	26	1.2	25.3	2.0
Total protein				48.6	3.1	45.1	2.7	48	2.4	47.8	2.4	46.1	3
Sodium	mmol/L	154	2	140.5	5.4	149.3	1.6	148.9	0.8	146.5	4	147.8	0.7
Potassium	mmol/L	3.8	0.2	2.97	0.14	3.67	0.34	3.26	0.2	3.28	0.27	3.79	0.53
Chloride	mmol/L			107	5	115	1	113	1	109	4	115	1
Calcium	mmol/L	2.47	0.03	2.41	0.12	2.18	0.28	2.37	0.12	2.38	0.11	2.27	0.13

*Data on CD-1 mice obtained from Charles River