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Research paper

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 labeling 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 unlabeled 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.

1. 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 tumor 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 tumors. However, the main research focus is on exploiting the intrinsic properties of tumors by labeling 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

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(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 radiolabeled zirconium-89 (^{89}Zr)-bevacizumab already indicated that VEGF-A is a valid target for imaging purposes in different tumor types [2–4], and a concise radiolabeling protocol has been published [5]. Moreover, the feasibility of ^{89}Zr-bevacizumab has been shown in cancer patients with several tumor types using a microdose; a single dose less than 30 nmol (~4.5 mg antibody) [6]. We labeled 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-millimeter lesions [2] using a real-time intraoperative fluorescence imaging system as described earlier [8].

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).

2. 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 2.4 and 2.5 an optimized formulation is described. This product is used in ongoing clinical studies.

2.1. 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, Hoffman La 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 filtrated 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 aluminum 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.

2.1.1. 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 × 7.8; 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.

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 labeling 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 unlabeled 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 the presence of 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 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 labeling 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%