European Journal of Pharmaceutics and Biopharmaceutics 78 (2011) 271–277

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Association of ranibizumab (Lucentis®) or bevacizumab (Avastin®) with dexamethasone and triamcinolone acetonide: An *in vitro* stability assessment

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ARTICLE INFO

Article history:
Available online 21 December 2010

Keywords:
Monoclonal antibodies
Asymmetrical flow field-flow fractionation
Aggregation state
Anti-inflammatory drugs
Neovascular age-related macular degeneration

ABSTRACT

The *in vitro* stability of monoclonal antibodies used for age-related macular degeneration, ranibizumab and bevacizumab, was investigated. The aggregation profile of the antibodies was compared, alone and after association with dexamethasone sodium phosphate or triamcinolone acetonide. Commercial formulations of ranibizumab and bevacizumab were dialysed into three different buffers. After dialysis, samples were stored at 4 °C, 25 °C and 40 °C during 35 days, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide phosphate solution or triamcinolone acetonide suspension. Combined formulations based on both commercial formulations were investigated as well. The aggregation state of the antibodies was measured by multi-angle light scattering (MALS) after separation by asymmetrical flow field-flow fractionation (AFFF) or size-exclusion chromatography (SEC). Ranibizumab results to be more stable than bevacizumab, alone and in combination with dexamethasone sodium phosphate or triamcinolone acetonide. Elevation in concentration, pH and temperature causes a decrease in stability of both antibodies. The association of triamcinolone acetonide phosphate solution with either ranibizumab or bevacizumab is observed to be the least stable combination of all samples tested. Dexamethasone sodium phosphate was shown to have a stabilizing effect on bevacizumab, although this is not the case for its combination with the commercial formulation Avastin®. The results demonstrate that the *in vitro* association of either ranibizumab or bevacizumab with dexamethasone sodium phosphate or triamcinolone acetonide suspension does not decrease the stability of these antibodies. Although ranibizumab is more stable than bevacizumab *in vitro*, further research has to point out how this affects their mechanism of action in vivo.

1. Introduction

Ranibizumab (Lucentis®) is a humanized monoclonal antibody fragment with a molecular weight of 48 kiloDalton (kDa) that inhibits vascular endothelial growth factor (VEGF). It has been registered since 2006 for the treatment of neovascular age-related macular degeneration (AMD) [1,2]. Bevacizumab (Avastin®) is a monoclonal humanized antibody with a molecular weight of 149 kDa that has a comparable mechanism of action, since ranibizumab is a fragment of the same antibody and currently, is widely used off-label for the treatment of AMD [1]. For ranibizumab, a monthly injection is recommended to maintain therapeutically effective drug concentrations [3], and the same frequency is generally reported for bevacizumab injections [2]. Nevertheless, a reduced frequency of injections would be favourable because of patient discomfort and risk of complications [4].

Combination therapy of VEGF-inhibitors with anti-inflammatory drugs such as dexamethasone or triamcinolone acetonide could possibly increase the therapeutic efficacy of the treatment. Anti-inflammatory drugs are well known for their positive effects on AMD [5–8], with a different mechanism of action compared to VEGF-inhibitors [9]. Therefore, combination of the two drugs could lead to a synergistic effect.

Several clinical trials report the co-administration of bevacizumab with triamcinolone acetonide or dexamethasone [5,10–13]. However, in these studies, the possible interaction between bevacizumab and triamcinolone acetonide or dexamethasone has never been taken into account. The antibody-based formulation may aggregate, resulting in serious clinical side-effects, since protein aggregates can reduce the efficacy and enhance the immunogenicity of the protein drug [14–17]. Thus, formation of dimers, trimers or higher order oligomers should be prevented where possible.

The present study focuses on the *in vitro* compatibility of ranibizumab and bevacizumab with dexamethasone sodium

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doi:10.1016/j.ejpb.2010.12.018

phosphate, triamcinolone acetonide phosphate solution and triamcinolone acetonide suspension. The purpose is to investigate whether the monomeric native state of the antibodies is influenced by addition of these anti-inflammatory drugs.

Samples are analysed by asymmetrical flow field-flow fractionation (AFFF). This analytical technique covers a large range of detectable protein sizes, from protein monomers up to subvisible particles [18], which makes it a good candidate for protein aggregation studies. As described by Demeule, AFFF offers the possibility to combine two methodologies [19]: in a first step, hardly any mechanical stress is applied allowing the detection of loose aggregates, followed by a second step in which the different fractions of monomers and aggregates are separated.

2. Methods

Four different series of samples were tested.

2.1. Series I

To obtain a baseline, the aggregation state of both commercial formulations of ranibizumab (Lucentis®, Novartis Pharma Schweiz AG, Bern, Switzerland) and bevacizumab (Avastin®, Roche Pharma, Reinach, Switzerland) was analysed during 35 days of storage at 4 °C, 25 °C and 40 °C. For both formulations, three different sample containers were stored, and for each container, two analyses were carried out directly after preparation (t_0) and after 7, 14 and 35 days. Because of the low inter-sample variability over time shown in these analyses (CV $\leqslant$ 0.5% for both ranibizumab and bevacizumab at all time points and temperatures), all other analyses were carried out in duplicate.

2.2. Series II

Concentrations of 5, 10, 18 and 25 mg/ml ranibizumab and bevacizumab were prepared to study the influence of concentration on the aggregation state. Ranibizumab and bevacizumab were dialysed overnight (Pierce Slide-A-Lyzer Dialysis Cassette, Reactolab, Servion, Switzerland) into a 10 mM histidine buffer pH 5.5 and a 50 mM phosphate buffer pH 6.2, respectively, since these are the buffers used in the commercial products Lucentis® and Avastin®. NaCl was added to the buffers to obtain isotonicity. After dialysis, all samples were concentrated by centrifugation or diluted with buffer to concentrations of 5, 10, 18 and 25 mg/ml. Samples were stored during 35 days at 40 °C.

2.3. Series III

The commercial products Lucentis® and Avastin® were associated with dexamethasone 21-phosphate disodium salt (Sigma–Aldrich, Lausanne, Switzerland), triamcinolone acetonide-21-phosphate dipotassium salt solution (Kenacort A solubile, Dermapharm AG Arzneimittel, Grünwald, Germany) or triamcinolone acetonide suspension (Kenacort A 40, Dermapharm AG Arzneimittel, Grünwald, Germany) to study the influence of a combined formulation on the stability of the antibody.

Both antibodies were stored at 40 °C, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide solution and triamcinolone acetonide suspension. Based on concentrations described in literature for combination therapy with bevacizumab, the following combined formulations were selected:

i. 1.5 mg bevacizumab and 0.8 mg dexamethasone sodium phosphate [5]
ii. 1.25 mg bevacizumab and 2 mg triamcinolone acetonide [10–13] (solution and suspension)
iii. 0.6 mg ranibizumab and 0.8 mg dexamethasone sodium phosphate
iv. 0.5 mg ranibizumab and 2 mg triamcinolone acetonide (solution and suspension)

The dosage of ranibizumab in the combined formulation was chosen to be 2.5 times lower than that of bevacizumab, based on the difference in therapeutic dosage, which is 0.5 mg for ranibizumab compared to 1.25 mg for bevacizumab. The pH of the combined formulations was not adjusted after addition of the anti-inflammatory drugs, in order to stay as close as possible to the clinical studies mentioned earlier.

2.4. Series IV

To evaluate the influence of anti-inflammatory drugs on the antibodies in different buffers and at different pH values, bevacizumab and ranibizumab were associated with dexamethasone 21-phosphate disodium salt, triamcinolone acetonide-21-phosphate dipotassium salt solution or triamcinolone acetonide suspension. Before addition of the anti-inflammatory drugs, both bevacizumab and ranibizumab were dialysed overnight in three different isotonic buffers to change the pH. For bevacizumab, 50 mM acetate buffer pH 5.0, 50 mM phosphate buffer pH 6.2 and 50 mM phosphate buffer pH 7.0 were used. For ranibizumab, 50 mM acetate buffer pH 5.0, 10 mM histidine buffer pH 5.5 and 50 mM phosphate buffer pH 7.0 were chosen. The buffer choice was based on a pH range and buffer capacity that is tolerated by the eye and that is acceptable for the stability of the antibodies [20,21]. A phosphate buffer pH 6.2 and histidine buffer pH 5.5 were selected because these buffers are used in the commercial products Avastin® and Lucentis®, respectively.

After dialysis, bevacizumab was analysed at a concentration of 19 mg/ml (pH 5.0 = 19.8 mg/ml, pH 6.2 = 19.2 mg/ml, pH 7.0 = 18.7 mg/ml) and ranibizumab at 6 mg/ml (pH 5.0 = 6.8 mg/ml, pH 5.5 = 5.9 mg/ml, pH 7.0 = 6.4 mg/ml). Both antibodies were stored at 4 °C, 25 °C and 40 °C, alone and in combination with dexamethasone sodium phosphate, triamcinolone acetonide solution and triamcinolone acetonide suspension. The ratio antibody : anti-inflammatory drug was the same as described in Series III. Again, no pH adjustments were made after addition of the anti-inflammatory drug, to mimic the clinical studies in which the combinations were applied.

2.5. Sample analysis

Samples with a sample volume of 0.5 μl for all bevacizumab samples and 1.0 μl for all ranibizumab samples were analysed directly after preparation (t_0) and after 7, 14 and 35 days. Before analysis, the samples containing triamcinolone acetonide suspension were filtered over a 0.2 μm filter to obtain a visible clear sample for measurement. The weight-averaged molar mass of the antibody fractions was measured by multi-angle light scattering (MALS) after separation by asymmetrical flow field-flow fractionation (AFFF) (Wyatt Technology Europe GmbH, Dernbach, Germany) [22–24]. Since the molar masses of both Avastin and Lucentis are known, the data obtained by MALS can be used to calculate the degree of aggregation. The concentrations of bevacizumab and ranibizumab were determined by UV spectroscopy at 280 nm, based upon an extinction coefficient of 1.7 and 1.8 cm ml/mg, respectively. Data were collected and analysed with Astra software (Wyatt Technology Europe GmbH, Dernbach, Germany). The aggregation state was expressed as the percentage of monomers versus time. For the aggregated fraction, a distinction