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RESEARCH PAPER

Breaking the Aggregation of the Monoclonal Antibody Bevacizumab (Avastin®) by Dexamethasone Phosphate: Insights from Molecular Modelling and Asymmetrical Flow Field-Flow Fractionation

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ABSTRACT

Purpose To investigate the mechanism behind the aggregation breaking properties of dexamethasone phosphate and related corticosteroids on the IgG1 antibody bevacizumab (Avastin®).

Methods An *in silico* 3D dimer model is developed to identify the bevacizumab-bevacizumab interface, and different corticosteroids are docked onto the model to distinguish preferred binding sites. *In silico* predictions are validated by *in vitro* stability studies, where the antibody is stressed in presence or absence of each corticosteroid and formed aggregates are quantified by asymmetrical flow field-flow fractionation.

Results The dimer model features one close crystal contact area: Lys445 on the Fc region interacts with one Fab arm of the second bevacizumab. Docking reveals an interaction between the phosphate group of dexamethasone phosphate and Lys445, while the rest of the molecule is hindering dimer formation. Predictions are confirmed *in vitro*, demonstrating that dexamethasone phosphate and betamethasone phosphate partly prevent antibody aggregation, whereas triamcinolone acetonide phosphate does not.

Conclusions Results suggest that bevacizumab monomers follow a specific mechanism to form dimers in which a protein-protein interaction hotspot can be distinguished. The dimer formation can be hindered by corticosteroids in a specific way. This approach allows a simple way to stabilize IgG1 antibodies.

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KEY WORDS aggregation breaker · asymmetrical flow field-flow fractionation · corticosteroids · molecular modelling · monoclonal antibody

ABBREVIATIONS

AF4	asymmetrical flow field-flow fractionation
Ig	immunoglobulin
MALS	multi-angle light scattering
PDB	protein data bank
SEC	size exclusion chromatography
VEGF-A	vascular endothelial growth factor A

INTRODUCTION

Therapeutic antibodies are currently the fastest growing area of biopharmaceuticals with an average yearly market growth rate of 35% since 2001 (1). The recent developments of chimeric and fully-humanized monoclonal antibodies have spawned an unprecedented interest in using these molecules as therapeutic agents, since they can specifically target molecules implicated in

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disease, thereby essentially side-stepping the secondary effects that may be associated with conventional drug therapies.

The development of stable antibody formulations is challenging, since they have the tendency to be physically and chemically instable in aqueous media. Antibodies are, for example, susceptible to denaturation, aggregation and covalent modifications (2,3). Degradation of antibody formulations due to aggregation phenomena is a particular problem. The formation of aggregates might lead to a reduced efficacy of the protein drug. Since aggregates can increase the immunogenicity of the antibody (4), clinical side-effects might occur. Antibody aggregation is also a source of batch-to-batch variations in the antibody production chain and its control leads to regulatory and quality control burdens with their associated costs. Furthermore, the propensity of antibodies to aggregate decreases the stability of the formulation, which negatively affects product shelf-life (5).

Like for most other proteins, antibody stability depends on many factors, like protein concentration, pH, ionic strength, temperature and agitation. Predicting whether an antibody will aggregate is challenging, notably because each antibody may have a very specific and characteristic stability profile (6). Moreover, due to the complexity of the antibody structure, identification of the precise nature of the antibody-antibody interaction and contact surfaces of aggregates remains challenging (7). A number of approaches have been investigated to improve antibody stability, including the addition of "stabilizing" agents to the protein formulation. Examples of these agents are polysorbate-based surfactants (8), amino acids (9), glucose (10), sorbitol (10), and dextran sulphate (9). Unfortunately, their success has been limited, mainly due to the fact that most of these agents are directed at optimizing the environment surrounding the antibody, and not specifically at interfering with the interaction hotspot involved in the formation of aggregates. Another concept to ameliorate the stability of the protein drug is to mutate single amino acids in Igs to specifically target hydrophobic patches implicated in aggregation (7). However, such an approach necessarily modifies the structure of the Ig, possibly affecting both the clinical efficacy and immunogenicity of the protein drug.

The humanized monoclonal IgG1 antibody bevacizumab (Avastin®) is registered for the treatment of different forms of cancer and has also been widely used off-label for the ophthalmic indication age-related macular degeneration. It acts by blocking vascular endothelial growth factor A (VEGF-A), thereby inhibiting neovascularisation and leakage of blood vessels (11). The present study is based on a clinical observation made earlier by our group (12), in which the combination of bevacizumab with the anti-inflammatory drug dexamethasone disodium phosphate led to a decrease in aggregate formation. The rationale behind the combination of bevacizumab with dexamethasone phosphate is the fact that the latter also suppresses the formation of new leaky blood vessels. Thus, a synergistic effect might be obtained through combination therapy (13).

The aims of this study are to clarify the mechanisms behind this protective process through a combination of *in silico* and *in vitro* studies. The formation of bevacizumab dimers is investigated by homology modelling and the interactions between several anti-inflammatory drugs and the antibody are studied by molecular docking. Outcomes of the *in silico* work are experimentally validated through *in vitro* stability studies on the combined formulations using Asymmetrical Flow Field-Flow Fractionation and Size Exclusion Chromatography coupled to Multi-Angle Light Scattering (AF4-MALS and SEC-MALS).

MATERIALS AND METHODS

Molecular Modelling

Template Identification and Alignment

Bevacizumab is a humanized antibody formed by a Fab region responsible for recognizing antigens and a Fc region derived from IgG1. The Protein Data Bank (PDB) (http://www.rcsb.org) (14,15) was surveyed for suitable crystal structures to be used as structural templates for homology modelling. Three structures were found: the bevacizumab Fab moieties in complex with the human VEGF (PDB id: 1BJ1) (16) and two full length IgG1 antibodies, a human (PDB id: 1HZH) and a murine one (PDB id: 1IGY). Because bevacizumab is a humanized antibody, the human antibody logically should be taken as template. However, the only full-length human IgG1 crystallized so far (PDB id: 1HZH) has an unusual crystal symmetry ($H3_2$) and strongly distorted orientations of the Fabs with respect to the axis of the Fc region (17). In contrast, the intact murine IgG1κ is a template with a more usual overall 3D structure. With a resolution of 3.2 Å (PDB id: 1IGY), this structure is acceptable as template. To humanize its hinge and Fc regions, the residues were mutated so as to match the sequence of the structure 1HZH. All sequence alignments were carried out with ClustalW (18,19) in the Biology Workbench 3.2 (San Diego Supercomputer Center; http://seqtool.sdsc.edu/CGI/BW.cgi). To nonetheless compare the symmetric murine configuration with an asymmetric model, the human template (1HZH) was used to build a second model.

3D Model Building and Validation

The initial 3D model of bevacizumab was carried out using Sybyl 8.0 (Tripos Inc., St. Louis, MO, USA). The Fabs of IgG1 (1IGY) were structurally superposed and thereafter replaced with the ones of bevacizumab (1BJ1). Then, the bevacizumab Fabs were connected with the hinge-Fc of IgG1 (1IGY) and finally, the hinge-Fc region was humanized using the sequence of 1HZH. The connecting region of the model was submitted to energy minimization using Sybyl 8.0 default parameters while keeping the disulfide bridges intact. The quality of the resulting model was assessed using Procheck (20). A Ramachandran plot analysis (in Procheck) of the amino acid conformations was carried out using a resolution mean between the crystal structures of both the Fc (resolution of 1IGY: 3.2 Å) and the Fabs (resolution of 1BJ1: 2.4 Å). Critical side chains were corrected for distortion in Sybyl 8.0 and the refinement procedure (manual adjustments and minimization) was repeated until reaching φ and ψ angles in the Ramachandran plot that were comparable to the input crystal structures. An analogous procedure was applied for the second model based on 1HZH.

3D (Dimer) Aggregation Model

A 3D aggregation model was built to identify the bevacizumab-bevacizumab interface, with the idea in mind that crystal contacts represent privileged protein-protein interfaces. Based on the crystal symmetries of the IgG1 structure 1IGY and subsequently on the IgG1 structure 1HZH, putative crystal contacts between two full length bevacizumabs were defined using SwissPDB-Viewer 4.0.1 (21). For the 3D aggregation model based on 1IGY, the crystallographic symmetry ($P2_1$) of the IgG1 crystal structure was applied to obtain a layer, which was translated along the unit cell, and to recreate crystal contacts. The first translation, featuring the only close crystal contact between the monomers in the order of 4 Å, was saved in pdb format and the bevacizumab model overlaid according to the carbon α atom positions in Sybyl 8.0. The second model based on the crystal structure of 1HZH was obtained by applying the same procedure. Its translation according to the crystal symmetry of 1HZH was also displayed to verify whether close crystal contacts would occur in the same region for this asymmetric conformer as for the symmetric 1IGY.

Docking Dexamethasone Phosphate and Similar Anti-Inflammatory Drugs onto the Bevacizumab Monomer

Dexamethasone phosphate and two similar, commercially available anti-inflammatory drugs (betamethasone phosphate and triamcinolone acetonide phosphate) were docked onto the bevacizumab monomer to see where they were binding preferentially, whether binding took place in the aggregation zone and if so, how the observed binding propensities could be related to properties of the small molecules and the binding site, respectively. The small molecule setup for docking was done in Sybyl 8.0. Hydrogens were added to the small molecular weight molecule, the phosphate group was left unprotonated and Gasteiger-Hückel charges were calculated and added. Each resulting molecule was minimized using 100 steps of the default Powell minimization protocol of Sybyl 8.0. Systematic flexible docking with standard parameters was then performed with FlexX 3.1.3 (Biosolveit GmbH) all over the bevacizumab antibody surface (monomer: Fabs, hinge and Fc), previously divided into several segments of 10 Å around each arginine and lysine. By adopting this strategy, roughly 90% of the whole antibody surface and all major cavities were included in the docking study.

Based on the binding propensities of dexamethasone phosphate at the bevacizumab-bevacizumab interface and the physicochemical properties of the ligand and the binding site on one monomer, a putative mechanism of action was postulated. This mechanism was subsequently verified by docking betamethasone phosphate and triamcinolone acetonide phosphate in the same way as described for dexamethasone phosphate, in order to find out whether they would bind in the antibody-antibody contact region. Ten docking poses per molecule and docking site were kept, as this number revealed later to be sufficient to distinguish between aggregation breakers and non-aggregation breakers. The poses were scored with the FlexX scoring function. Evaluation of docking results was based on the attributed bevacizumab-small molecule interactions score, and the subsequent visual inspection was performed by considering the context of both the monomer and the dimer antibody models, using a cylindric "volume of interference" (Supplementary Material, Figure S1). The best-ranked poses projecting roughly orthogonally outwards from the bevacizumab surface and thus enclosed by this volume of interference, were retained.

***In Vitro* Experiments**

Sample Preparation

Three anti-inflammatory drugs were tested for their aggregation breaking properties, following the modelling outcomes:

i. Dexamethasone 21-phosphate disodium salt (Sigma-Aldrich, Lausanne, Switzerland),
ii. Betamethasone 21-phosphate disodium salt (Sigma-Aldrich, Lausanne, Switzerland), and

iii. Triamcinolone acetonide-21-phosphate dipotassium salt solution (Kenacort A Solubile, Dermapharm AG Arzneimittel, Grünwald, Germany).

All were combined with bevacizumab (Avastin®, Roche Pharma, Reinach, Switzerland) in a 1:150, 1:15 and/or 1:1.5 molar ratio (antibody : anti-inflammatory drug). Ratios were derived from clinical studies in which bevacizumab was combined with dexamethasone disodium phosphate in a molar ratio of 1 mol bevacizumab *versus* 150 mol dexamethasone disodium phosphate (13).

Bevacizumab was used as commercial formulation, hereinafter referred to as Avastin®, in which the antibody is formulated in a 51 mM phosphate buffer pH 6.2 with 60 mg/ml α,α-trehalose dihydrate and 0.04% polysorbate 20, or was dialyzed overnight (Pierce Slide-A-Lyzer Dialysis Cassette, Reactolab, Servion, Switzerland) into 50 mM phosphate buffer at pH 7, hereinafter referred to as bevacizumab. The concentration of bevacizumab was kept at 25 mg/ml in all series to simulate the concentration of the commercial formulation, since dilution may lead to reversion of small aggregates into monomers (6). After dialysis, the antibody formulation was combined directly with one of the three anti-inflammatory drugs or incubated at 40°C for one week, before addition of the anti-inflammatory drugs. Temperature and pH were chosen in order to accelerate the formation of aggregates, since an ongoing aggregation process is needed to observe a stabilizing effect of the different anti-inflammatory drugs in a reasonable time frame. Osmolality and pH were controlled and stayed constant over the duration of the study. The pH and osmolality of the samples containing antibody alone were similar to those in which the antibody was combined with a corticosteroid. Over time, all samples remained clear solutions and no insoluble aggregates were observed upon visible inspection.

It should be noted that the commercially available product Avastin® contains 0.04% polysorbate 20. Although the product was dialysed for a part of the studies, this does not lead to the complete removal of polysorbate (22). Thus, it should be taken into account that all observed effects of the corticosteroids on the antibody occur in the presence of this surfactant.

Inter-sample variability was observed when different batches of Avastin® were dialysed, with a maximum variability of 11%. In other words, the same treatment and stress did not always lead to the same amount of aggregates, possibly due to an inter-batch variability in the polysorbate concentration (22). Therefore, comparisons were always made on the same batch, meaning that the sample to which the corticosteroid was added was always compared with a sample of the antibody alone from the same batch.

Series I: Effect of Dexamethasone Phosphate on Aggregation Formation Induced by Temperature Stress

Three different concentrations of dexamethasone phosphate in 51 mM phosphate buffer pH 6.2 were added to the commercial formulation of Avastin® to obtain Avastin:dexamethasone disodium phosphate molar ratios of 1:1.5, 1:15 and 1:150. To the sample containing the antibody alone, the same volume of 51 mM phosphate buffer pH 6.2 was added, to avoid differences in antibody concentration between the samples with and without anti-inflammatory drug. After addition, Avastin® alone and the combined formulations were all stored for 35 days at 40°C. Samples were analysed directly after preparation (t_0) and after 7, 14 and 35 days. Based upon the outcomes of this series, in which the 1:15 molar ratio showed to be optimal, it was decided to perform all other series with this particular molar ratio.

Series II: Effect of Dexamethasone Phosphate on Aggregation Formation Induced by Mechanical Stress

Bevacizumab was combined with dexamethasone phosphate in a 1:15 molar ratio, after dialysis of the antibody into 50 mM phosphate buffer at pH 7. The same volume of buffer was added to the sample containing antibody alone. Both samples were placed vertically in a Thermomixer (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany) and were horizontally shaken at controlled room temperature for 48 h at 1,000 rpm. Samples were analysed at t_0 and at 1, 4, 24 and 48 h.

Series III: Effect of Three Anti-Inflammatory Drugs on Prestressed Antibody

Bevacizumab was dialysed into 50 mM phosphate buffer at pH 7 and stored at 40°C for 7 days. After 7 days, dexamethasone phosphate, betamethasone phosphate and triamcinolone acetonide phosphate were added to the antibody in a 1:15 molar ratio. The same volume of buffer was added to the sample containing antibody alone. All samples were stored for 28 days at 40°C and analysed at t_0 and after 1, 7, 14 and 28 days.

Series IV: Effect of Dexamethasone Phosphate on Prestressed Fab Fragment

The stabilizing properties of dexamethasone phosphate on the commercially available Fab-fragment ranibizumab (Lucentis®, Novartis Pharma Schweiz AG, Bern, Switzerland) were investigated as well. Ranibizumab was dialysed overnight into a 50 mM phosphate buffer at pH 7 and concentrated to 10 mg/ml (based on the concentration of the commercial formulation). Since the fragment is very stable, the sample was stored for 1 year at 25°C before the

addition of dexamethasone disodium phosphate in a 1:15 molar ratio. To the sample that contained the Fab fragment alone, a similar volume of buffer was added. Both the sample of ranibizumab alone and the combined formulation were stored for 28 days at 40°C. Samples were analysed at t_0 and after 1, 7, 14 and 28 days.

Sample Analysis

Asymmetrical Flow Field-Flow Fractionation. All samples were analyzed by asymmetrical flow field-flow fractionation (AF4) (Wyatt Technology Europe GmbH, Dernbach, Germany), coupled to multi-angle light scattering (MALS) and UV spectroscopy at 280 nm (23). This technique allows the measurement of aggregates up to the subvisible range (24). Samples were injected undiluted; the injection volume per run depended on the concentration of the formulation and was 0.5 μl for bevacizumab (25 mg/ml) and 1.0 μl for ranibizumab (10 mg/ml). The applied mobile phase was similar to the buffer of the analyzed formulations, i.e. 51 mM phosphate buffer pH 6.2 for the commercial formulation and 50 mM phosphate buffer pH 7.0 for the dialysed samples. MALS detection allowed calculating the weight-average molar mass of the antibody fractions that were separated by AF4, thus providing information on the aggregation state of the particular fraction (a fraction with a molar mass of e.g. twice the monomer contains dimers, etc.). Bevacizumab and ranibizumab concentrations were determined by UV spectroscopy, based upon an extinction coefficient of 1.7 and 1.8 cm*ml/mg, respectively. Data collection and analysis were done using Astra software (version 5.1.9.1, Wyatt Technology Europe GmbH, Dernbach, Germany) and the different fractions were classified as monomers, dimers, trimers or higher order aggregates. Because of the low inter-sample variability observed over time using this method (12), one sample container was stored for all samples and analyses were carried out in triplicates. All data were reported as average percentage of monomers/aggregates ± standard deviation (SD) (n=3). Pair-wise comparisons were carried out to compare the antibody alone with the combined formulations using a Mann–Whitney U test (exact one-tailed significance, $p \leq 0.05$).

Size Exclusion Chromatography. In order to confirm the data obtained by AF4 using an orthogonal technique, part of the samples was analysed by size exclusion chromatography (SEC) as well. A TSK G3000 SW_{XL} column, 7.8×300 mm (Tosoh Bioscience GmbH, Stuttgart, Germany) was combined with the same UV and MALS detectors used for the AF4 analyses. Separation was performed at 25°C at a flow rate of 0.5 ml/min, using a 200 mM potassium phosphate buffer with 250 mM KCl pH 7.0 as a mobile phase. 2 μl undiluted sample were injected per run. Data collection and analysis were performed as described for AF4.

RESULTS

Molecular Modelling

3D Monomer- and Aggregation Model

The geometric quality of the final "symmetric" monomer model based on 1IGY (Fig. 1) consisting of the bevacizumab Fabs and the humanized hinge-Fc is comparable to that of the input template structures, with 74.9% of the residues in the model found in most favoured, 21.4% in additionally allowed, 2.2% in generously allowed and 1.5% in disallowed areas of the Ramachandran plot. The aggregation model obtained by replicating the monomer model according to the crystal symmetry ($P2_1$) of the murine IgG1 (PDB id: 1IGY) (Fig. 1a) shows a single specific close contact area (least distance in the order of 4 Å) between the two monomers (Fig. 1b). The bottom of one Fc comes to lie in between the two Fab regions of the other bevacizumab monomer. More specifically, this specific contact is characterized by the interaction of Ser202 (1BJ1 naming and numbering), belonging to one Fab arm of one bevacizumab, and Lys445 (1HZH and 1IGY naming and numbering), which is part of the Fc region of the second bevacizumab. Lys445 and Ser202 form the major interaction between the monomers and are fully conserved in engineered IgG1-based sequences (Supplementary Material, Figures S2-S6). The amino acids lining the contact area include for the Fab region His198 to Pro204 (1BJ1 naming and numbering) and for the Fc region Met381 to Val386 and Val443 to Glu449 (1IGY naming and numbering). Comparing the murine (PDB id: 1IGY) with the human Fc (PDB id: 1HZH), the residues surrounding and including Lys445 are well conserved from a sequence (Supplementary Material, Figure S2A) and structural (Supplementary Material, Figure S5) point of view. The non-conserved amino acids are not involved in inter-antibody interactions and are thus thought to only marginally affect the prediction outcomes for potential aggregation breakers. The aggregation model based on the human framework shows, similar to the murine model, a close contact area between the region around Lys445 and the Fab fragment, although due to the asymmetric Fab arrangement, another region on the Fab is involved. As expected for a Fab region, this region is not fully conserved among pharmaceutically relevant antibodies (Supplementary Material, Figure S7), but the hydrophilicity of the corresponding residues is conserved.

Docking of Anti-Inflammatory Drugs

Concerning the docking of the steroids all over the monomer antibody model, highest docking scores (reflecting the antibody-small molecule interaction energy) are obtained for phosphate-containing steroids binding to Lys445 at the

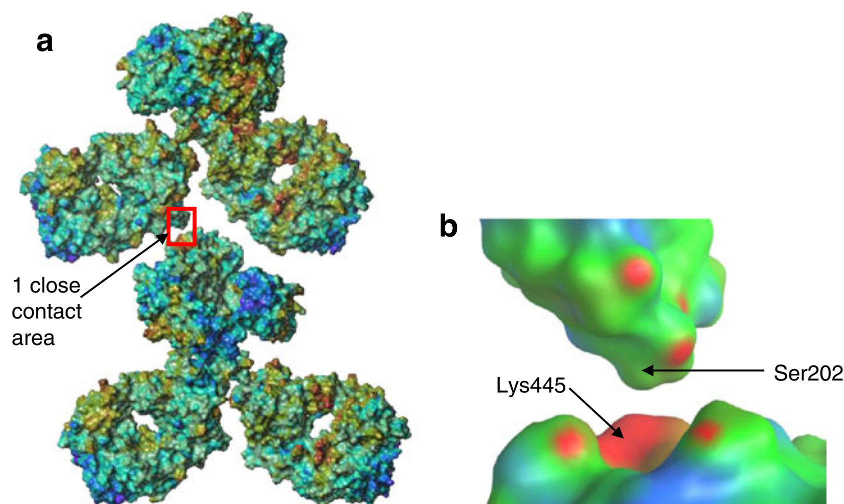

Fig. 1 The 3D aggregation model (**a**) of bevacizumab and a zoom on the contact region (**b**). The aggregation model was obtained by replicating the monomer model according to the crystal symmetry ($P2_1$) of the template IgG1. The least distance in the contact region between Lys445 (mouse and human IgG1 naming and numbering, PDB ids: 1IGY and 1HZH) and Ser202 (bevacizumab Fab naming and numbering, PDB id: 1BJ1) is in the order of 4 Å. The colouring gradient of the electrostatic potential surface goes from blue for negatively charged to red for positively charged atoms. (**a**) and (**b**) were prepared using Sybyl 8.0 (Tripos Inc.) and MOE 2011 (Chemical Computing Group), respectively.

aggregation interface. Interactions with other lysines are less favourable, and the docking poses around different arginines receive even lower scores. For all shown docked compounds, a strong electrostatic interaction between the phosphate of the ligand and the Lys445 side chain is noted (Fig. 2d for dexamethasone phosphate, Fig. 2e for betamethasone phosphate and Fig. 2f for triamcinolone acetonide phosphate). To provide an order of the strength of this interaction, an exposed salt bridge is known to be as strong as a neutral H-bond (5 ± 1 kJ/mol) (25,26). Additional H-bonds between residues adjacent to Lys445 and the phosphate further stabilize the steroid in the binding pocket. Among the steroids docked to this region, the propensity of the 10 generated poses to interfere with the adjacent antibody was highest for dexamethasone phosphate and betamethasone phosphate, with the higher scored docking poses all interfering. For triamcinolone acetonide phosphate, this propensity was only at 3 out of 10 poses. Because in our case, the docking scores were not informative with respect to their antibody-antibody breaking ability, we first displayed the docking poses of each small molecule on the aggregation model and then visually defined a « volume of interference » (Supplementary Material, Figure S1). The simplest geometric shape including all the dexamethasone phosphate or betamethasone phosphate aggregation breaking poses was defined using a cylinder. The centre of its base was put to the Cα atom of Lys445, with the plane of the base including the N atom of Lys445, and the radius was set to 7 Å. A height of 12 to 15 Å was drawn orthogonally from the base using Fc atoms situated approximately on the surface of the circle in vicinity of the adjacent Fab of the other antibody monomer. All docked steroids overlap in vicinity to the Fc, with their phosphates binding to the Lys445 side chain. While dexamethasone phosphate and betamethasone phosphate are interfering with the Fab of the adjacent antibody, triamcinolone acetonide phosphate is generally not. A score of 5 was obtained for dexamethasone phosphate and betamethasone phosphate, while a score of 2 was obtained for triamcinolone acetonide phosphate. The same scores were also achieved when generating 100 docking poses. Figures 2a, b and c are representative figures showing how dexamethasone phosphate, betamethasone phosphate and triamcinolone acetonide phosphate are positioned on the monomer. The moieties of dexamethasone phosphate and betamethasone phosphate that are clashing with the second monomer in the aggregation model are clearly visible in Fig. 2g and h, respectively. In contrast, triamcinolone acetonide phosphate does not clash into the second monomer (Fig. 2i), which is probably due to the acetonide part that confers rigidity and a different spatial orientation to the compound.

In Vitro Experiments

Series I: Effect of Dexamethasone Phosphate on Aggregation Formation Induced by Temperature Stress

Over time, storage at 40°C leads to differences in the decrease in monomer percentage for Avastin® alone or in presence of dexamethasone phosphate (Supplementary Material, Figure S8). The association of Avastin® with dexamethasone phosphate after 35 days of storage at 40°C is depicted in Fig. 3 (and Supplementary Material, Table SI). The commercial product alone comprises 88.3 ± 0.2% of monomers, e.g. 11.7 ± 0.2% of the antibody has formed

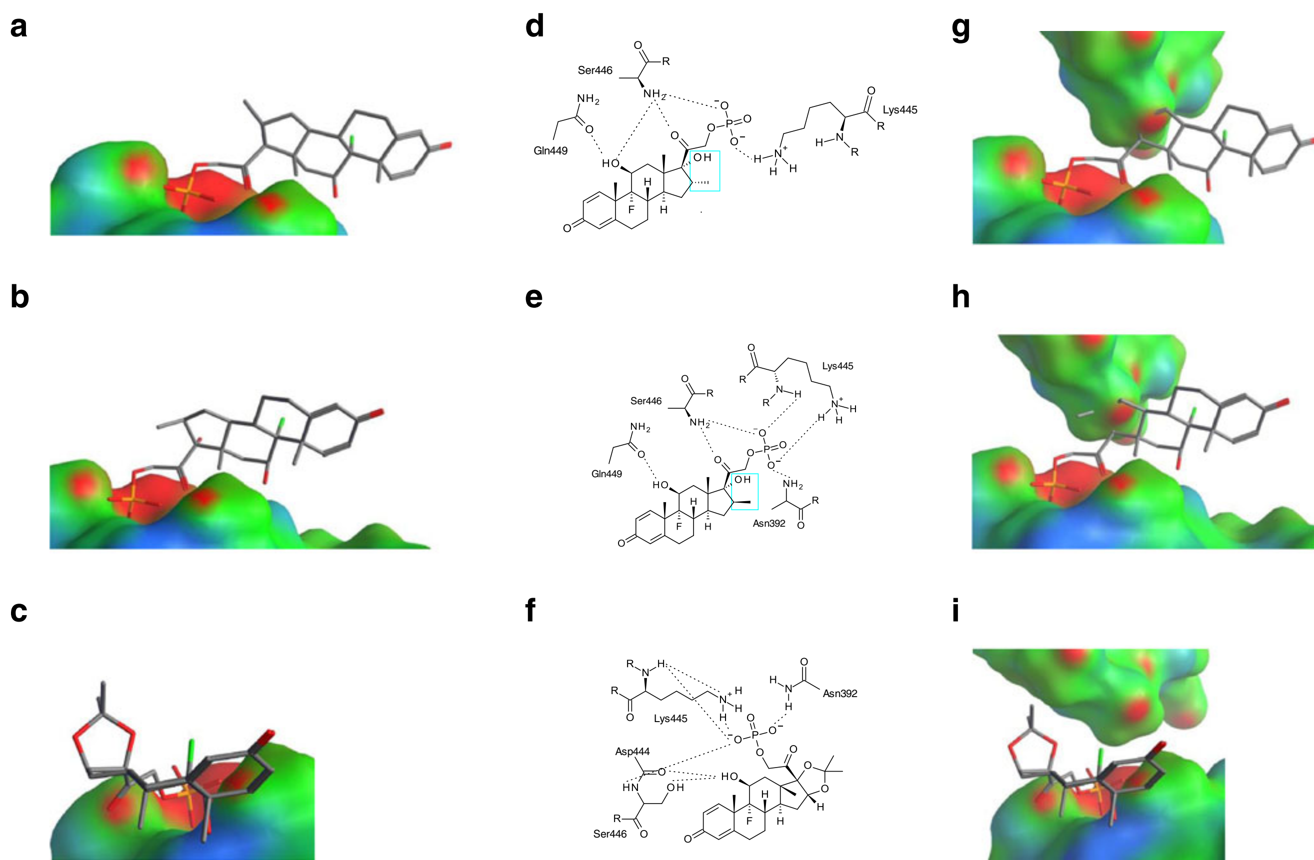

Fig. 2 Representative docking poses of dexamethasone phosphate (**a**, **d** and **g**), betamethasone phosphate (**b**, **e** and **h**) and triamcinolone acetonide phosphate (**c**, **f** and **i**), respectively. Docking poses on the bevacizumab monomer are displayed in **a**, **b** and **c** using MOE 2011 (Chemical Computing Group). Interactions between the docked steroid and the bevacizumab monomer are drawn in a schematic way in **d**, **e** and **f** using SymyxDraw 3.3. The *dotted lines* represent H-bonds or electrostatic interactions and the *cyan box* the parts of the steroid that clash with the second monomer of the aggregation model. The same poses are also represented in the aggregation model (**g**, **h**, **i**). MOE (Chemical Computing Group) was used for producing the monomer and dimer views, where the carbon atoms of the steroids are drawn in grey. The colour gradient for the electrostatic potential surface is the same as for Fig. 1.

aggregates, consisting of dimers (10.16±0.04%) and trimers (1.5±0.2%). The stabilizing effect of dexamethasone phosphate is observed for all three molar ratios, being most pronounced for the 1:15 combination, which contains only 4.3± 0.5% dimers and 0.2±0.2% trimers. All three combined formulations show a significant difference in aggregation percentages compared to Avastin® alone (Mann–Whitney U, one-tailed $p \leq 0.05$). Very low percentages of higher order aggregates (0.1±0.2% decamers) were only observed for the 1:150 molar ratio.

Series II: Effect of Dexamethasone Phosphate on Aggregation Formation Induced by Mechanical Stress

Figure 4 shows the stabilizing effect of dexamethasone phosphate on bevacizumab during stress through agitation. Mechanical stress of the sample of bevacizumab alone leads to increased aggregation: The total percentage of aggregates doubles from 9.2±0.6% after one hour of agitation to 18± 1% after 48 h. In contrast, after one hour of agitation, the combined formulation (with a 1:15 molar ratio) shows an aggregation breaking effect, attributed to the presence of dexamethasone phosphate: Instead of 9.2±0.6%, a total aggregate percentage of 4.2±0.2% is measured. This percentage even slightly decreases to 3.8±0.2% after 48 h of agitation, indicating a stabilizing effect as well. The aggregation species that are observed are dimers and trimers for both samples. Differences in the amount of aggregates between the samples are significant (Mann–Whitney U, one-tailed $p \leq 0.05$) at all time points.

Series III: Effect of Three Anti-Inflammatory Drugs on Prestressed Antibody

The addition of betamethasone phosphate in a 1:15 molar ratio to the prestressed antibody shows aggregation breaking properties of the corticosteroid (Fig. 5). The results are comparable to the formulation in which dexamethasone phosphate is combined with the prestressed antibody (Table I, Fig. 5). After 28 days, 63.3±0.5% of monomers

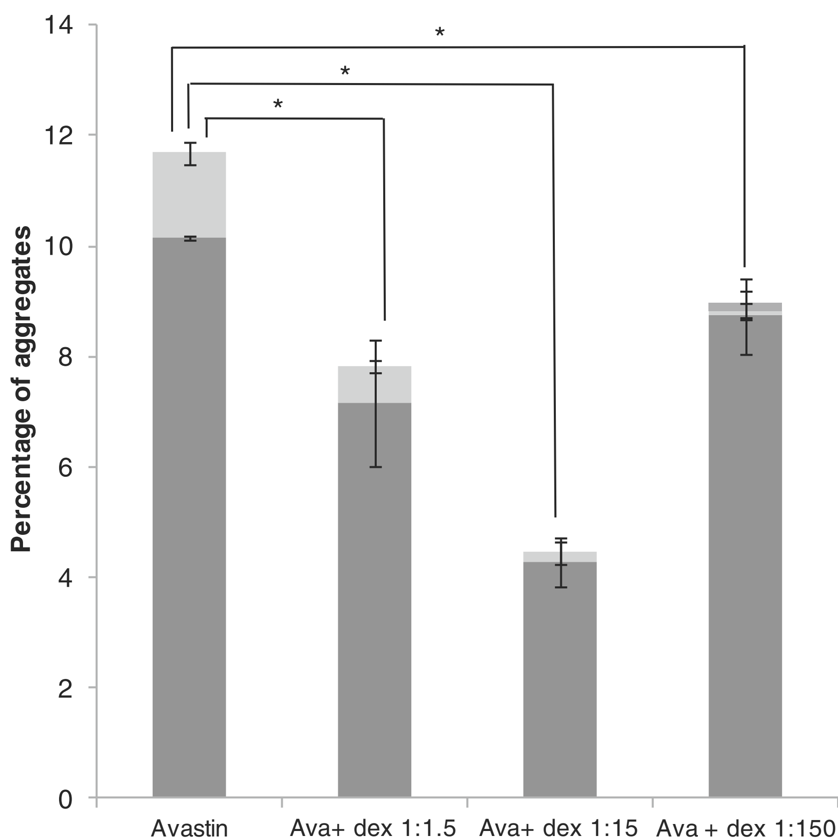

Fig. 3 Avastin® commercial formulation alone (Ava) and in combination with dexamethasone phosphate (dex) in a 1:1.5, 1:15 and 1:150 molar ratio after 35 days of storage at 40°C. Quantification of aggregates was performed by AF4 coupled to MALS. Percentages of aggregates are expressed as average ± SD (n=3), *dark grey* = dimers, *light grey* = trimers, *intermediate grey* = higher order aggregates. * Significantly different from the percentage of aggregates of Avastin® alone (Mann–Whitney U test, $p \leq 0.05$, exact one-tailed significance).

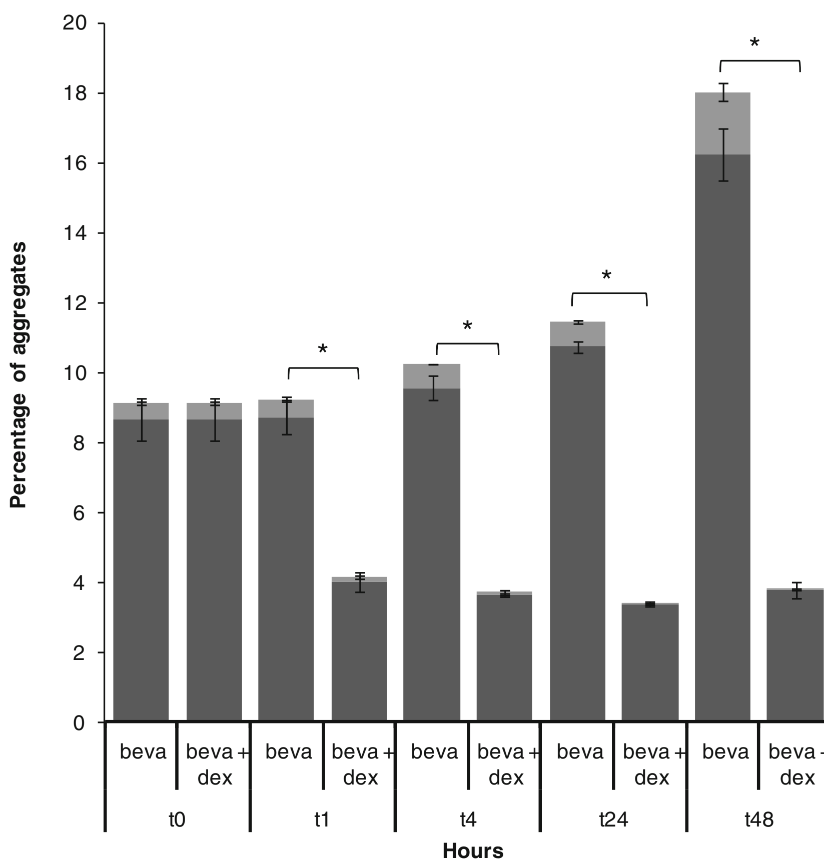

Fig. 4 Bevacizumab alone (beva) and in combination with dexamethasone phosphate in a 1:15 molar ratio (dex). Samples were stressed by agitation during 48 h. Quantification of aggregates was performed by AF4 coupled to MALS. Percentages of aggregates are expressed as average ± SD (n=3), *dark grey* = dimers, *light grey* = trimers. *Significantly different from the percentage of aggregates of bevacizumab alone (Mann–Whitney U test, $p \leq 0.05$, exact one-tailed significance).

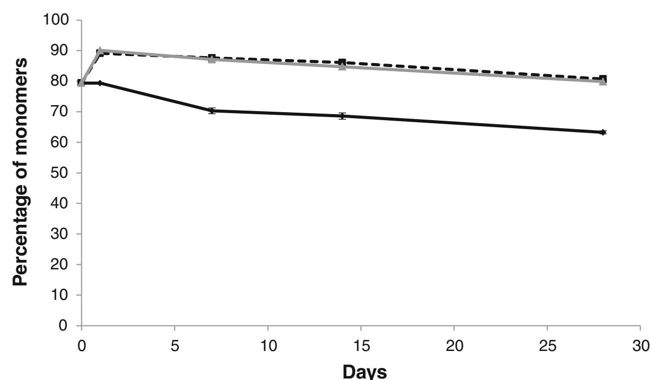

Fig. 5 Percentage of monomers for bevacizumab alone (*black curve*) and in combination with dexamethasone phosphate (*black dashed curve*) and betamethasone phosphate (*grey curve*) in a 1:15 molar ratio after 28 days at 40°C. Quantification of aggregates was performed by AF4 coupled to MALS. Bevacizumab was stressed for 7 days at 40°C before the addition of the steroid drugs. Percentages are expressed as average ± SD, shown as vertical bars (n=3).

are present in the bevacizumab sample, compared to 80.73 ± 0.06% for the combination with dexamethasone phosphate (Table I) and 79.9 ± 0.3% for betamethasone phosphate (results not shown). Differences in aggregation between the antibody alone and the combined formulations are significant (Mann–Whitney U, one-tailed $p \leq 0.05$). Moreover, the aggregated fractions of both combined formulations only consist of dimers (19.27 ± 0.06% and 20.1 ± 0.3%, respectively), while the antibody alone forms both dimers (26.5 ± 0.1%) and trimers (10.3 ± 0.4%) (As depicted for bevacizumab alone or in combination with dexamethasone phosphate in Supplementary Material, Figure S9).

In order to check the data obtained by AF4 with an orthogonal technique, the combination of the prestressed antibody with dexamethasone phosphate was investigated by SEC. The percentages of monomers found by SEC are in agreement with those detected by AF4, especially considering the fact that both analyses were carried out on different vials (Table I). Thus, these data confirm the aggregation breaking properties of dexamethasone phosphate on the IgG1-based antibody. It should be noted that SEC detects more separate fractions than AF4: Fragments and higher order aggregates are present in the samples analysed by SEC, but absent when the analyses are performed by AF4. It is unclear whether these fractions are created during the analysis, or whether SEC enables better separation.

In contrast to the aggregation breaking effect seen with dexamethasone phosphate and betamethasone phosphate, the addition of triamcinolone acetonide phosphate only has an effect at t_1, thereafter the combination does not significantly affect the aggregation profile of the antibody (Supplementary Material, Figures S10 and S11). The samples with and without steroid drug show similar percentages of aggregates after 28 days of storage: In the bevacizumab sample, 20.8 ± 0.6% of dimers, 3.5 ± 0.2% of trimers and 0.9 ± 0.2% of higher order aggregates are present, compared to 20.2 ± 0.2% of dimers, 3.5 ± 0.2% of trimers and 0.95 ± 0.09% of higher order aggregates for the combined formulation.

Table I Prestressed Bevacizumab (7 Days at 40°C) Alone and in Combination with Dexamethasone Phosphate in a 1:15 Molar Ratio After 28 Days at 40°C

AF4	Bevacizumab	Bevacizumab + dexamethasone phosphate 1:15
Fragments (%)	Not detected	Not detected
Monomers (%)	63.3 ± 0.5	80.83 ± 0.06*
Dimers (%)	26.5 ± 0.1	19.27 ± 0.06
Trimers (%)	10.3 ± 0.4	Not detected
Higher order aggregates (%)	Not detected	Not detected
SEC	**Bevacizumab**	**Bevacizumab + dexamethasone phosphate 1:15**
Fragment (%)	1.7 ± 0.1	2.3 ± 0.1
Monomers (%)	60.7 ± 1.1	82.9 ± 0.3*
Dimers (%)	26.5 ± 0.5	12.3 ± 0.3
Trimers (%)	8.5 ± 0.4	1.93 ± 0.04
Higher order aggregates (%)	2.6 ± 0.2	0.62 ± 0.02

Quantification of monomers/aggregates was performed by AF4 coupled to MALS and by SEC coupled to MALS. Percentages are expressed as average ± SD (n=3).

*Significantly different from the percentage of monomers of bevacizumab alone (Mann–Whitney U test, $p \leq 0.05$, exact one-tailed significance).

Series IV: Effect of Dexamethasone Phosphate on Prestressed Fab Fragment

The percentages of formed aggregates after 28 days are represented in Table II, both for the prestressed

Table II Prestressed Ranibizumab (365 Days at 25°C) Alone and in Combination with Dexamethasone Phosphate in a 1:15 Molar Ratio

AF4	Ranibizumab	Ranibizumab + dexamethasone phosphate 1:15
Monomers (%)	51.8 ± 0.5	50.0 ± 0.4
Dimers (%)	15.4 ± 0.4	14.9 ± 0.9
Trimers (%)	8 ± 1	7 ± 1
Higher order aggregates (%)	25 ± 1	27.6 ± 0.4

Quantification of monomers/aggregates was performed by AF4 coupled to MALS. Percentages are expressed as average ± SD (n=3). No significant difference was observed between the samples with and without dexamethasone phosphate (Mann–Whitney U test, $p \leq 0.05$, exact one-tailed significance).

ranibizumab alone and for the combination with dexamethasone phosphate in a 1:15 molar ratio. The addition of dexamethasone phosphate has no stabilizing effect on the Fab fragment; the monomer percentages that are present in both samples are comparable ($51.8 \pm 0.5\%$ for ranibizumab alone *vs.* $50.0 \pm 0.4\%$ for the combined formulation). All measured aggregation species (dimers, trimers and higher order aggregates) are also comparable between both groups of samples.

DISCUSSION

In this study, the aggregation breaking properties of dexamethasone phosphate on the monoclonal antibody bevacizumab are investigated. Construction of a 3D aggregation model and small molecule docking allow comprehending the process through which aggregation might occur and how an aggregation breaker might interfere with this mechanism. Furthermore, the requirements that have to be met by a small molecule to be a breaker can be predicted (structure-activity relationship). The combination with *in vitro* stability studies provides a validation of the observations made *in silico*.

It should be noted that the 3D model focuses on the onset of aggregation; only the formation of dimers was investigated. Moreover, the native structure of the antibody was used, while it is generally assumed that especially partly unfolded antibodies are prone to aggregation (27,28). Computational prediction of unfolding would however require time- and resource-intensive molecular dynamics, which would go beyond the scope of this work. Besides, reversible self-association of native state proteins is possible through hydrophobic or electrostatic interactions; with or without subtle conformational changes (6,29). The term "aggregation" that is used throughout the paper therefore refers to "self association of native state monomers" in this particular context.

Based on the symmetry and space group found in the crystal structures of 1IGY and 1HZH, respectively, a « symmetric » (replicated according to the crystal symmetry of 1IGY) and an « asymmetric » (replicated according to the crystal symmetry of 1HZH) bevacizumab model were built. Both aggregation models reveal a contact zone situated on the Fc tip region around Lys445 of the first bevacizumab and a variable interacting region on one Fab of the second bevacizumab. The contact zone on the Fab depends on the overall antibody configuration (symmetric *vs.* asymmetric). To understand the stabilizing effect of dexamethasone phosphate, the ligand was docked all over the monomer antibody model. The highest docking scores were obtained for the binding of the phosphate group to Lys445 at the aggregation interface. Additional H-bonds between residues adjacent to Lys445 and the phosphate further stabilize the steroid in the binding pocket. Thus, dexamethasone phosphate masks the interaction interface between two monomers and consequently hinders dimer formation through the strong electrostatic interaction between the phosphate and the side chain of Lys445. *In vitro* investigations support these observations: Dexamethasone phosphate is able to partly prevent or reverse aggregation of the antibody during the stability studies.

It is shown that the 1:15 molar ratio bevacizumab:dexamethasone phosphate is optimal to stabilize the protein in the commercial formulation at pH 6.2. Apparently, in a formulation with a 1:1.5 ratio, the number of dexamethasone molecules is too low to interact with all antibodies present, whereas the 1:150 ratio leads to the formation of higher order aggregates. For betamethasone phosphate, the 1:15 combination was also observed to be the optimal molar ratio (results on other molar ratios are not shown). A similar importance for molar ratios was reported by Cleland *et al.*, who studied the stabilizing effects of sugar on a lyophilized monoclonal antibody: The molar ratio between stabilizing agent and antibody was found to be a critical parameter in the stabilizing process (30). It should be noted that the 1:15 molar ratio was observed to be optimal only for this particular formulation. Since the dialysed formulation at pH 7 is different from the commercial one, it might not be the optimal ratio at pH 7. This should be investigated in further work.

Because an aggregation breaking effect of dexamethasone phosphate is observed during both thermal stress and agitation, the assumption can be made that the form of stress applied has no influence on the ability of the steroid drug to hinder dimer formation. This observation may suggest that the formation of bevacizumab aggregates is similar for both thermal and mechanical stresses, at least in the initial process of aggregation. *In vitro* measurements show that the first aggregates observed are dimers (and low percentages of trimers in the case of agitation) for both stressing processes; higher order aggregates are only measured in later stages of the stress studies. Thus, in addition to the 3D dimer model, these data imply that aggregation takes place through a primary formation of dimers, which might act as active nuclei for further aggregation. This said, it should be noted that there is an important difference between aggregation formation due to thermal stress and agitation. In the case of thermal stress, the corticosteroid is able to cause reversion of formed aggregates back to monomers (Fig. 5). However, the slope of the curve is comparable to that of bevacizumab alone, i.e. the aggregation kinetics are similar and the addition of the breaker does not slow down the rate at which aggregates are formed. In contrast, during agitation a clear stabilization of the aggregation kinetics is observed: Fig. 4 shows that over time, the

percentage of aggregates increases for the sample containing bevacizumab only, whereas it stays almost the same when dexamethasone phosphate is present.

To validate the aggregation model and to verify whether the Fc region is indeed indispensable for aggregation, as shown in the aggregation model, a negative control is performed *in vitro*. Ranibizumab is a Fab fragment that has the same sequence as a single Fab fragment of bevacizumab. If the prediction of the aggregation model is correct, than the assumption could be made that ranibizumab is less prone to aggregation than the complete antibody bevacizumab, since it lacks the Fc region that is involved in the aggregation process. This is confirmed by comparing the aggregation profiles of bevacizumab and ranibizumab induced by temperature stress: Ranibizumab is already more stable than bevacizumab at t_0 and stays more stable over the whole study period, as was observed in earlier work (12). Furthermore, the absence of the Fc region implies that dexamethasone phosphate is unable to stabilize ranibizumab: If dexamethasone phosphate interacts with Lys 445 as proposed in the 3D model, than it would be impossible to stabilize the aggregated Fab fragment, which lacks this particular and surrounding amino acids. Indeed, no significant difference is observed between the aggregate percentages of ranibizumab alone and in combination with dexamethasone phosphate, thereby confirming the assumption.

A second validation of the aggregation model and the assessment of the capacity of the procedure to distinguish between aggregation breakers and non-breakers in a predictive way were performed by docking several steroids onto the Fc contact region of the monomer. Subsequently, the second monomer of the aggregation model was displayed and the molecules were selected or rejected based on their propensity of interfering with dimer formation while binding to the first monomer. It seems that phosphate is fundamental for the interaction, since this negatively charged functional group can form an electrostatic interaction with the (at physiological pH) positively charged terminal amide of Lys445. Of the tested steroids, betamethasone phosphate is predicted to be a bevacizumab-bevacizumab interaction breaker, since its phosphate group is in a similar configuration as in dexamethasone phosphate. Indeed, betamethasone phosphate shows a similar binding mode as dexamethasone phosphate. The *in vitro* examination of betamethasone phosphate shows that this molecule has a similar aggregation breaking effect on bevacizumab as was observed for dexamethasone phosphate, supporting the predictability of the model.

Triamcinolone acetonide phosphate serves as a further control for corroborating the model and the predictiveness of the compound selection procedure. In comparison with both dexamethasone phosphate and betamethasone phosphate, it is observed *in vitro* that triamcinolone acetonide phosphate lacks the protective properties on this particular antibody. By just looking at the presence of a phosphate group, one might expect that triamcinolone acetonide phosphate also has an aggregation breaking effect on bevacizumab. However, when comparing the docking results on the Fc contact region of triamcinolone acetonide phosphate with the aggregation-breaking steroids, it is shown that (i) the presence of the acetonide moiety restricts the conformational flexibility so that Lys445 is still able to interact with the phosphate, but that (ii) the steroid part is oriented parallel to the Fc surface and therefore (iii) not interfering with the adjacent antibody, since only the acetonide group is pointing towards the other monomer, not being voluminous enough to hinder dimer formation.

CONCLUSION

In this paper, the mechanism behind the aggregation breaking properties of dexamethasone phosphate on the monoclonal IgG1-based antibody bevacizumab was investigated. In the proposed *in silico* model, interfering with dimer formation is based on the ability of dexamethasone phosphate (or betamethasone phosphate) to hinder the interaction between the Fc part of the first, and the Fab arm of the second bevacizumab monomer. *In vitro*, bevacizumab dimers revert partly into monomers after addition of dexamethasone phosphate, independently of the form of stress applied. This observation supports the idea that the mechanism behind the initial dimer formation of bevacizumab is similar for these two forms of stress testing. As we have shown, it is possible to predict whether a molecule has aggregation breaking properties: In order to hinder the dimer formation, a flexibly bending phosphate group is required to accurately position the rest of the steroid. Such flexibility is guaranteed in dexamethasone phosphate and betamethasone phosphate, but impossible in triamcinolone acetonide phosphate due to the rigidity conferred by its acetonide moiety.

This approach allows a simple way to stabilize therapeutic antibodies without the need to modify the Ig structure through mutation of specific amino acids, which might lead to a loss in efficacy. The therapeutic activity of the corticosteroids might be an advantage in the development of a combination product. However, further research will focus on the discovery of safe and therapeutically inactive molecules with similar aggregation breaking properties. Besides, supplementary studies on unformulated bevacizumab need to be carried out to investigate the contribution of polysorbate to the aggregation breaking effect of the anti-inflammatory drugs. The effects shown

for the specific antibody bevacizumab might be applicable to other IgG1 antibodies as well, which might be advantageous for the future development of stable IgG1-derived antibodies.

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