SHORT COMMUNICATION

Identification of aggregation inhibitors of the human antibody light chain repertoire by phage display

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Protein aggregation hinders the development of biologics and underpins the molecular basis of many human diseases. Considerable variation of aggregation propensity exists not only between different proteins, but also within a single homologous family, which complicates analyses. A classic example is observed among human antibody light chains, which aggregate in a clonally specific manner, driven by sequence diversity within their variable domains. Here, we utilise a library versus library strategy, based on phage display and a chemical library of FDA approved drugs, to overcome this limitation. Our approach allowed the identification of small molecule drugs that inhibit the aggregation of the human light chain repertoire. It also provides a general template for the small molecule targeting of diverse protein families.

Keywords: phage display/antibodies/protein aggregation/light chain/human antibody variable domains

Antibodies are effector molecules of the human immune system and key players in the defence against bacteria and viruses. Antibodies also represent a rapidly growing class of human therapeutics and now represent the majority of new drugs entering clinical trials (Nelson *et al.*, 2010). Unfortunately, many antibodies are unstable and have a propensity to aggregate both *in vitro* and *in vivo*. This represents an important bottleneck in the development and production of human antibody therapeutics (Lowe *et al.*, 2011). It also represents an area of unmet medical need due to the difficulty of treating antibody deposition associated with lymphoproliferative disorders, with therapies relying on chemotherapy or stem cell transplantation, generally with limited success (Mahindra *et al.*, 2012). In particular, deposition of kappa light chain is observed in patients, while deposition of other chains is less common (Buxbaum, 1992; Pozzi *et al.*, 2003). The propensity of light chain to aggregate also is a problem in the production of human antibody therapeutics (Lowe, *et al.*, 2011).

While there is currently little understanding of the determinants of human light chain aggregation, it is generally believed that aggregation propensity of the chain is almost exclusively determined by its variable domain (V_L) (Myatt *et al.*, 1994; Demarest and Glaser, 2008; Lowe, *et al.*, 2011). Although the aggregation propensity of human V_L can be improved through mutation (Dudgeon *et al.*, 2012), increasing aggregation resistance in a non-genetic manner has proven to be a difficult and persistent problem (Lowe, *et al.*, 2011). Difficulties are related to the high level of sequence diversity observed in this protein family, which complicates the development of general approaches (Ewert *et al.*, 2003; Ewert *et al.*, 2004). In the clinical setting, sequence diversity results in patients presenting with light chain deposits that differ in their clonal origins, while in the development of human antibody therapeutics it manifests in high failure rates during production and formulation.

Results and discussion

As a starting point, we used a high-throughput method on phage (Jespers *et al.*, 2004a) to assess protein aggregation. The method is based on the display of aggregation-prone domains on phage, followed by transient heating of the phage particle. Mutants that resist aggregation can then be captured by conformation-dependent superantigens, such as protein L, which binds to folded but not to aggregated V_L domains (Jansson *et al.*, 1998; Jespers, *et al.*, 2004b).

As the method described by Jespers *et al.* was capable of identifying mutations, we speculated that it could also be utilised to identify small organic molecules that improve the aggregation propensity of human antibody V_L domains (Supplementary Fig. S1). To investigate this hypothesis, we used a chemical library of approximately 1200 compounds (Prestwick library). This library is dominated by FDA approved drug compounds to ensure a high level of chemical diversity, low toxicity and accelerated lead discovery. The library, and the strategy of drug repositioning in general, have a proven track record of generating leads against a diverse set of protein targets (Kawahara *et al.*, 2011; Cabrera *et al.*, 2012; Spiros *et al.*, 2013; Bharadwaj *et al.*, 2014; Liu *et al.*, 2014).

For the initial screen, an aggregation-prone human V_L domain (O12/O2/DPK9) was displayed on the tip of filamentous bacteriophage. The domain was then heated and captured using protein L. DPK9 readily aggregates under such conditions (80°C) (Dudgeon, *et al.*, 2012). However, this was not observed when adding select organic molecules from a range of drug classes, for which retention of superantigen binding after heating was considerably improved (Fig. 1A). To further validate the compounds identified on phage, a secondary screen based on turbidity measurements using soluble V_L domains was performed. This analysis revealed that, while minor improvements were observed for a range of compounds,
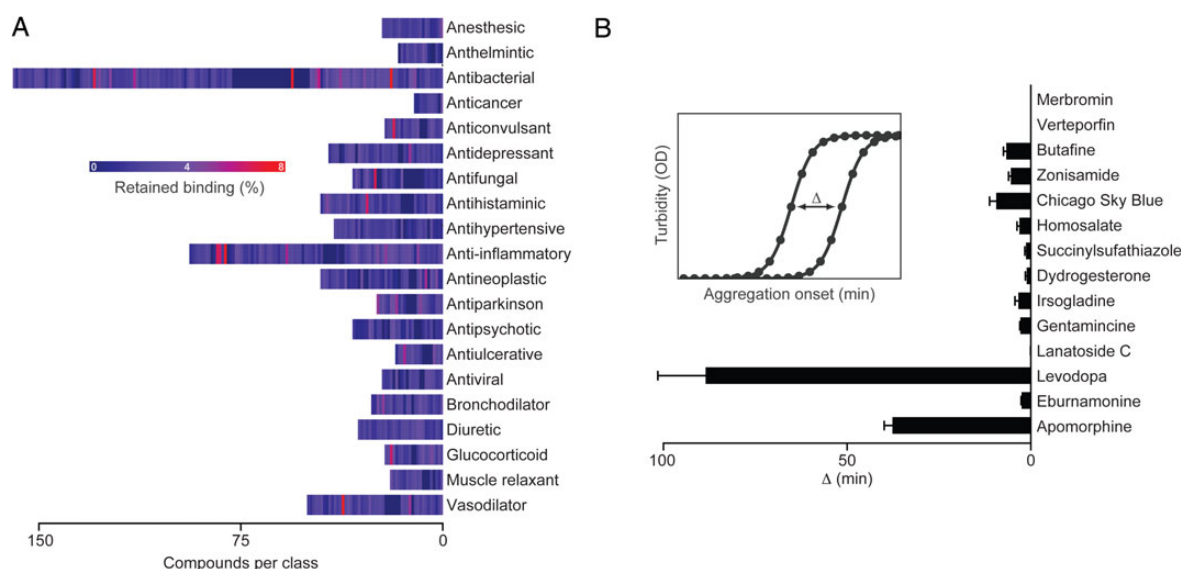
Fig. 1. Identification of chemical inhibitors of aggregation. (**A**) Human antibody V_L was displayed on phage, and effects of drug compounds on aggregation were determined by measuring retained binding to protein L superantigen after heating for 10 min at 80°C. A chemical library was utilised with compounds falling into a range of drug classes (anesthesic to vasodilator). (**B**) Secondary screen using soluble V_L protein and turbidity at 85°C. Delays in the onset of aggregation (in minutes) in the presence of compound are shown (mean, SD, $n = 2$, see Materials and Methods).

by far the largest effects were observed for a pair of antiparkinson drugs (levodopa and apomorphine, Fig. 1B). These two drugs are closely related catechol compounds. As a next step we tested further related chemicals, including amino acids (phenylalanine and tyrosine), their derivatives (tyrosol, hydroxytyrosol and caffeic acid), naturally occurring polyphenols (oleuropein, resveratrol, nordihydroguaiaretic acid and 7,8-dihydroxyflavone) and an additional antiparkinson drug (benzeraside). These experiments revealed that the effects (as measured as a delayed onset of V_L aggregation—in minutes) were critically dependent on the presence of a catechol moiety, with even limited chemical modification resulting in a loss of aggregation inhibition (Fig. 2).

To demonstrate that our findings were generally applicable, we next investigated the effect of the compounds on the human V_L repertoire. For this purpose, we utilised a previously reported synthetic phage display library, with diversity closely mimicking the amino acid distribution in the human repertoire (Dudgeon, *et al.*, 2012). This was achieved through the use of common Vkappa germline segments (O12/O2/DPK9—also utilised in the primary aggregation screen reported here) and TRIM codon mutagenesis (Knappik *et al.*, 2000) for the randomisation of complementarity determining region 3 (CDR3). In contrast, the DPK9 CDR2 region was not randomised, to ensure the presence of a high proportion of aggregation-prone domains as previous work in our laboratory had identified hotspots mutations in this region that render human V_L domains aggregation resistant (Dudgeon, *et al.*, 2012).

Further experiments revealed that many of the domains within the synthetic V_L repertoire were indeed prone to aggregation (as indicated by poor retention of superantigen binding after heating) (Fig. 3A). Representative domains covering a range of aggregation propensities were selected from the repertoire. The domains were expressed as soluble proteins and tested against a set of chemical compounds, and inhibition of aggregation was measured (Fig. 3B). This analysis revealed that aggregation of many of the domains (DPKA6, DPKB6, DPKE4, DPKF6, DPK9) was potently inhibited by catechol

compounds. However, other domains (DPKD3, DPKB2, DPKD2, DPKF2) aggregated under all analysed conditions, despite the fact that the proteins only differed at a limited number of CDR1 and CDR3 positions, indicating that the effects of the chemical inhibitors were not dominant.

To further investigate the potential of the identified compounds as excipients for drug manufacture and formulation (Ladiwala *et al.*, 2012), we studied variable light domains derived from trastuzumab (Herceptin®) and adalimumab (Humira®), two blockbuster therapeutic monoclonal antibodies directed against human epidermal growth factor receptor 2 (HER2) (Carter *et al.*, 1992) and TNF-alpha (Jespers *et al.*, 1994; Scheinfeld, 2003), respectively. We observed considerable effects on the aggregation behaviour of the Herceptin and Humira variable light domains (Supplementary Fig. S2). However, again the extent of inhibition was clearly different for each domain, despite the fact that they differ almost exclusively in CDR positions (Table S1).

Taken together, our results demonstrate that small molecule aggregation inhibitors can be identified using a phage display strategy. The identified chemical class (catechols) has been shown to potently inhibit the aggregation of other proteins, including alpha-synuclein (Zhou *et al.*, 2009) and amyloid beta (Sato *et al.*, 2013). Here, we demonstrate that this is also the case for human antibody variable light domains, including domains derived from existing antibody therapeutics. Previous studies (Zhou, *et al.*, 2009) had indicated that catechols promote the formation of oligomeric protein species (which resist aggregation into larger oligomers and particulate matter); this was also observed for human antibody variable light domains (Supplementary Fig. S3).

Some of the compounds identified here display relatively high IC_{50} values, within a range relevant to human therapy (micromolar) (Supplementary Fig. S4). However, our data also reveal that the chemical approach outlined here is not generally applicable to the human repertoire, as it is significantly dependent on CDR diversity. This is in marked contrast to previously identified mutations (aspartate at Kabat positions
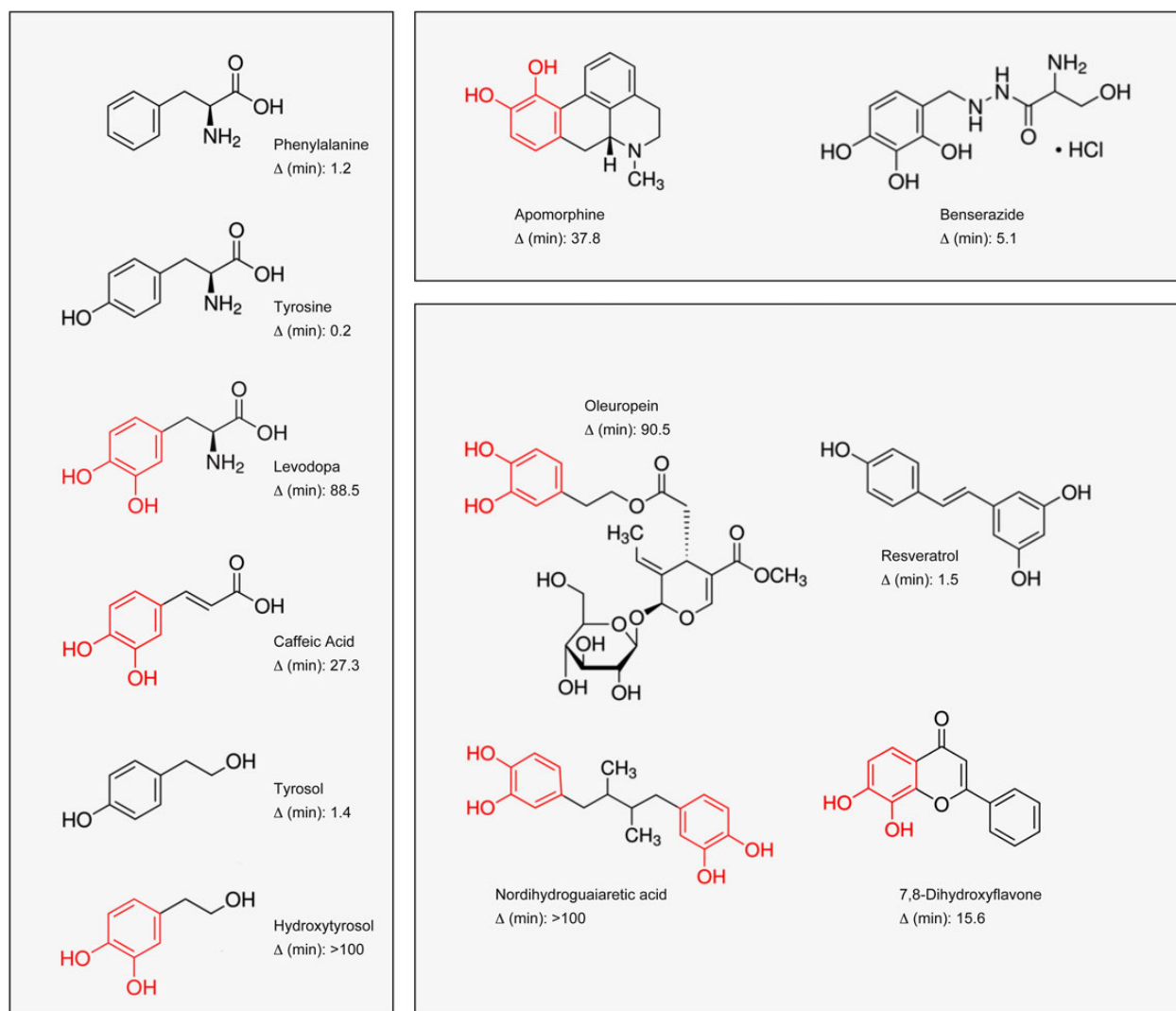
Fig. 2. Catechols (in red) inhibit the aggregation of human antibody variable light domain. The effect of chemical compounds on the aggregation propensity of human V_L was analysed by measuring turbidity at 85°C (see Material and methods). This revealed delayed onsets of protein aggregation (in minutes) for catechols from a range of compound classes, including plant-derived (lower right panel), antiparkinson (upper right panel) and phenylalanine-related (left panel).

49–53/56 of human V_L), which have been shown to improve aggregation resistance independent of diversity at other CDR positions (Dudgeon, *et al.*, 2012). Consequently, it is unlikely that the identified catechol compounds will be directly suitable for human therapy (although they are potentially highly useful excipients for drug manufacture and formulation). Rather, the conclusion of our study is actually a sobering one, namely that inhibition of aggregation in diverse protein families, such as the human light chain repertoire, may require customised therapeutic interventions, reflecting the underlying clonal diversity of the aggregation-prone proteins. This is reflected by our observation that even the most active of the identified compounds failed to prevent the aggregation of a considerable proportion of the human light chain repertoire, despite the fact that they effectively inhibited the aggregation of other family members (Fig. 3B).

Although more potent molecules may be discovered in the future, it is likely that such compounds would have to fall outside the chemical space currently utilised by drug-like molecules. This is indicated by our unbiased analysis of approximately 1200 existing FDA-approved drugs, which revealed a single class of highly active compounds (catechols), while other molecules were considerably less active (including doxycycline, which had previously been reported to inhibit light chain aggregation (Ward *et al.*, 2011)). Unfortunately, it is not clear that novel and customised molecules can be developed in an effective manner, based on current cost structures of drug development. Consequently, sequence diversity in aggregation-prone protein families may require novel approaches, such as the targeting of conserved hotspots (Thanos *et al.*, 2006) or the use of multispecific biologics (monoclonal antibodies or other) (James *et al.*, 2003).

In summary, the phage display strategy outlined here allows the rapid identification of aggregation inhibitors within large chemical libraries, in the context of extensive target sequence diversity.

Material and methods

Cloning and expression of antibody variable light domains

For phage display studies, segments encoding the human DPK9 variable light domain were cloned into the phage vector FdMyc. For soluble expression, mutant genes were inserted into
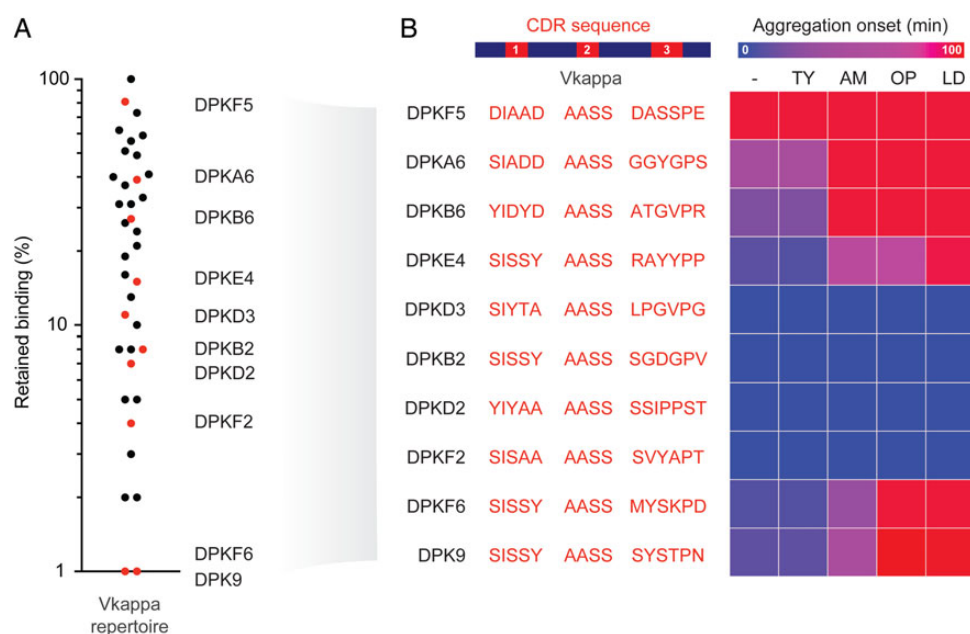
Fig. 3. *Library versus library* screen for chemical inhibition of aggregation. (**A**) A synthetic library of human antibody V_L domains was displayed on phage. Aggregation propensity was determined by measuring retained binding to protein L superantigen after heating for 10 min at 80°C. (**B**) A representative set of library members were expressed as soluble proteins and the effect of drug compounds on their aggregation propensity analysed (CDR sequences shown; TY: tyrosine, AM: apomorphine, OP: oleuropein, LD: levodopa). Aggregation was monitored by turbidity at 85°C (see Materials and Methods).

the periplasmic expression vector pET12a (Novagen) and protein was expressed in *Escherichia coli* BL21-Gold (Stratagene) at 30°C essentially as previously described (Lee *et al.*, 2007; Rouet *et al.*, 2012). The filtered supernatant was added to protein L resin (GE Healthcare) and the resin was washed with phosphate buffered saline (PBS). Protein was eluted by adding 0.1 M glycine–HCl pH 2.7, neutralised with 100 mM Tris–HCl pH 8.0 and dialysed against PBS.

Heat refolding on phage

Aggregation resistance was analysed by measuring the retention of signal after heating in a phage enzyme-linked immunosorbent assay (ELISA) format essentially as described by Jespers *et al.* (2004). Wells of a Nunc Maxisorp Immunoplate were coated overnight with protein L superantigen at a concentration of 5 μg/ml in carbonate buffer. The plate was washed with PBS and blocked with 4% (w/v) milk powder diluted in PBS. Single colonies were picked from agar plates and grown overnight (2xTY medium supplemented with 15 μg/ml tetracycline; shaking at 250 rpm at 30°C). Cells were removed by centrifugation and phages were biotinylated directly in the culture supernatant through addition of biotin-PEO4-N-hydroxysuccinimide to a final concentration of 50 μM (Pierce) and the reaction was quenched after 2 h by addition of Tris–HCl pH 7.5 to a final concentration of 100 mM. Supernatant was supplemented with 100 μM of the respective compound of the Prestwick Chemical Library and then incubated at 80°C for 10 min, followed by incubation at 4°C for 10 min. The supernatant was added to the blocked ELISA wells for 1 h at room temperature followed by three washes with PBS supplemented with 0.1% Tween-20. Bound phage particles were detected using an Extravidin-HRP conjugate (Sigma) and 3,3′,5,5′-tetramethylbenzidine substrate. Finally, the level of retained protein L binding after heating was calculated as a percentage of the unheated phage sample.

Turbidity measurements

Aggregation resistance of antibody domains was determined by measuring the turbidity of concentrated protein samples at elevated temperatures. For this purpose, absorbance at 350 nm was measured and V_L domains were heated as soluble proteins at 100 μM concentration in 20 mM phosphate buffer (pH 7.4) at 85°C. Measurements were made on a Varian Cary 100 Bio UV-Vis spectrophotometer (Agilent Technologies) using a quartz cuvette with a 1-cm path length. Aggregation onset was determined through sigmoidal fitting of turbidity curves and determination of midpoints (in minutes).

Supplementary data

Supplementary data are available at *PEDS* online.

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