

# Physical and biological properties of homophilic therapeutic antibodies

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**Abstract** Homophilic antibodies have been discovered in mice and primates and can also be engineered. Compared to conventional antibodies, homophilic antibodies form lattices on targets leading to enhanced binding via polyvalent attachment. Previously, we have observed a paradoxical dose/potency effect with an engineered homophilic antibody against a human lung cancer tumor. Here, we have investigated some biophysical properties of homophilic antibodies and also studied the inhibition of human tumor growth in a xenograft model using homophilic Herceptin. Dimerization and viscosity of two homophilic antibodies are greater at physiological temperature than at 4°C. Similarly, binding to solid-phase antigen is greater at 37°C than at room temperature or 4°C. Dimer formation is higher at therapeutic concentration, supporting the notion that preformed dimers in solution are the effective molecular species responsible for polyvalent target binding and enhanced therapeutic potency.

**Keywords** Homophilic antibody · Dimerization · Xenograft · Polyvalency

## Introduction

Homophilic antibodies are characterized by their ability to self-bind and form homodimers/polymers. To date, only

five naturally occurring homophilic antibodies have been reported, and thus it appears that this type of antibody is infrequent [1–5]. Their behavior in physiological solutions cannot easily be distinguished from conventional antibodies, as they fail to produce aggregates or precipitates. However, under non-physiological conditions, such as in low salt concentration or PEG-containing solutions, dimer formation can be demonstrated [6, 7]. In solid-phase assays, homodimerization is readily observed [8]. Furthermore, a paradoxical therapeutic potency has been observed whereby homophilic Herceptin is more effective at lower than at higher concentration.

The first discovered homophilic antibody was the murine TEPC-15 anti-phosphocholine antibody, which is considered to be the prototype of self-binding antibodies [8, 9]. We have identified the domain in TEPC-15 that is responsible for its homophilic property [10]. The domain is located in the heavy chain, covering CDR2 and FR3. This homophilic domain can be conferred to conventional antibodies by chemical [11–13] and molecular biology methods (unpublished). These engineered antibodies exhibit similar homophilic properties as the naturally occurring homophilic antibodies. The biological hallmarks of homophilic antibodies are enhanced target binding, effector functions and apoptosis induction in target cells when compared to their corresponding conventional antibodies (for summary see [14]). This enhancement is not due to increased affinity but is the result of increased avidity caused by dimer-induced polyvalency. In contrast to the polyvalency of IgM antibodies, homophilic antibodies achieve polyvalency through non-covalent lattice formation on targets.

Recently, we have studied the potency of an engineered homophilic Herceptin and observed an inverse dose/effect relationship [15]. We found that targeting of a human lung

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cancer tumor is more effective at a lower concentration of homophilic Herceptin. A similar dose/effect relation exists in the induction of apoptosis of tumor cells. Independently, Bingaman et al. [16] reported enhancement of an engineered anti-CD20 to induce apoptosis of human B-cell tumors. These *in vitro* and *in vivo* studies demonstrate that conversion of therapeutic antibodies into homophilic antibodies can increase their therapeutic potency.

In this report, we compare the physical properties as well as the biological activity of a set of homophilic and non-homophilic antibodies. We identified the key parameter that controls homophilic effects.

## Materials and methods

### Antibodies and cell lines

HPC-G9 and HPC-G11 mouse anti-PC antibodies were obtained from Patricia Gearhart [17]. S107 was obtained from John Kearney (University of Alabama at Birmingham); Trastuzumab (Herceptin) was a gift from Genentech (Genentech, Inc., 1 DNA Way South San Francisco, CA 94080). Homophilic Herceptin was prepared as described [15]. 1F7 (IgM, kappa) was a gift from Sybille Muller [18], (NCI-H1650 NSCLC cells were obtained from ATCC catalog # CRL-5883. (PO Box 1549 Manassas, VA 20108).

### ELISA reagents

PC-BSA was obtained from Biosearch Technologies (Novato, CA). Donkey anti-mouse IgG1-HRP was obtained from SouthernBiotech. (160A Oxmoor Blvd. Birmingham, AL 35209). ELISA assay was performed as described [7].

### Size-exclusion chromatography

HPC G9 and G11 were chromatographed as described [15]. A 70-ml column containing Sephacryl S300 HR (Sigma-Aldrich, St. Louis, MO) was equilibrated in PBS. One milliliter of antibody samples was applied and 1 ml fractions were collected. Chromatography was performed at 4°C, 20°C, and 37°C. The fractions were monitored at 280 nm and/or with aliquots by Ig-capture ELISA.

### Xenograft studies

Mouse studies were performed under the University of Kentucky Department of Lab Animal Resources (DLAR) Protocol #929M2005. Daily care was provided by the UK DLAR. Hsd Athymic Nude-Foxn1<sup>TM</sup>/foxn1 + strain mice,

6–7 weeks old, were obtained from Harlan Labs (Indianapolis, IN). Mice were injected subcutaneously on the upper back with  $\times 10^6$  H1650 cells that had been harvested from culture, washed two times twice in PBS, and resuspended in 500 ml sterile PBS. Twenty-four hours after inoculation, the mice were divided into two groups consisting of 6 mice each. The first group was treated with 100 ml of PBS containing 110  $\mu$ g of naked Herceptin, and the second group was treated with PBS containing 110  $\mu$ g of homophilic Herceptin. The antibody treatments were administered twice weekly. Tumor measurements were taken three times weekly.

### Antibody viscosity

Microdilution tubes (USA Scientific, Ocala, FL containing HPC G9 or HPC G11 at 1 mg/ml in PBS) were mounted on standard microscope slides. They were equilibrated at 4°, 20°, and 37°C, sealed and then positioned vertically and then horizontally then photographed at each temperature.

### Time lapse measurement of viscosity equilibrium

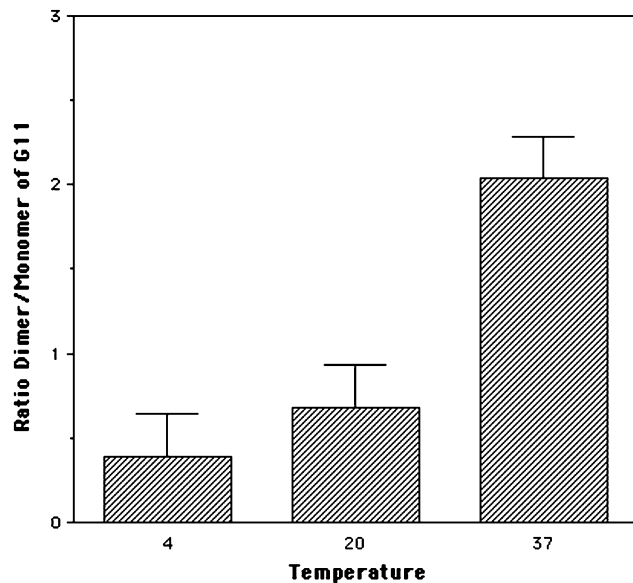
Microdilution tubes containing antibodies at 1 mg/ml in PBS were equilibrated at either 4 or 37°C. Tubes were positioned vertically for 3 s then horizontally and filmed. The time required for the meniscus to cease movement was measured using a stopwatch.

## Results

### Temperature-dependent equilibrium of homodimers

HPC G9/HPC G11 are isoforms of IgG anti-phosphocholine antibodies whereby HPC G11 shares idiootype and sequence with CDR2/FR3 in TEPC-15[17]. Accordingly, HPC G11 is homophilic and HPC G9 is not. Since temperature is known to affect the physical properties of proteins, including antibodies, we compared the behavior of HPC G9 and HPC G11 at 4, 20, and 37°C using size-exclusion chromatography. We compared the amount of antibody eluted within the exclusion volume with that of the inclusion volume.

Figure 1 shows the ratio of excluded/included HPC G11 at different temperatures. There was no significant change in the ratio of excluded protein with HPC G9 at 4, 20, and 37°C (data not shown), but the ratio for HPC G11 increased with temperature. These findings support the notion that the degree of dimerization of homophilic G11 in solution is higher at physiological temperature than at non-physiological temperatures.

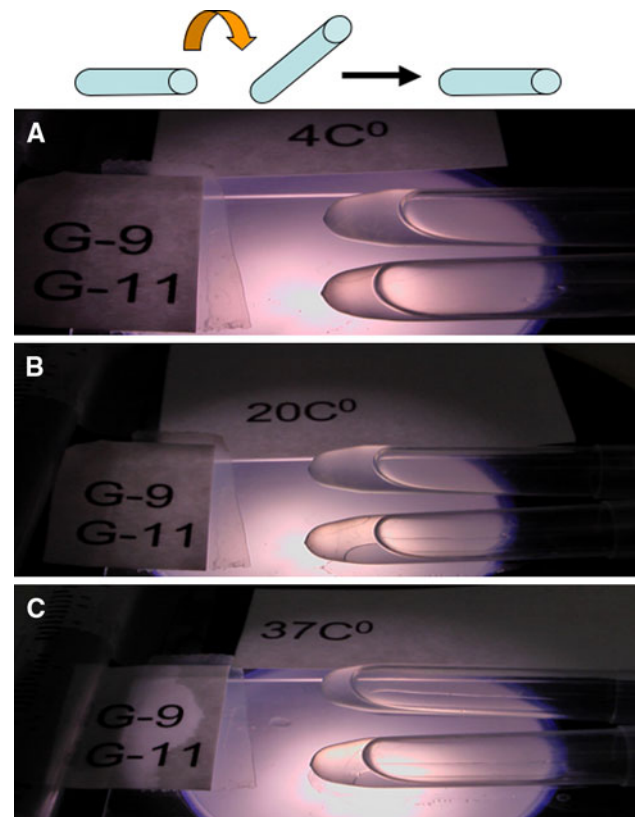


**Fig. 1** Ratio of G11 Dimer to Monomer. G11 (100  $\mu$ g) was chromatographed on a Sephacryl S300 HR at 4, 20 and 37°C. Protein, measured at 280 nm, eluted at the dimer and monomer position was used to calculate the ratio of dimer to monomer. The error bars represents the SD of two or more chromatograms

#### Viscosity differences

Next, we compared the viscosity of HPC G9 and HPC G11 at different temperatures. A volume of 500  $\mu$ l of each antibody was placed into racked tubes and allowed to equilibrate in a horizontal position at 4, 20, and 37°C for 30 min. The tubes were then moved into a top-up, vertical position for 1 s and then returned to the horizontal position and photographed. Figure 2 shows the level of the fluids near the bottom of the tubes in the final horizontal position. We measured the position of the meniscus and calculated the ratios of these measurements with HPC G9 or HPC G11. The ratio at 4°C was 1.45 (Fig. 2a), at 20°C 0.92 (Fig. 2b), and at 37°C it was 0.54 (Fig. 2c). In Fig. 3, the tilting of the tubes after equilibration was reversed (i.e. the bottom of the tube was raised vertically for 1 s and then returned to a horizontal position and photographed). The ratio of HPC G9 to HPC G11 at 4°C was 1.5 (Fig. 3a), 1.41 at 20°C (Fig. 3b), and 2.06 at 37°C (Fig. 3c).

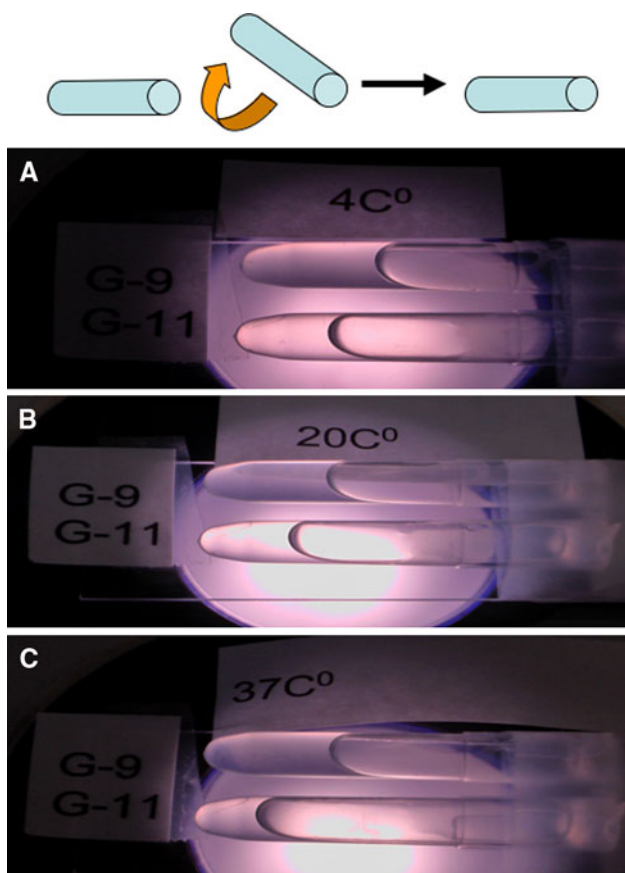
To exclude the possibility that the viscosity of G11 is unique, we tested S107 a murine monoclonal IgA antibody known to be homophilic (6). For comparison, another murine antibody G9 was included in the viscosity analysis. The tubes were positioned first bottom-up for 3 s and then returned to a horizontal position. The time required for the meniscus to cease movement was measured for each antibody. In Fig. 4a, we show the seconds recorded for the meniscus of these antibodies to cease movement at 4 and 37°C. The more time required for movement to cease, the



**Fig. 2** Top-up test tube viscosity assay. In racked microdilution tubes (USA SCI, Inc., Ocala, FL), 500  $\mu$ l of an HPC G9 or HPC G11 antibody solution (1 mg/ml in PBS) was placed. These tubes were equilibrated at 4°C (a) 20°C (b) or 37°C (c) for 30 min in a horizontal position. The tubes were then moved into a top-up, vertical position for 1 s and then returned to the horizontal position. Photographs of the meniscus of the liquid were taken

higher the viscosity of the solution. The time differences recorded at both temperatures shown in Fig. 4a are smaller for G11 and S 107 than for G9. In Fig. 4b, we present the ratio of time at 4°C divided by time at 37°C. The ratio for G 11, S 107, and 1F7 are identical, while the ratio for G9 is more than 2 times greater. G11 and S107 are facultative homophilic polymers, while 1F7 IgM is a covalent pentamer. It is interesting to note that the homophilic polymers and the covalent IgM polymer have similar viscosity but different viscosity from the monomeric G9 bivalent antibody. This unique viscosity of homophilic antibodies is independent of Ig class and might be part of their unique dimerization potential responsible for their superior targeting.

Previously we have compared the therapeutic potency of homophilic Herceptin with naked Herceptin in a xenograft experiment [15]. In Fig. 4c, we are comparing the time required to reach equilibrium in the tilting tube setup for Herceptin and homophilic Herceptin (S-Herceptin). At 37°C, both antibodies show the same time, while at 4°C the



**Fig. 3** Top-down test tube viscosity assay. The same as Fig. 2 except the tubes, after equilibration, were moved into a top-down position for 1 s and then returned to the horizontal position and photographed

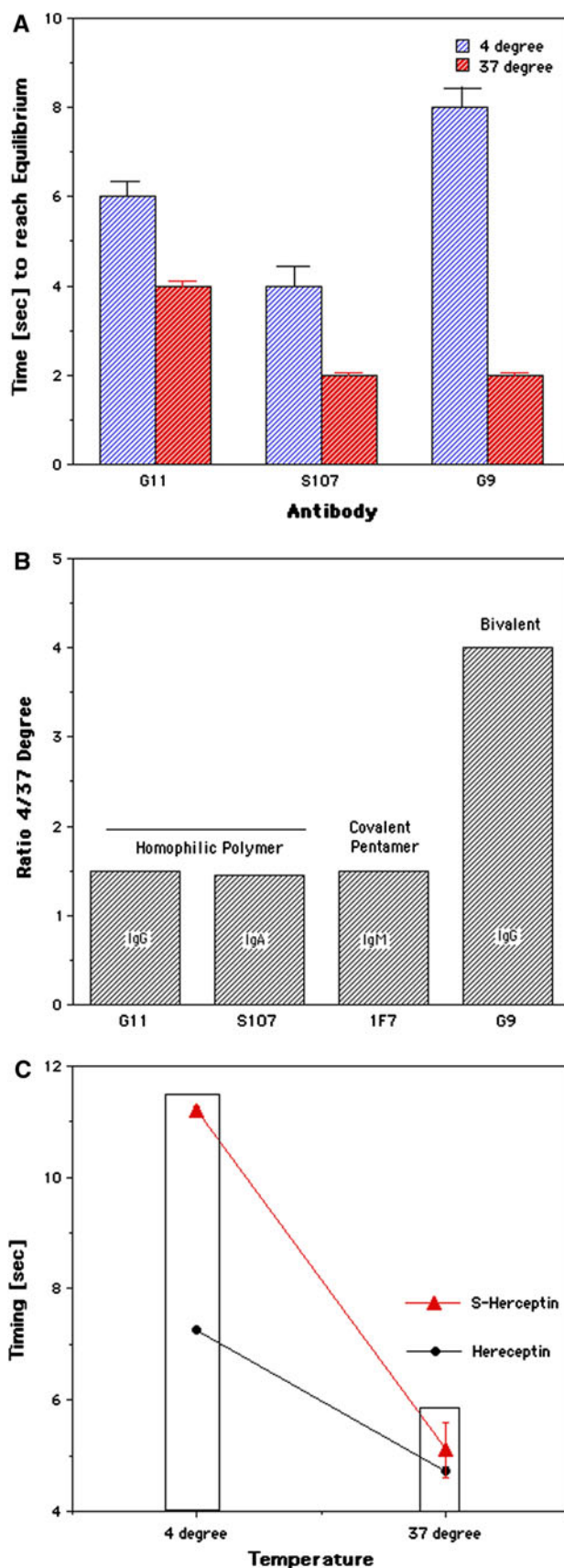
homophilic Herceptin requires significantly more time than the Herceptin.

These fluidity observations indicate that the viscosity of HPC G11 increases with increasing temperature, while the viscosity of HPC G9 decreases. These findings contrast with the typical decrease in protein viscosity with increasing temperature and thus indicate a unique biophysical property for homophilic antibodies.

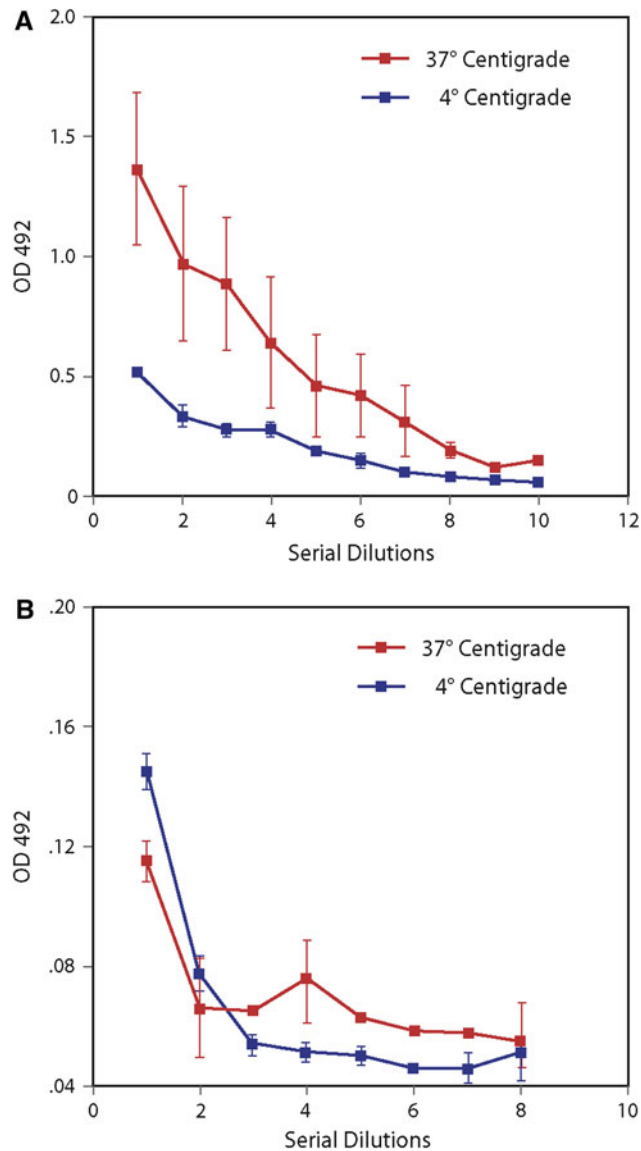
**Binding to PC-BSA at different temperatures**

Next, we evaluated the potency of target binding for the anti-PC antibodies HPC G9 and HPC G11 in solid-phase

**Fig. 4 a** Comparison of time required for meniscus to reach equilibrium after top-down to horizontal movement. The time required for equilibration was measured at 4°C and 37°C for G11, S107, and G9. Error bars represent percent variations of two runs. **b** The ratio of time to reach equilibrium as in **a** is calculated: time at 4°C divided by time at 37°C for G 9, G11, 1F7 and S107. **c** Comparison of time lapsed to reach equilibrium for Herceptin and homophilic Herceptin (S-Herceptin) at 4 and 37°C. See **a**



assays. Serial dilutions of both antibodies were added to ELISA plates coated with PC-BSA at either 4 or 37°C, and binding was detected with HRP-anti-mouse IgG. Figure 5a compares the binding of HPC G11 at 4 and 37°C. There was significantly more binding at 37 than at 4°C. At both temperatures, the binding of HPC G9 is not significantly different (Fig. 5b).



**Fig. 5** PC binding ELISA. Duplicate 96-well plates were coated with PC-BSA (9 µg/ml), then blocked with Blotto. G11 or G9 (1 mg/ml) was then added and serially diluted. The plates were then incubated at 4 or 37°C overnight. Goat anti-mouse IgG1 HRP (Sigma), diluted 1:1000, was added and incubated at room temperature. Color was developed with OPD (Sigma) and read at 492 nm using a microplate reader (Multiskan MCC/340, Fischer Scientific). **a** Shows the binding of G11 at 4 and 37°C. **b** PC binding of G9 at 4 and 37°C as in **a**

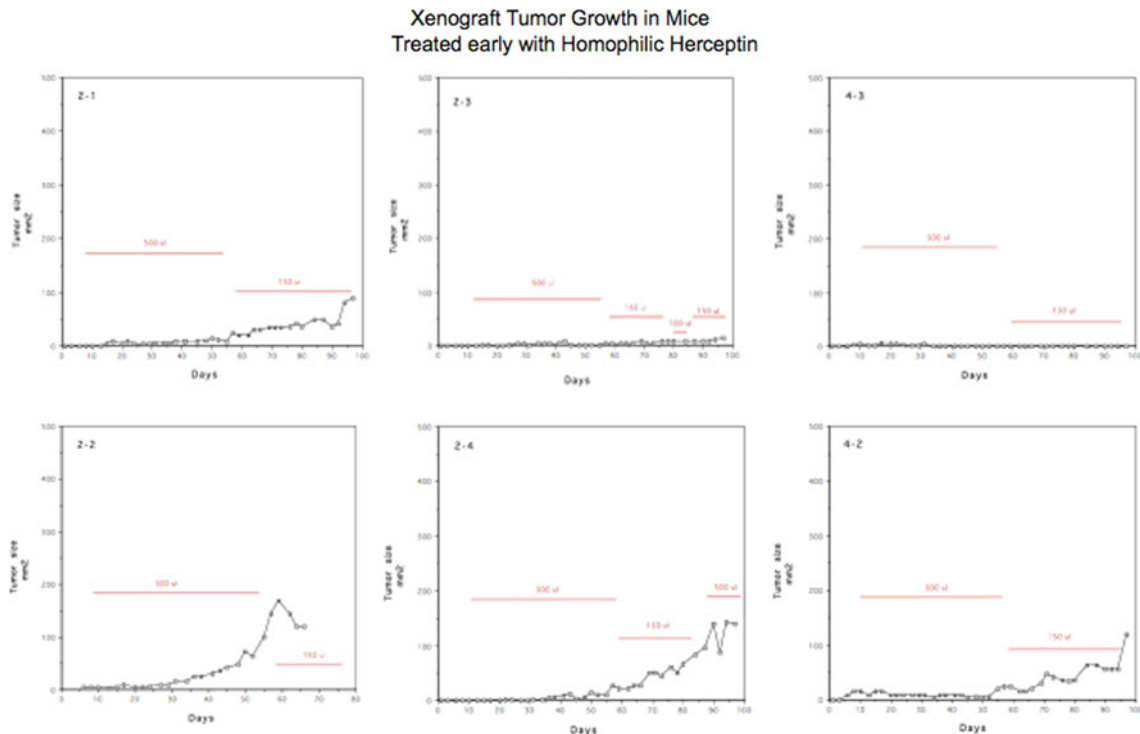
## Tumor growth inhibition in a xenograft model

Nude Balb/c (Nu-Nu) mice were injected into the upper dorsal region with  $5 \times 10^5$  NSCLC H 1650 cells to induce tumor formation. After the appearance of tumors, the mice were treated with homophilic Herceptin at different doses. In Fig. 6, the tumor growth of individual mice treated with different amounts of homophilic Herceptin is shown. A dose of 500 µg bi-weekly was maintained for 55 days at which point the dose was reduced to 150 µg. Reducing the dose appears to allow the tumor to grow except in mouse 4-3. Interestingly, the tumor in mouse 2-2 began to shrink with the lower dose. In mouse 2-4, the tumor growth accelerated when the dosage was decreased and then plateaued when the dosage was returned to 500 µg. Despite the individual variability, the tumor growth in this group was significantly reduced compared with the tumor growth of the group treated with Herceptin (see Fig. 8). In Fig. 7 after the appearance of tumors, the mice were treated with Herceptin initially, and then switched to homophilic Herceptin. Mouse 3-1 (control mouse) only received Herceptin and showed the fastest tumor growth in this group. Changing the treatment from Herceptin to homophilic Herceptin reduced the tumor growth. In Fig. 8, we show the compiled tumor growth of Herceptin and homophilic Herceptin-treated groups. The mean tumor growth in the homophilic Herceptin group is less than the Herceptin-treated group.

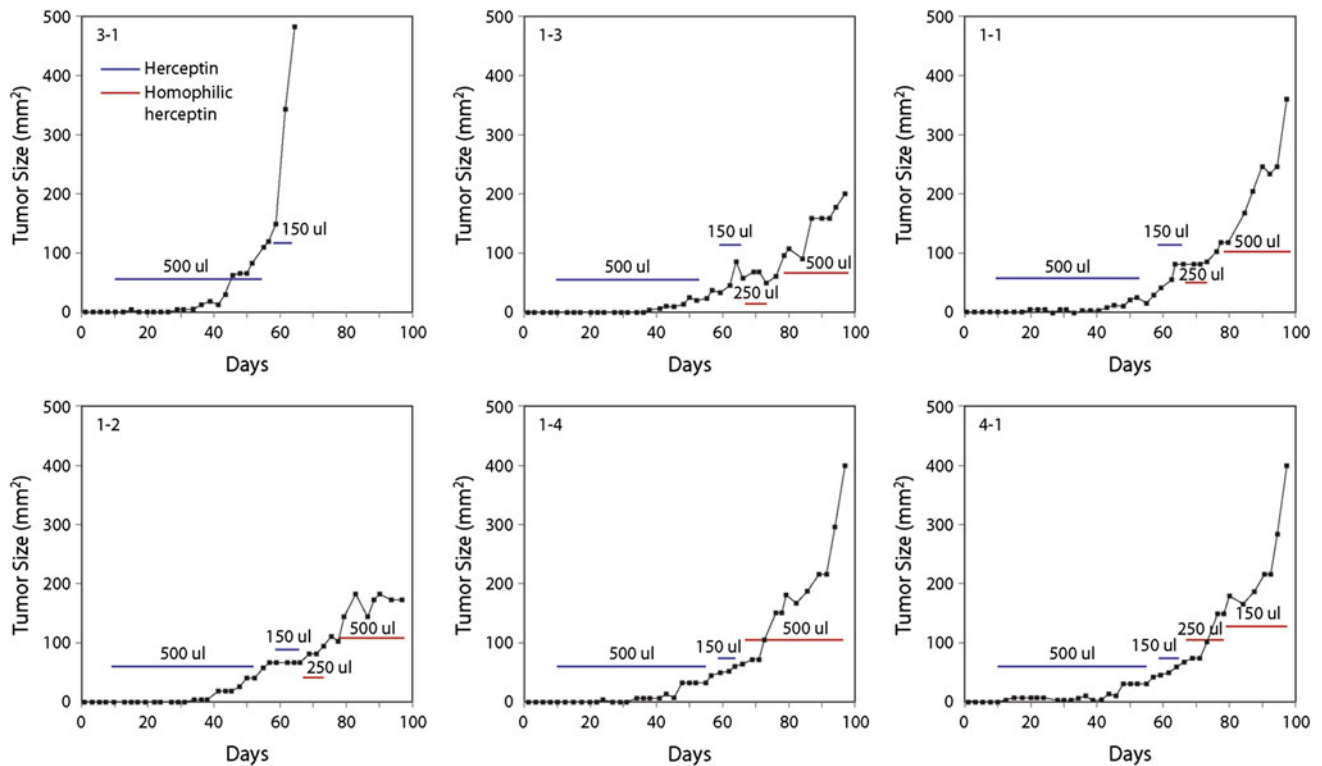
## Discussion

Homophilic antibodies belong to a small group of naturally occurring antibodies [1] (Kohler, *Current Trends in Immunology*, in press). Their hallmark is the potential to self-bind after attaching to antigen and in solution [6]. The self-binding domain was identified in the VH domain of the murine antibody TEPC-15 [10]. The TEPC-15 has a several fold higher potency in an infectious animal model than another antibody with identical affinity but different idio-type [19].

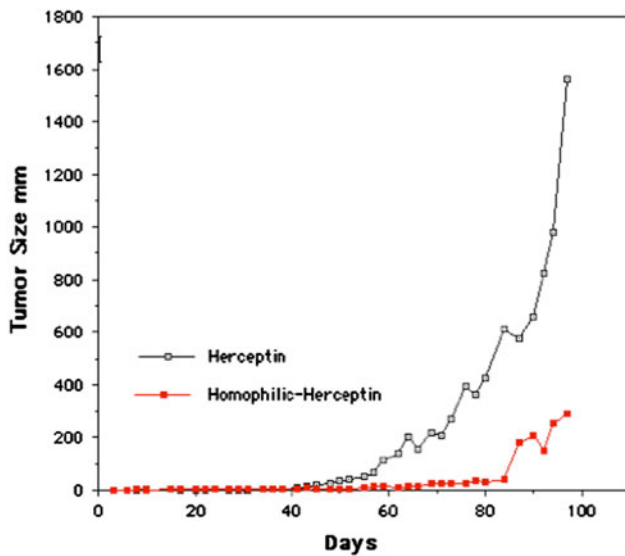
Subsequently, our laboratory developed methods to attach the Self-binding (homophilic) domain to antibodies thereby generating homophilic proteins [11, 20, 21]. Naturally occurring homophilic antibodies appear to be specific for non-protein antigens. Since affinity maturation requires T-helper and non-protein antigens cannot induce T-helper, carbohydrate-specific antibodies cannot be improved by increasing affinity. However, lattice formation by homophilic antibodies increases the polyvalency and the avidity of antibodies producing improved target binding. The restriction of naturally occurring antibodies for carbohydrate antigen does not apply for engineered



**Fig. 6** Xenograft using NSCL H1650 cells. Tumor bearing nude mice were treated with homophilic Herceptin using different doses as indicated. Individual mice are shown



**Fig. 7** Xenograft using NSCL H1650 cells. Tumor bearing nude mice were treated with Herceptin. Dose of Herceptin and homophilic Herceptin was changed as indicated. Individual mice are shown



**Fig. 8** Mean tumor size in the xenograft mice (6 mice/group) was treated with either Herceptin or homophilic Herceptin

homophilic antibodies [14]. Our laboratory has converted antibodies against murine and human tumor using commercially available antibodies (for summary see [13, 14]). This approach has also been used by Bingamen et al [16]. Using homophilic-converted Herceptin, we have observed a paradoxical dose/effector relationship *in vitro* [15]. As the concentration of the homophilic Herceptin increased, binding and induction of apoptosis decreased. Titrating the amount of homophilic antibodies, we observed a bell-shaped dose–response pattern in fluorescence signal in FACS and the amount of induced apoptosis. We also observed a concentration-dependent elution of antibody dimer in size-exclusion chromatography, whereby more dimers than monomers were eluted at lower concentrations. It is tempting to conclude that the increased dimerization in solution is the effective molecular conformation responsible for the observed enhancement antibody potency.

In the current study, we compared the biophysical properties of homophilic and conventional antibodies. We used two non-engineered murine homophilic antibodies against non-tumor target with respect to changes at different temperature. Comparing the fluidity of two homophilic antibodies (S107/TEPC 15 and G11) with non-homophilic antibodies (G9 and Herceptin) at different temperature indicated a unique pattern. While normal antibodies follow the protein typical increase in viscosity at lower temperature, the homophilic antibodies show increase in viscosity at higher temperature.

We also analyzed the effect of different temperatures on the efficiency of binding in solid-phase assays. For these studies, we used a pair of hybridoma antibodies against phosphorylcholine (PC): one being homophilic (HPC G11)

and the other non-homophilic (HPC G9). Binding at 37°C to PC was higher with HPC G11 than at 4°C, but temperature did not affect the binding of HPC G9 to PC.

The objective of this study was to demonstrate a correlation between the biophysical properties, such as the effect of temperature on oligomer/polymer state and antibody potency. We find that target binding is enhanced at physiological temperature and correlated with increase viscosity. Together with the earlier demonstration of a reversed dose/effector relation, homophilic antibodies have unusual properties that clearly separate them from conventional antibodies. It is therefore tempting to hypothesize that the increased potency of homophilic antibodies is due to dimer formation in solution prior to targeting. It appears that there is a critical ratio of antibody concentration to tumor target density in order to produce maximum homophilic antibody potency. This relationship of antibody to target expression can be implied from the earlier *in vivo* xenograft tumor growth reported [15] and from that observed in the current study.

Aggregation of antibodies can lead to pathological conditions recognized as cold agglutinins and cryoglobulins [22]. As the term indicates, aggregation and precipitation occur at low temperatures. In contrast, dimerization without precipitation is the unique property of homophilic antibodies suggesting the term warm agglutinin for this class of antibodies. It is interesting to note that the dual property of antibodies with either pathological or beneficial properties is reminiscent of  $\beta$ -sheet amyloid that can induce disease but also can serve as an intracellular storage form of proteins [23].

The functional properties of homophilic antibodies proposed here need to be confirmed in further *in vivo* studies that will explore how the target density controls the tumor cell response to treatment with homophilic antibody. The unique properties of homophilic antibodies may lead to a novel concept in immunotherapy in which the antibody dose will be optimized to the tumor antigen presentation.

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