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Design, Construction, and In Vitro Analyses of Multivalent Antibodies

Kathy Miller,† Gloria Meng,‡ Jun Liu,§ Amy Hurst,§ Vanessa Hsei,¶ Wai-Lee Wong,‡ Rene Ekert,‖ David Lawrence,§ Steven Sherwood,§ Laura DeForge,‡ Jacques Gaudreault,¶ Gilbert Keller,‖ Mark Sliwkowski,§ Avi Ashkenazi,§ and Leonard Presta*‡

Some Abs are more efficacious when being cross-linked to form dimers or multimers, presumably as a result of binding to and clustering more surface target to either amplify or diversify cellular signaling. To improve the therapeutic potency of these types of Abs, we designed and generated Abs that express tandem Fab repeats with the aim of mimicking cross-linked Abs. The versatile design of the system enables the creation of a series of multivalent human IgG Ab forms including tetravalent IgG1, tetravalent F(ab’)(2), and linear Fab multimers with either three or four consecutively linked Fabs. The multimerized Abs target the cell surface receptors HER2, death receptor 5, and CD20, and are more efficacious than their parent mAbs in triggering antitumor cellular responses, indicating they could be useful both as reagents for study as well as novel therapeutics. The Journal of Immunology, 2003, 170: 4854–4861.

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Materials and Methods

Construction, expression, and purification of Abs

The parent mAbs included two humanized Abs, anti-HER2 mAb hu4D5 (1) (Genentech, South San Francisco, CA) and anti-DR5 mAb 16E2 (Genentech), and two mouse-human chimeric Abs, anti-CD20 mAb C2B8 (2) (Genentech; and IDEC, La Jolla, CA) and anti-DR5 mAb 3H3 (Genentech), which was used to generate TA-DR5b. The H chains for L3 and L4 were engineered by fusing two Fabs head-to-tail (23). We modified this approach (Fig. 1A) by linking three (L3) or four (L4) Fabs in a linear, head-to-tail manner: by using linear Fabs to generate tetra- valent F(ab’)(2) forms (TFP); and by fusing the linear Fab to an IgG Fc (TA).

The multivalent Abs in this report were derived from parent mAbs targeting the following: HER2, a receptor overexpressed on ∼30% of breast cancers (24, 25); CD20, a B cell differentiation Ag important in proliferation and differentiation and associated with many lymphomas (26); and death receptor 5 (DR5), an apoptosis-inducing receptor that is a member of the TNFR superfamily (27).

Abbreviations used in this paper: scFv, single-chain Fv; L3, linear Fab multimer with three consecutively linked Fabs; L4, linear Fab multimer with four consecutively linked Fabs; TFP, tetravalent F(ab’)(2); TA, tetravalent IgG1; DR5, death receptor 5; RE, restriction endonuclease; HMVEC, human microvascular endothelial cell; ECD, extracellular domain; APO, ligand; PI, propidium iodide; SE, sedimentation equilibrium; FcRn, neonatal FcR.
were created by subcloning tandem repeats of PCR-generated Vp/CH1 DNA encoding residues 1–236 as defined by Kabat et al. (Eu nomenclature) (28) via unique restriction endonuclease (RE) sites incorporated into the 5' and 3' PCR primers (BamHI, Nhel, BspEI, NotI) into a previously described pRK expression vector (29) (Fig. 1B). The PCR primers for the final Vp/CH1 repeat included a termination codon. The TA H chains were made first by modifying a human IgG1 H chain (from mAb hu4D5) in a pRK expression vector (29). Site-directed oligonucleotide (30) was used to delete the sequence encoding residues 1–236 (28) encompassing the Vp/CH1 region and to insert unique RE sites (BamHI, Nhel, and BspEI). The Vp/CH1 repeats from either L3 or L4 were then subcloned into the H chain backbone by rapid digestion and ligation (Fig. 1B). TEP was generated from TA by replacing the Fc region with a leucine zipper (17) via site-directed mutagenesis.

Separate plasmids encoding the H and L chains were transiently expressed in human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA) and purified using protein A (TA; mAb) or protein G (TTP; L3, L4) chromatography (Amershams Pharmacia Biotech, Piscataway, NJ) as described previously (31). The Abs were analyzed by SDS-PAGE and quantitated by $A_{280}$ and by amino acid composition analysis.

Cell lines
All cell lines used were human in origin: colon carcinoma COLO105; lung carcinoma SK-MES-1; breast adenocarcinomas SK-BR-3, MDA-MB-361, MDA-MB-453, MDA-MB-231, MCF7, and BT474; kidney epithelial cell line 293; B lymphoma cell line WIL2-S. The cell lines SK-BR-3, MDA-MB-361, MDA-MB-453, MDA-MB-231, MCF7, HUVEC, 293, and WIL2-S were from American Type Culture Collection; human microvascular endothelial cell (HMVEC) line was from Clonetics (San Diego, CA); and the B cell lymphoma cell line BJAB (32) was a gift from E. Humke (Genentech). Cells were cultured in either RPMI 1640 with 10% FBS or RPMI 1640 with 10% FBS or 50:50 medium (50% Ham's F-12:50% DMEM) with 10% FBS.

Binding affinity measurements
Binding interactions were measured by surface plasmon resonance using a Biacore 3000 (Biacore, Uppsala, Sweden) as described previously (33, 34). Briefly, Abs were immobilized to the sensor chip through primary amine groups, and then the carboxymethylated sensor chip surface matrix was treated a single i.v. dose at 5 mg/kg of either TA-HER2, L4-HER2, or L3-HER2, all 115I labeled (lactoperoxidase method). The cells were washed in cold medium and allowed to continue growing for 0–24 h. TCA precipitation of the supernatant separated intact from catabolized Ab. Cell surface bound Ab was removed by acid washes. Intracellular Ab was recovered after cell lysis. Samples were quantitated by gamma counter (Iso-Data 500/100 series; Iso-Data, Oakland, NJ). SK-BR-3 cells grown on glass coverslips were prepared as above and incubated at 37°C for 0 or 5 h with 115I-labeled TA-HER2 or hu4D5 and processed for ultrastructural autoradiography as previously described (40).

Pharmacokinetic studies
Eighteen male Sprague Dawley rats (Charles River Laboratories, Hollister, CA) were divided into six groups of three, and each group was administered a single i.v. dose of either TA-HER2 or hu4D5, hu4D5, TA-DR5, L3-DR5, or anti-DR5 mAb. Serum samples were harvested at the following times: predose; 5 h; 2, 4, 8, and 24 h; and days 2, 4, 7, 10, 14, and 21. L3-DR5 and L4-HER2 were collected at the additional times of 30 min and 12 and 32 h. Samples were quantitated by ELISA (41), and standard curves were generated by plotting absorbance vs concentration using a four-parameter logistic curve fitting. Pharmacokinetic parameters were determined by noncompartmental and compartmental analysis as described (42).

Results
Generation of multivalent mAbs
When expressed as protein, the H chains of TA dimerize through the hinge/Fc region, whereas TFP is joined via the hinge/leucine zipper to form the Fab’2, as shown in Fig. 1A. As compared with the two Fab domains of bivalent IgG, L3 has three Fabs, and TA, TFP, and L4 have four Fabs, resulting in Abs that are potentially trivalent or tetravalent. Although up to four Fab repeats were constructed, more Fab units could be added, limited only by the number of unique restriction sites available. The RE sites joining the VH/CH1...
domains encode two amino acids with small side chains (alanine-serine or alanine-glycine) to provide for some flexibility between the Fab units. Expression levels of the multivalent mAbs in transiently transfected mammalian 293 cells were similar to the parental native mAb, (1–15 mg/L supernatant). Each Ab appeared as a single species when evaluated by SDS-PAGE, with molecular mass in agreement with those calculated from amino acid composition (Fig. 1C).

Affinity and stoichiometry measurements of anti-HER2 multivalent mAbs

Binding kinetics of anti-HER2 mAbs to HER2-ECD were measured by surface plasmon resonance using a Biacore 3000. To avoid avidity effects, mAbs were immobilized on sensor chips and serial dilutions of HER2-ECD were injected. Kinetic data were fit using a 1:1 binding model with the $K_a$ and $K_d$ determined simultaneously for all concentration curves. After adjusting for molecular mass and the number of binding sites, all mAbs were found to bind an equivalent amount of HER2-ECD. The $K_a$, $K_d$, and $K_D$ were similar for all mAb forms, including the parental native mAb, indicating no striking difference in affinity between them (Table I).

SE ultracentrifugation analysis was used to determine whether all Fab domains in the multivalent mAbs were capable of binding HER2-ECD. The molecular masses of the mAbs determined by SE were in agreement with those calculated from amino acid composition (Table II). However, the molecular mass of HER2-ECD determined by SE was significantly higher than predicted from amino acid composition (Table II), suggesting a high degree of glycosylation, an observation consistent with HER2-ECD having eight putative N-linked glycosylation sites (43).

Ab:HER2-ECD complexes were measured by SE analysis after the tetravalent anti-HER2 mAb (TA-HER2) was mixed with HER2-ECD at molar ratios ranging from 1:2.7 to 1:4.5 (TA-HER2:HER2-ECD). The experimental molecular masses of complexes were similar to but slightly less than those calculated for theoretical complexes containing four functional binding sites, and significantly greater than those calculated to have only three functional sites (Fig. 2; Table II). This suggests that, although all four Fabs of TA-HER2 are capable of binding HER2-ECD, they may not bind HER2-ECD equivalently, possibly due to steric hindrance. Likewise, the average molecular masses of the complexes formed by the linear, tandem Fab forms L3-HER2 and L4-HER2 were closer in size to theoretical complexes calculated to have fully functional binding sites ($A_3$) than to those having one inactive site ($B_3$) (Table II). As with the full-length mAb, the discrepancy between the experimental average molecular mass and the calculated average molecular mass of the fully bound forms suggests that the Fabs of L3-HER2 and L4-HER2 may not all be able to bind HER2-ECD equivalently.

Functional analyses of multivalent anti-HER2 mAbs

The anti-HER2 multivalent mAbs were compared with the parental bivalent anti-HER2 (hu4D5) in cytostasis assays. hu4D5 has been previously shown to be efficacious in cytostasis assays when applied to adenocarcinoma cell lines expressing HER2 at relatively high levels ($>2.5 \times 10^6$ HER2/cell) but not on cell lines with

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Table II. **SE analysis**

<table>
<thead>
<tr>
<th>Multivalent Ab</th>
<th>Molecular Mass (AA)</th>
<th>Molecular Mass (SE)</th>
<th>Molar Ratios Ab-ECD</th>
<th>Calculated Molecular Mass [-] $A_1$</th>
<th>Calculated Molecular Mass [-] $B_2$</th>
<th>Experimental Molecular Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA-HER2</td>
<td>241,431</td>
<td>240,000 ± 7,000</td>
<td>1:3.6</td>
<td>542,300</td>
<td>441,500</td>
<td>514,000 ± 18,150</td>
</tr>
<tr>
<td>L3-HER2</td>
<td>143,313</td>
<td>148,700 ± 16,000</td>
<td>1:3.0</td>
<td>386,300</td>
<td>260,380</td>
<td>369,800 ± 20,200</td>
</tr>
<tr>
<td>L4-HER2</td>
<td>191,472</td>
<td>193,300 ± 12,000</td>
<td>1:4.0</td>
<td>510,000</td>
<td>376,300</td>
<td>464,000 ± 22,500</td>
</tr>
<tr>
<td>HER2-ECD</td>
<td>69,221</td>
<td>79,200 ± 3,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

AA, Amino acid analysis; $A_1$, Theoretical molecular mass of complexes assuming fully functional binding sites; $B_2$, Theoretical molecular mass assuming one non-functional binding site.
moderate (∼0.5 × 10^4 HER2/cell) or low (∼1.0 × 10^4 or less HER2/cell) expression (36). All four forms of multivalent mAb (TA-HER2, TFP-HER2, L3-HER2, and L4-HER2) elicited cytostasis comparable to that of hu4D5 on high-expressing cell lines such as BT474 (not shown) and SK-BR-3 (Fig. 3A), while not effecting growth of low-expressing cell lines MDA 231 or MCF7 (data not shown). Significantly, the multivalent mAbs inhibited the growth of the moderately expressing cell line MDA 361 more effectively than hu4D5 (Fig. 3B).

Assays measuring the internalization and catabolism of the native and multivalent mAbs showed that the multivalent mAbs were processed at twice the rate of the native hu4D5. TA-HER2 was internalized more rapidly than native hu4D5 on both the high-expressing BT474 cell line (Fig. 4A) and the moderately expressing MDA 453 cell line (Fig. 4B). For the BT474 cells, the amount of unbound mAb was similar for hu4D5 and TA-HER2; however, the amount of unbound TA-HER2 was consistently less than that of hu4D5 for MDA 453 cells. This more pronounced effect of TA-HER2 on cells with moderate expression of HER2 (compared with cells with high expression levels) is in agreement with the cytostasis assays and underscores the greater potential of the multivalent mAbs to cluster HER2 when HER2 is expressed at moderate levels. The linear Fab construct L3-HER2 was also internalized and catabolized similar to TA-HER2, i.e., at twice the rate of hu4D5 (data not shown).

Ultrastructural autoradiography confirmed that both TA-HER2 (Fig. 5) and hu4D5 (data not shown) followed the same intracellular pathway to binding to HER2 and being internalized. As apparent from the decay-trace patterns, iodinated TA-HER2 bound to cell surface HER2 expressed on microvilli (Fig. 5A) and was subsequently associated with coated pits (Fig. 5B), indicating that the mAb-directed internalization was an active process (9). The internalized TA-HER2 continued along the degradation pathway into smooth cytosolic vesicles (Fig. 5, C and D) and into acidic endosomes for catabolism (Fig. 5, E and F). The autograph across for hu4D5 was equivalent to that shown for TA-HER2 and to previous observations (40).

**Functional analyses of multivalent anti-CD20 mAbs**

In vitro studies on mAbs targeting CD20 on B cells show that the mAbs require cross-linking to trigger apoptosis (5–7). Multivalent anti-CD20 mAbs were compared with the parental anti-CD20 Rituximab (2) for their ability to induce apoptosis of the B lymphoma cell line WIL2-S. Apoptosis was measured using flow cytometric light scatter analysis of cells stained with FITC-annexin V combined with PI incorporation (Fig. 6). Native bivalent anti-CD20 Fab per molecule may be able to bind, thus reducing the inherent cross-linking effect of TA-CD20. TFP-CD20 and L3-CD20 multivalent mAbs also induced apoptosis of WIL2-S cells without requiring cross-linking (Fig. 6B).

**Functional analyses of multivalent anti-DR5 mAbs**

DR5 triggers cellular apoptosis after binding to and being oligomerized by its ligand, APO2/ TRAIL (27, 37, 44). Viability of tumor cell lines derived from colon (COLO 205; Fig. 7, A and C) and lung (SK-MES-1; Fig. 7B) after exposure to concentrations representing equimolar amounts of APO2/ TRAIL, the parental anti-DR5 mAb, and its multivalent derivatives was evaluated. The results show that TA-DR5, TFP-DR5, L3-DR5, and L4-DR5 triggered levels of cell death similar to those of APO2/ TRAIL (Fig. 7, but was much more effective than cross-linked bivalent anti-CD20 mAb at 0.5 and 1.0 µg (Fig. 6A). The reduced efficacy of TA-CD20 at 10 µg compared with lower concentrations may be due to competition among the TA-CD20, i.e., at 10 µg, only one or two Fab per molecule may be able to bind, thus reducing the inherent cross-linking effect of TA-CD20. TFP-CD20 and L3-CD20 multivalent mAbs also induced apoptosis of WIL2-S cells without requiring cross-linking (Fig. 6B).

**FIGURE 2.** Plot of SE analysis of TA-HER2. Experimentally determined TA-HER2/HER2-ECD complexes (●) calculated molecular mass of TA-HER2/HER2-ECD complexes containing four functional binding sites (○) or three functional binding sites (□).

**FIGURE 3.** Characterization of the anti-HER2 multivalent Abs in cytostasis assays. Representative plots of n = 4 crystal violet cytostasis assays with TA-HER2 (○), TFP-HER2 (●), L3-HER2 (■), and L4-HER2 (□) compared with huMab4D5 (◆) on the HER2 moderate-expressing cell line MDA361. Each point is the average of duplicate measurements. Abs were added at equimolar concentrations, and results are presented as the percentage of growth compared with cells to which no Ab is added.

**FIGURE 4.** Internalization analyses of TA-HER2 in comparison to huMab4D5. Equimolar concentrations of TA-HER2 and huMab4D5 were added to BT474 cells (A; n = 4 assays) and MDA453 cells (B; n = 3 assays) and analyzed in internalization assays, each point in triplicate. Ab measurements include the following: cell surface bound (●, TA-HER2; ◆, huMab4D5), internalized (△, TA-HER2), A, huMab4D5), catabolized (□, TA-HER2; ■, huMab4D5), and unbound (○, TA-HER2; ●, huMab4D5).
A–C). TA-DR5 triggered apoptosis at ~100-fold lower concentration than the parental, bivalent mAb (Fig. 7, A and B), and TFP-DR5, L3-DR5, and L4-HER2 rapidly decreased. TA-DR5, like APO2L/TRAIL (32), did not kill human endothelial cell lines HMVEC (Fig. 7D) or HUVEC (data not shown).

Both APO2L/TRAIL and TA-DR5 effected cell death via activation of the caspase signaling pathway (Fig. 7, E and F). Apoptosis mediated by ligand or mAb was inhibited by addition of Z-VAD, a caspase inhibitor (45). Both the caspase adaptor Fas-associated death domain and the signaling initiator caspase 8 associate with DR5 on the lymphoma-derived cell line BJAB, previously characterized for DR5 expression (32), after incubation with two different anti-DR5 tetravalent Abs, TA-DR5 and TA-DR5b (Fig. 7F). These are the same signaling molecules that associate with DR5 after incubation with APO2L/TRAIL to form the death-inducing signaling complex (32) (Fig. 7F).

TA-DR5 was also tested on the National Cancer Institute Human Tumor Cell Line panel (46). It inhibited cell growth comparable to APO2L/TRAIL on most of the tumor cell lines (data not shown). However, on some cell lines, e.g., the leukemia cell line RPMI 8226, the ligand performed better; whereas on other cell lines, e.g., two renal cancer cell lines, RXF 393 and ACHN, the TA-DR5 performed better. Such selective sensitivity indicates that TA-DR5 could be a useful reagent for gaining more insight into DR5-directed apoptosis on various tumor cell lines.

Pharmacokinetic analysis of multivalent mAbs

The pharmacokinetics of several anti-HER2 and anti-DR5 multivalent mAbs were measured in rats and compared with their parental mAbs. The concentration-time profiles and pharmacokinetic parameters (Fig. 8A; Table III) showed that similar concentrations of hu4D5 and TA-HER2 remained in serum over time, whereas that of L4-HER2 rapidly decreased. TA-HER2 and hu4D5 (Fig. 8A) had similar mean clearance and mean volumes of distributions (volume of distribution of the central compartment; steady-state volume of distribution) resulting in comparable terminal half-lives. In comparison to hu4D5, the mean clearance of L4-HER2 was ~20-fold greater and the mean steady-state volume of distribution 1.8-fold lower, with a shorter terminal half-life.

The concentration-time profile and pharmacokinetic parameters showed that TA-DR5 was eliminated more rapidly than the anti-DR5 mAb, with a 4.3-fold greater mean clearance and a shorter terminal half-life (Fig. 8B; Table III). Because both TA-DR5 and native anti-DR5 mAb were cleared more rapidly than TA-HER2, the Abs were tested for binding to human neonatal FcR (FcRn), a receptor involved in prolonging IgG half-life (47, 48). FcRn bound the anti-DR5 mAb and TA-DR5 less well than did TA-HER2, although both were human IgG1 (data not shown). Although the reason for the reduced binding is not clear, this provides at least a possible explanation for the shorter half-life. The mean clearance of L3-DR5 was ~16-fold greater and the mean steady-state volume of distribution was lower than the anti-DR5 mAb, resulting in a terminal half-life of hours instead of days (Fig. 8B; Table III).

Discussion

Based on the observation that cross-linking increases the efficacy of some mAbs (5–7, 49), a set of three types of multivalent mAbs was designed: linear with tri- and tetravalent Fab repeats, a tetravalent F(ab’)_2, and a tetravalent full-length IgG1. All three classes can be built from a basic linear Fab repeat. Any V_h/CH1 domain of interest can be incorporated into the system through the use of unique restriction sites situated between the Fab repeats, i.e., separate PCR fragments of the V_h/CH1 domain with the various restriction sites at the 5’ and 3’ ends allow for construction of two, three, four, or more tandem repeats of the V_h/CH1. This can be expressed with a single L chain (V_L-C_L) to generate the multivalent Ab forms.
FIGURE 7. Analyses of anti-DR5 multivalent Abs. Equimolar concentrations of TA-DR5 (○), APO, L (△), and DR5-mAb (□) were added to COLO 05 (A), SK-MES-1 (B), and HMVEC (D), and viability was analyzed by crystal violet assay, each point in triplicate. C. Equimolar concentrations of DR5-mAb (○), TFP-DR5 (●), TA-DR5 (□), L3-DR5 (▲), and L4-DR5 (□) were analyzed as in A, B, and D, in a representative plot of n = 4 assays, each point assayed in duplicate. E, SK-MES-1 cells were assayed by crystal violet after adding TA-DR5 (●) and APO, L (△), without (dashed line) and with (solid line) the caspase inhibitor Z-VAD. Ill, Z-VAD alone. F, The B cell line BJAB was incubated with two different anti-DR5 Abs, TA-DR5 and TADF5-b, after which DR5 was purified from cell lysates, and the associated proteins were visualized by Western blot analyses (32).

The multivalent constructs evaluated in this study included mAbs targeted against the HER2 (24), CD20 (26), and DR5 (27) proteins. All mAbs expressed as single species at levels similar to their parental, native IgG1 mAbs. Affinity measurements designed to avoid avidity effects suggest that these constructs bind their target similar to the parental mAb. Evidence from sedimentation ultracentrifugation experiments indicates that all F(ab’)_2 on the multivalent Abs are capable of binding; however, there may be some binding inhibition, potentially due to steric hindrance.

One important attribute of these multivalent mAbs may be their ability to more efficiently cluster cell-bound targets (compared with bivalent mAbs) without requiring additional cross-linking.

Despite being structurally diverse, the linear Fab, F(ab’)_2, and full-length IgG1 appear to function similarly in this respect. The bivalent anti-HER2 mAb hu4D5 was originally chosen as a therapeutic candidate due to its cytostatic character (50). Compared with the bivalent hu4D5, the multivalent mAbs effected slightly increased levels of cytostasis on cells expressing high levels of HER2 and, in addition, induced a higher level of cytostasis on cells expressing only moderate levels of HER2. The multivalent Abs may induce cytostasis through similar mechanism(s) as hu4D5 (51); however, the more pronounced effect of the multivalent mAbs on moderately expressing HER2 cells may be due to their ability to cluster more HER2 per mAb (three to four receptors per mAb) compared with hu4D5 (only two receptors per mAb). On high-expressing HER2 cells, clustering of HER2 by native hu4D5 may be sufficient to elicit a minimal cytostatic signal, and additional clustering by the multivalent mAbs may not appreciably augment this signaling. This suggests that, in addition to being useful as in vitro reagents to study the relationship of clustering and growth inhibition, the multivalent mAbs might be therapeutically efficacious on a wider range of HER2-expressing tumors than a bivalent anti-HER2 mAb.

Another therapeutic, anti-CD20 mAb, Rituximab, has been shown previously to require cross-linking to effect the apoptosis of CD20-bearing B cells (5–7). In vivo, the cross-linking agents could be complement, FcγR-bearing cells, or both. Evidence for the role of FcRs in vivo comes from studies in which binding to these receptors has been voided (4, 52) and a study correlating human FcγRIII polymorphism with patient response (53). Trivalent linear Fab, tetravalent F(ab’)_2, and tetravalent IgG1 forms of anti-CD20 expressed apoptosis on WIL2-S cells equivalent to or more efficiently than cross-linked bivalent anti-CD20 IgG1. A third system in which oligomerization of receptors is required for effect is that of DR5. Anti-DR5 multivalent mAbs were as potent as the native ligand, APO, L (TRAIL), (and more potent than bivalent mAb) in effecting the apoptosis of cancer-derived cell lines, did not kill normal endothelial cells, and used the same intracellular caspase pathway as the natural ligand.

In concert with previous studies showing the increased avidity and increased effect of multivalent mAbs, including those based on scFv, Fab, and IgG (13–23), the mAb forms evaluated in this study underscore the potential for enhanced therapeutic utility of multivalent mAbs. Although functionally similar with regard to clustering of target molecules, the multivalent mAb forms vary in size.

FIGURE 8. Pharmacokinetic analyses of multivalent Abs. Serum pharmacokinetic profiles and parameters (mean ± SD) over time of TA-HER2, L4-HER2, and huMab4D5 (A), and TA-DR5, L3-DR5, and DR5-mAb (B), following 5 mg/kg i.v. administration in Sprague Dawley rats.
and half-life. The larger IgG form may be the choice when a functional Fc is warranted, although cellular effector functions triggered by the multivalent IgG form in comparison to bivalent IgG have yet to be studied. For mAbs that require cross-linking to exert their effect, e.g., apoptosis resulting from cross-linking of anti-CD20, in vivo the tetravalent IgG1 may be able to enhance apoptosis by two mechanisms. First, multivalent mAb would cluster CD20 more effectively than would bivalent mAb. Second, if Fcγ and/or FcγR-bearing cells act as cross-linking agents in vivo, then the presence of the Fc on the multivalent IgG form could increase cross-linking even more. If a smaller mAb form is required, e.g., CD20, in vivo the tetravalent IgG1 may be able to enhance apoptosis, although cellular effector functions triggered by binding to the target cell, followed by internalization of the mAb/target complex into the cell, release of the conjugated toxin, and simultaneously reduce the cytokine release syndrome-related toxicity.

Two other attributes of these mAbs may also provide therapeutic benefit. First, in some cases, the presence of an Fc may lead to infusion-related cytokine release syndrome (58–60). Because the linear multi-Fab and F(ab')2 mAbs can function in clustering target molecules as efficaciously as the full-length tetravalent mAb, these forms might enhance the efficacy of a therapeutic bivalent mAb and simultaneously reduce the cytokine release syndrome-related side effects. Second, a relatively new class of toxin-conjugated mAbs have been designed, including one approved for cancer, Mylotarg (3), and others in clinical trials (61). These mAbs function by binding to the target cell, followed by internalization of the mAb/target complex into the cell, release of the conjugated toxin, and subsequent cell death due to the action of the toxin (61, 62).

The anti-HER2 linear multi-Fab and IgG forms described in this study both showed a 2-fold increase in internalization compared with that of the native bivalent mAb. Whether this increase is therapeutically relevant must still be tested, but if more rapid internalization is advantageous, then the conjugation of toxins to these multivalent mAbs might increase efficacy and safety.

The multivalent TA-DR5 exhibited reduced binding to FcRn and a shorter half-life compared with its TA-HER2 counterpart (the latter had a half-life equivalent to that of bivalent anti-HER2). One difference between TA-DR5 and TA-HER2 is that TA-DR5 has a human L chain, whereas TA-HER2 has a κ L chain. Whether this difference in L chain class affected half-life or whether some other structural difference is the culprit must be investigated. The relatively short half-life of the linear multi-Fab forms also warrants further investigation. Although not necessarily a disadvantage for some applications such as solid tumor penetration (18), the half-life of the linear multi-Fabs could be extended by known techniques such as conjugation with polyethylene glycol (42, 63).

The multivalent mAb approach described in this report offers the versatility to tailor the Ab form to potentially enhance its therapeutic utility. Along with previously described engineered multivalent mAbs, these will hopefully provide an increased choice in molecular forms available for therapeutic mAbs.

### References


