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Higher cytotoxicity of divalent antibody-toxins than monovalent antibody-toxins

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ABSTRACT

Recombinant antibody-toxins are constructed via the fusion of a "carcinoma-specific" antibody fragment to a toxin. Due to the high affinity and high selectivity of the antibody fragments, antibody-toxins can bind to surface antigens on cancer cells and kill them without harming normal cells [L.H. Pai, J.K. Batra, D.J. FitzGerald, M.C. Willingham, I. Pastan, Anti-tumor activities of immunotoxins made of monoclonal antibody B3 and various forms of *Pseudomonas* exotoxin, Proc. Natl. Acad. Sci. USA 88 (1991) 3358–3362]. In this study, we constructed the antibody-toxin, Fab-SWn-PE38, with SWn (n = 3, 6, 9) sequences containing n-time repeated (G_4S) between the Fab fragment and PE38 (38 kDa truncated form of *Pseudomonas* exotoxin A). The SWn sequence also harbored one cysteine residue that could form a disulfide bridge between two Fab-SWn-PE38 monomers. We assessed the cytotoxicity of the monovalent (Fab-SWn-PE38), and divalent ([Fab-SWn-PE38]$_2$) antibody-toxins. The cytotoxicity of the dimer against the CRL1739 cell line was approximately 18.8-fold higher than that of the monomer on the ng/ml scale, which was approximately 37.6-fold higher on the pM scale. These results strongly indicate that divalency provides higher cytotoxicity for an antibody-toxin.

Introduction

Recombinant antibody-toxins are comprised of a "carcinoma-specific" antibody fragment fused to a toxin. They are protein-based reagents used to treat cancer. Antibody fragments bind specifically to the surface antigens on cancer cells with high affinity, and the toxin kills the cells. In recent years, several approaches have been utilized to generate recombinant antibody-toxins. Two frequently used binding moieties for antibody-toxins are the variable domain fragment (Fv) and the antigen binding fragment (Fab).

Fv is the smallest functional module of an antibody required for antigen binding. It is comprised of a heterodimer of the variable heavy chain domain (V_H) and variable light chain domain (V_L). V_H and V_L are typically connected by a flexible peptide linker resulting in a single chain Fv (scFv), where the linker prevents the dissociation of the two domains [2]. The scFv-toxin was expected to evidence a high degree of penetration into tumor tissue due to its small size. However, it evidenced low stability in the blood circulatory system as shown by renal clearance assays, and also had a very short half-life, $t_{1/2}\beta \approx 15$–20 min, in murine models [3].

Fab is a larger antibody fragment than scFv. It is comprised of the Fd chain (V_H and C_{H1}) and light chains (V_L and C_L). The Fd chain and light chains are connected by a disulfide bond between the cysteines at the end of each chain. The refolding yields of Fab-toxins were shown in previous studies to be 10-fold that of the scFv-toxin [4,5]. For the Fab-toxin, the C_{H1} domain and C_L domain harbor hydrophobic patches on their surfaces that bring, and fit, the Fd and light chains together, and a disulfide bond between them can be readily formed [6].

Furthermore, the Fab-toxin evidenced a longer half-life in animal plasma, close to the half-life of the whole IgG-toxin [5,7–9]. The disulfide bond between the Fd and light chain makes Fab-toxin more resistant to proteolytic attack than the scFv-toxin, which is more vulnerable to proteolytic attack because the molecule is not in the form of a stable dimer for the V_H and V_L domains [5].

Here, we utilized Fab fragments of the monoclonal antibody (MAb) B3 for antibody-toxin construction. MAb B3 is a murine antibody directed against a carbohydrate antigen in the Le^Y family. When assessed via peroxidase immunohistochemical techniques using frozen human tumors, the antigen was determined to be present on many mucin-containing carcinomas—including those of the colon, stomach, ovary, breast, and esophagus [10]. B3 is one of the most extensively studied antibodies, and it has a very strong characteristic for specific binding to target cell lines, whereas it evidences very little binding to non-target cell lines.

We have selected a mutant form of PE (PE38) for this study. PE (*Pseudomonas* exotoxin A) is a 66 kDa protein comprised of three domains: an amino-terminal cell surface binding domain (domain Ia: 1–252), a middle translocation domain (domain II: 253–364) along with a middle nonessential domain (domain Ib: 365–404),

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and a carboxyl-terminal toxicity domain (domain III: 405–613). PE38 lacks domain Ia and a part of domain Ib (365–380). Domain III of PE38 catalyzes the transfer of an ADP-ribose moiety of oxidized nicotinamide adenine dinucleotide (NAD^+) to a modified histidine of protein synthesis elongation factor 2. This terminates all peptide chain elongation within a target cell [3,11], and induces programmed cell death [12].

In our previous study, we constructed a Fab-PE38 monomer, which featured a modified hinge without a cysteine residue between Fab and PE38 [13]. In an attempt to construct an antibody-toxin with higher binding valency, we have been developing divalent antibody-toxins that harbor two Fab-PE38 monomers bridged by a disulfide bond [6,14–16]. Among these antibody-toxins, Fab-ext-PE38 harbored a long extension sequence (ext) between Fab and PE38, and the Cys residue on the ext formed a disulfide bond between two monomers. The long sequences after the disulfide bridge cysteine made the dimerization of Fab-ext-PE38 easier to generate [Fab-ext-PE38]$_2$, as it reduced the collisions and the steric hindrance between the two PE38s [15].

In this study, three recombinant antibody-toxins—[Fab-SW_3-PE38]$_2$, [Fab-SW_6-PE38]$_2$ and [Fab-SW_9-PE38]$_2$—were designed and produced. They have an SWn sequence between Fab and PE38 to allow for more space between the two Fab domains. An SWn sequence harbors one cysteine which forms a disulfide bond bridge between two monovalent antibody-toxins. An SWn sequence also harbors the flexible $(G_4S)n$ (n = 3, 6, 9) sequence before the disulfide bridge cysteine residue, which allows more space between the two Fab domains and the freedom to bind to surface antigen.

Each antibody-toxin was obtained as a dimer and a monomer. In order to assess the effects of divalency, we evaluated the cytotoxic activity against four human cancer cell lines: A431, CRL1739, MCF7 (all Le^Y antigen positive) and KB3-1 (Le^Y antigen negative). The cytotoxicity of divalent molecules was approximately 5.4- to 18.8-fold higher for CRL1739 cells as compared to monovalent molecules on a ng/ml scale, 1.9- to 5.6-fold higher for A431 cells, and 0.7- to 3.1-fold higher for MCF7 cells. These results indicate that a divalent molecule can be utilized as a therapeutic agent in target-specific antibody therapy with higher efficacy.

Materials and methods

Construction of plasmids for expression of the Fab-SWn-PE38 (n = 3, 6, 9). The plasmids for expression of Fd-SWn-PE38 (n = 3, 6, 9) were constructed based on the pCW1 plasmid (Fd-ext-PE38 = Fd-SKPSIST-KAS-$G_4C(G_4S)_2$-GGPE-PE38) [15] and the light chain was expressed from the pMCH75 plasmid [17]. The pSWn series (n = 1–9) plasmids have $(G_4S)n$ (n = 1–9) units inserted between a modified hinge (SKPSIST) and a spacer sequence (QAS-$G_4C(G_4S)_2$-GGPE). For the insertion of G_4S, we employed a PCR method. Primer 1 (5′-TAA TAC GAC TCA CTA TAG GGA GA-3′) was annealed to the 5′-end of the DNA fragment encoding for the Fd fragment, and primer 2 (5′-CCC AAG CTT GAG ACC CGC CTC CAC CGG TAC TTA TGC TAG GCT TAC TAC-3′) containing G_4S, coupled with the AgeI and HindIII restriction enzyme sites, was annealed to the modified hinge region. PCR fragments were digested with NdeI/HindIII and cloned into an NdeI/HindIII-digested pCW1. The resultant plasmid, pSW1, encodes for Fd-SW_1-PE38 (Fd-SKPSIST-G_4S-QAS-$G_4C(G_4S)_2$-GGPE-PE38) and harbors the AgeI site. pSW2 was constructed to express Fd-SW_2-PE38 by PCR. Primer 1 and primer 3 (5′-CCC TCC GGA CCC GCC TCC ACC GGT ACT TAT GCT AGG CTT ACT AC-3′) containing G_4S, coupled to the AgeI and BspEI restriction enzyme sites, were used. PCR fragments were digested with NdeI/BspEI and cloned into the NdeI/AgeI-digested pSW1. BspEI and AgeI produce compatible cohesive ends, and can be ligated to each other to generate the pSW2 plasmid. For the construction of pSW2, the AgeI site from the PCR fragment is retained, but the ligation of the ends of BspEI and AgeI destroys the AgeI site of pSW1. The same PCR fragment was repeatedly used for further G4S insertion. Thus, pSW3-9 was constructed via the insertion of the PCR fragment into an NdeI/AgeI-digested pSW2-8, as described above.

Production of antibody-toxins. Fd-ext-PE38, Fd-SW_3-PE38, Fd-SW_6-PE38, Fd-SW_9-PE38, and L chain were expressed in *Escherichia coli* BL21(λDE3) cells containing pCW1, pSW3, pSW6, pSW9 and pMCH75, respectively. Transformed cells were grown in 1 L of culture and induced by 1 mM IPTG when the OD_{600} reached 2.0 [6]. The inclusion bodies were isolated as previously described [13]. Inclusion bodies were solubilized in buffer containing 6 M guanidine–HCl, 0.1 M Tris–Cl and 2 mM EDTA (pH 8.0). The quantities of proteins were determined via a Bradford assay with Coomassie Plus protein assay reagent (Pierce). The purity of each polypeptide chain was analyzed via SDS–PAGE and densitometry (TINA ver. 2.0). The purity was determined by averaging the purities of three different serially diluted samples.

The solubilized Fd-ext-PE38 or Fd-SWn-PE38 (n = 3, 6, 9) chain was combined with the light chain at a 1:1 molar ratio, making a total of 80–100 mg/10 ml. Refolding was conducted as described previously [6]. The divalent and monovalent antibody-toxins were purified via Q-sepharose, Source-Q, Protein G HP chromatography and Superdex 200 HR 26/60 chromatography (Amersham, UK). After purification, the amounts of properly folded antibody-toxins were measured using a BCA (Bicinchoninic Acid) Protein Assay Kit (Pierce). The purity of each of the mono- and divalent antibody-toxins were determined in the same fashion as described above.

Cytotoxicity assays toward B3 antigen-expressing cancer cells. The cytotoxicity of mono- and divalent molecules was assessed via the inhibition of protein synthesis [1]. The inhibition of protein synthesis was determined by measuring the incorporation of [^{3}H]Leucine into the cellular protein after 24 h of exposure to antibody-toxin and 14 h of labeling. Incorporated [^{3}H] was counted with a Microbeta TriLux Liquid Scintillation Counter (Wallac EG&G Co.). The assay was repeated three times with triplicate samples. The ID_{50} was defined as the concentration of antibody-toxin necessary to reduce the incorporation of [^{3}H]Leucine into target cells by 50%. The ID_{50} values of the antibody-toxins were calculated using Origin 7.0 software (Origin Lab Corporation) at the ng/ml concentration scale.

Results

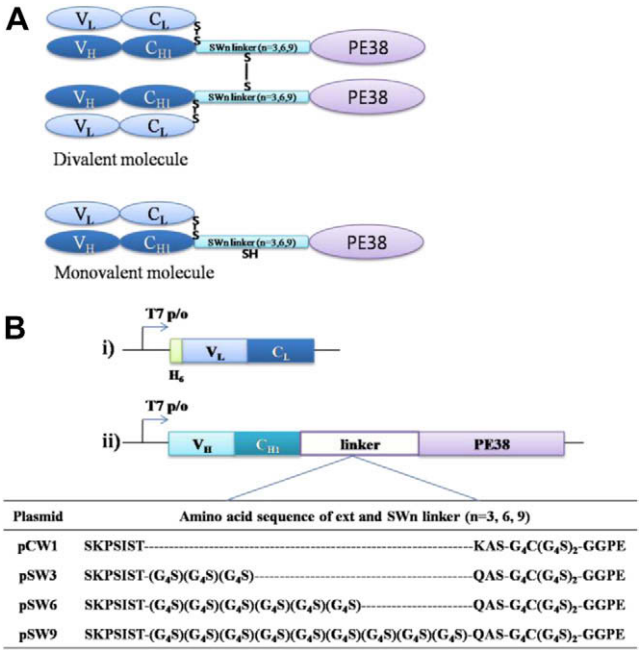
Construction and expression of Fab-SWn-PE38 (n = 3, 6, 9)

The schematic structures of di- and monovalent molecules are presented in Fig. 1. The DNA sequences of the constructed plasmids were confirmed via sequencing analysis.

Escherichia coli BL21(λDE3) cells harboring the pCW1, pSW3, pSW6, pSW9, or pMCH75 plasmids were utilized for the production of Fd-ext-PE38, Fd-SW_3-PE38, Fd-SW_6-PE38, Fd-SW_9-PE38 or Light chain, respectively. The yields of inclusion bodies were 31.2–97.8 mg per 1 L cell culture. The purity of inclusion bodies was approximately 28–51%.

Refolding and purification of antibody-toxins

The CW1, SW3, SW6, or SW9 polypeptide chains were refolded with the MCH75 chain by mixing at a 1:1 molar ratio to a final total protein amount of 80–100 mg/L. The refolding was conducted via a redox shuffling method [18]. The refolded proteins were then puri-



Plasmid	Amino acid sequence of ext and SWn linker (n=3, 6, 9)
pCW1	SKPSIST----KAS-$G_4C(G_4S)_2$-GGPE
pSW3	SKPSIST-$(G_4S)(G_4S)(G_4S)$----QAS-$G_4C(G_4S)_2$-GGPE
pSW6	SKPSIST-$(G_4S)(G_4S)(G_4S)(G_4S)(G_4S)(G_4S)$----QAS-$G_4C(G_4S)_2$-GGPE
pSW9	SKPSIST-$(G_4S)(G_4S)(G_4S)(G_4S)(G_4S)(G_4S)(G_4S)(G_4S)(G_4S)$-QAS-$G_4C(G_4S)_2$-GGPE

Fig. 1. Structure of the antibody-toxin fusion proteins used in this study. (A) Schematics of divalent and monovalent molecules. (B) Overview of the expression constructs for the (i) light, (ii) Fd-ext-PE38 and (iii) Fd-SWn-PE38 (n = 3, 6, 9) chains. The table shows the encoding plasmids and linker sequences between Fab and PE38. The Fab domain of monomer is formed by disulfide bonding between the cysteines at the ends of the Fd and light chains. The divalent molecule is constructed by a disulfide bond bridge between the cysteines in the SWn linker of each monomer. (G_4S) represents a repeating unit of amino acids = GGGGS, $G_4C(G_4S)_2$ = GGGGCGGGGSGGGGS, H_6 = HHHHHH, p/o = promoter/operator.

fied via Q-sepharose, Source-Q, and Protein G HP affinity chromatography and Superdex 200 HR 26/60 size exclusion chromatography. Refolding yields at the different steps and the SDS–PAGE analysis of the final preparation are summarized in Fig. 2. The final yields of $[Fab\text{-}ext\text{-}PE38]_2$, $[Fab\text{-}SW_3\text{-}PE38]_2$, $[Fab\text{-}SW_6\text{-}PE38]_2$ were 0.26–0.56%. These are approximately 18.6- to 40-fold that of the previously constructed $[FabH1\text{-}PE38]_2$ (0.014%) [14]. For monovalent molecules, Fab-ext-PE38, Fab-SW_3-PE38, and Fab-SW_6-PE38 evidenced yields of 3.41–4.79%, and these were similar to the FabH1-PE38 monomer (3.8%) [14]. However, the refolding yields of $[Fab\text{-}SW_9\text{-}PE38]_2$ and Fab-SW_9-PE38 were quite low, at 0.02% and 0.37%, respectively. The purified antibody-toxins were analyzed via SDS–PAGE under both non-reducing and reducing conditions.

The calculated molecular weights of the divalent molecules were 183.6–193.6 kDa. The results of SDS–PAGE analysis demonstrated that the non-reduced divalent molecules moved more slowly than the 250 kDa size marker, which is larger than the calculated molecular weights of the divalent molecules. Monovalent molecules with calculated molecular weights of 91.8–96.8 kDa also moved more slowly than the 95 kDa size marker. This is expected for the branched configurations of divalent and monovalent molecules. However, the di- and monovalent molecules resolved into two bands of approximately 72 kDa (calculated 66.3–71.3 kDa) and 28 kDa (calculated 25.5 kDa) when the proteins were reduced prior to electrophoresis. This result demonstrated that di- and monovalent antibody-toxins were properly formed.

Cytotoxicity of antibody-toxins for B3-antigen-expressing cancer cells

The cytotoxicity of antibody-toxins was measured on the basis of the ability to inhibit protein synthesis using $[^3H]$Leucine-incorporation. Using this assay, we compared the mono- and divalent antibody-toxins. The antigen-positive cancer cell lines utilized were A431, CRL1739, and MCF7. The KB3-1 cancer cell line was utilized as the negative control (Fig. 3). The ID_{50} for antibody-toxins was determined at ng/ml concentrations, then converted to pM concentrations (Table 1).

The ID_{50} of divalent antibody-toxins ranged from 0.07 to 1.4 ng/ml for the CRL1739 cell line, 0.5–9.3 ng/ml for the A431 cell line, and 6.6–15.0 ng/ml for the MCF7 cell line. The monovalents had ID_{50} values of 0.9–11.3 ng/ml for CRL1739, 3.4–29.7 ng/ml for A431, and 8.9–30.3 ng/ml for MCF7. The cytotoxicities of divalent molecules were approximately 5.4- to 18.8-fold higher than that of monovalent molecules for CRL1739, 1.9- to 6.8-fold higher for A431, and 0.7- to 3.1-fold higher for MCF7. For this study, ng/ml concentrations were converted to pM concentration in order to compare the cytotoxicities, as the molecular weights of divalent antibody-toxins were twice that of the monovalents. At pM concentrations, divalent antibody-toxins showed 1.4- to 37.6-fold higher cytotoxicity for the A431, CRL1739 and MCF7 cell lines than was observed in conjunction with the monovalent antibody-toxins. These results show that an increase in binding valency can provide higher cytotoxicity. The ID_{50} values for the di- and monovalent antibody-toxins were >1000 ng/ml for the KB3-1 negative control cell line.

Discussion

We have prepared three divalent molecules and their monomers in order to compare their cytotoxicities. To quantitatively compare them, the cytotoxicity of each mono- and divalent antibody-toxin was assessed. The divalent antibody-toxins evidenced cytotoxicity 1.4- to 37.6-fold higher against antigen-positive cell lines than was noted with the monovalent antibody-toxins at pM concentrations. These results show that increased cytotoxicities are attributable to increased binding valency. We also compared the cytotoxicity of a previously reported dimer, $[Fab\text{-}ext\text{-}PE38]_2$, and its monomer, Fab-ext-PE38. Our findings demonstrated that the cytotoxicity of the dimer was 0.9- to 12.8-fold higher than the monomer. The differences between the dimers and monomers are similar for the new and old molecules.

In addition, three different molecules were designed to increase the distance between the binding domains. Previously-constructed $[Fab\text{-}ext\text{-}PE38]_2$ has 14 amino acids between Fab and cysteine on the ext sequence [15]. A cysteine residue forms a disulfide bond between two monovalent antibody-toxins, and this disulfide bridge physically ties two monomers together. The maximum distance that can be achieved between Fab binding domains by spreading the two Fab domains is limited by the number of amino acids between the Fab and the disulfide bridge. This determines the maximum allowable distance between two antigens for which the dimer can have effective divalent binding.

In a previous report regarding $[Fab\text{-}ext\text{-}PE38]_2$, we suggested that cytotoxicity might be dependent on the antigen environment on the cell surface. If the antigens are on a long flexible structure, and if a divalent antibody-toxin can simultaneously bind two of them, it might be expected that a divalent molecule would have higher avidity for divalent binding than a monovalent molecule. In this study, we constructed $[Fab\text{-}SW_3\text{-}PE38]_2$, $[Fab\text{-}SW_6\text{-}PE38]_2$ and $[Fab\text{-}SW_9\text{-}PE38]_2$ by using the flexible amino acid G_4S sequence. These divalent antibody-toxins harbor 29, 44, and 59 amino acids between Fab and the cysteine residue. We expected that if the distance between binding domains was wider, then the avidity of divalent antibody-toxins should increase. However, the differences in cytotoxicity between the three dimers were not significant within the experimental error range. This implies that the 14–59 amino acid insertion harboring the $(G_4S)n$ sequence does

A pair of polypeptide chain for refolding	Refolding Total amount [a] (mg/L)	Antibody-toxins	After protein G-column		After Superdex 200	
			Yield (mg) [b]	%[c]	Yield (mg) [b]	%[c]
Fd-ext-PE38 Light chain	80	$[Fab\text{-}ext\text{-}PE38]_2$	1.4	1.8	0.28	0.35
		Fab-ext-PE38	6.1	7.7	3.83	4.79
Fd-SW_3-PE38 Light chain	80	$[Fab\text{-}SW_3\text{-}PE38]_2$	1.3	1.6	0.21	0.26
		Fab-SW_3-PE38	4.5	5.7	3.03	3.79
Fd-SW_6-PE38 Light chain	100	$[Fab\text{-}SW_6\text{-}PE38]_2$	1.7	1.7	0.56	0.56
		Fab-SW_6-PE38	5.2	5.2	3.41	3.41
Fd-SW_9-PE38 Light chain	100	$[Fab\text{-}SW_9\text{-}PE38]_2$	0.2	0.2	0.02	0.02
		Fab-SW_9-PE38	1.5	1.5	0.37	0.37

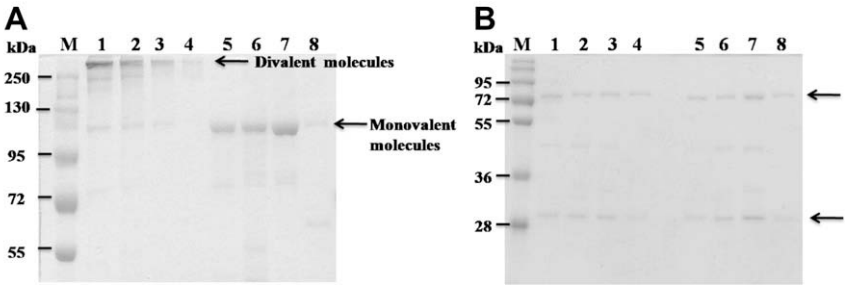


Fig. 2. Determination of antibody-toxins yields and SDS–PAGE analysis. The total amount[a] of protein in each mixture from 1 L refolding material. After the sample was purified, the total quantity of protein was measured via BCA assay. Purity was determined by densitometry (TINA ver. 2.0) with SDS–PAGE analysis and calculated by averaging the measurements of three different dilution of the samples. The refolding yields were averages of three repeated test results. % was calculated yield[b]/total amount[a] × 100. (A,B) SDS–PAGE of purified antibody-toxins utilized for the cytotoxicity assay. (A) Gel (8%) with non-reducing conditions. (B) Gel (12%) with reducing conditions. The upper arrow indicates Fd-ext-PE38 or Fd-SWn-PE38 (n = 3, 6, 9) and the lower arrow indicates the H_6-light chain. Lanes 1–4 represent divalent molecules and 5–8 show monovalent molecules. Lane M, protein size marker; lane 1, $[Fab\text{-}ext\text{-}PE38]_2$; lane 2 $[Fab\text{-}SW_3\text{-}PE38]_2$; lane 3 $[Fab\text{-}SW_6\text{-}PE38]_2$; lane 4 $[Fab\text{-}SW_9\text{-}PE38]_2$; lane 5, Fab-ext-PE38; lane 6, Fab-SW_3-PE38; lane 7, Fab-SW_6-PE38; lane 8, Fab-SW_9-PE38.

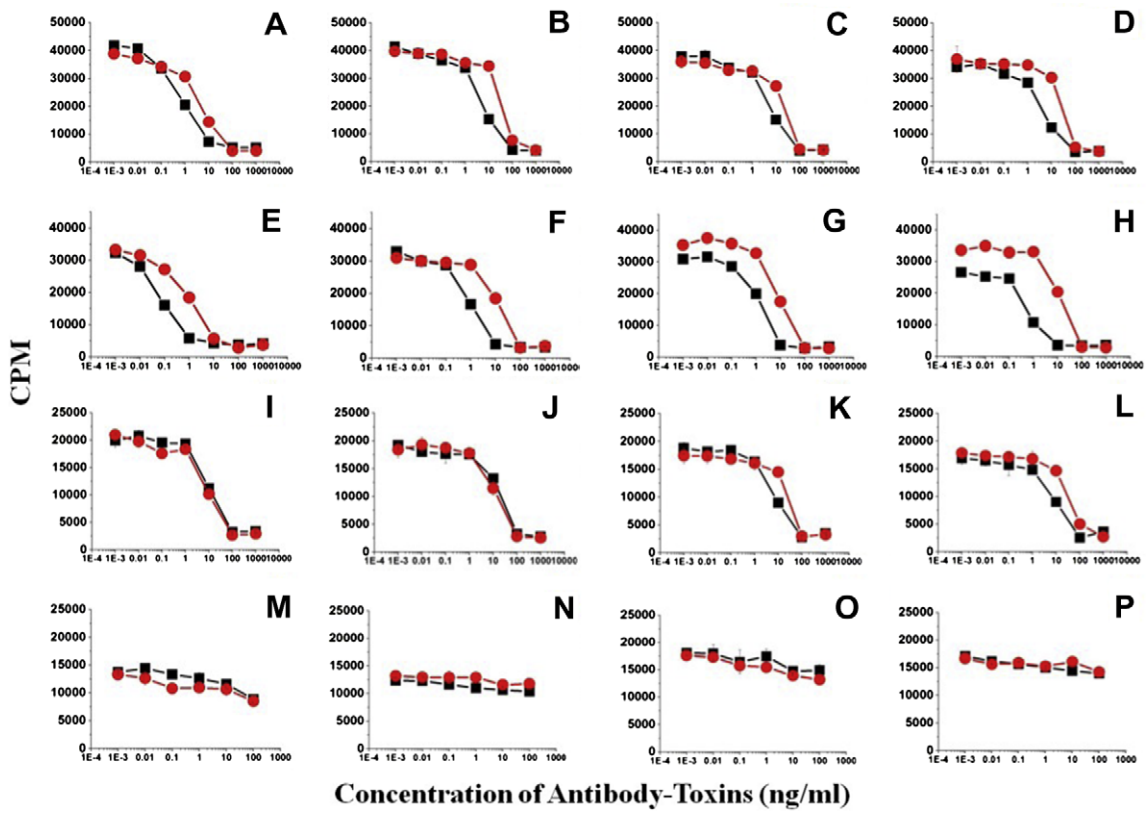


Fig. 3. Cytotoxic activity comparison of di- and monovalent antibody-toxins on human cancer cell lines. (A–D) A431, (E–H) CRL1739 and (I–L) MCF7 express B3 antigen, but (M–P) KB3-1 does not. Tenfold serial dilutions of divalent (■) and monovalent antibody-toxins (●), starting at 1000 ng/ml and going to 0.001 ng/ml, were added to human cancer cell lines in 96-well plates. Incorporation of [^{3}H]Leucine into cell proteins was measured after 24 h of exposure to antibody-toxins. The first column (A, E, I, M) shows $(Fab\text{-}ext\text{-}PE38)_2$ and Fab-ext-PE38, the second column (B, F, J, N) shows $(Fab\text{-}SW_3\text{-}PE38)_2$ and Fab-SW_3-PE38, the third column (C, G, K, O) shows $(Fab\text{-}SW_6\text{-}PE38)_2$ and Fab-SW_6-PE38 and the fourth column shows $(Fab\text{-}SW_9\text{-}PE38)_2$ and Fab-SW_9-PE38. Each plotted point is the average value of triplicate samples, and each assay was repeated three times. The average ID_{50} values from three assays obtained in ng/ml were converted to pM concentrations; these are provided in Table 1. CPM, counts/min.

Table 1
Average ID50 values of antibody-toxins from cytotoxicity assays.

Cell line Cancer type (B3 antigen expression) Antibody-toxin	A431 Epidermoid +++ ID_{50}		CRL1739 Gastric +++		MCF7 Breast +++		KB3-1 Epidermoid cervix –
	ng/ml	pM	ng/ml	pM	ng/ml	pM	ng/ml
$[Fab\text{-}ext\text{-}PE38]_2$	0.5	2.9	0.07	0.4	9.9	56.6	>10,000
Fab-ext-PE38	3.4	38.9	0.9	10.2	8.9	101.7	>10,000
$[FabSW_3\text{-}PE38]_2$	5.5	30.9	1.0	5.6	15.0	84.2	>10,000
$Fab\text{-}SW_3\text{-}PE38$	29.7	333.7	10.3	115.7	11.1	124.7	>10,000
$[Fab\text{-}SW_6\text{-}PE38]_2$	9.3	51.6	1.4	7.7	6.6	36.6	>10,000
$Fab\text{-}SW_6\text{-}PE38$	17.7	196.6	7.5	83.3	12.6	140.0	>10,000
$[Fab\text{-}SW_9\text{-}PE38]_2$	4.2	23.0	0.6	3.3	9.9	54.3	>10,000
$Fab\text{-}SW_9\text{-}PE38$	23.5	288.2	11.3	124.0	30.3	333.0	>10,000

not create binding avidity differences. The finding of similar cytotoxicities compels us to surmise that the insertion of the SWn sequence is not sufficiently long to catch two antigens at the same time, or that the SWn sequence does not spread the Fab binding domain widely enough to achieve divalent binding.

However, as we reported previously, divalent antibody-toxins evidenced higher cytotoxicity than monovalents. Obviously, a divalent antibody-toxin has a greater chance to bind to an antigen than a monovalent because it has two cell-binding domains; even if each of these binds only to one antigen; the two toxin domains would induce enhanced cytotoxicity. Additionally, in case of clinical application the dimer could have the advantage of higher structural stability, resulting in a longer half-life in the blood circulation as the disulfide bond bridges two monomers, which more closely resembles the structure of immunoglobulin. Whether or not two Fab domains in these molecules actually participated in divalent binding will require further investigation.

With regard to refolding yields, those of $[Fab\text{-}SW_9\text{-}PE38]_2$ and $Fab\text{-}SW_9\text{-}PE38$ were much lower than the other molecules, which demonstrates that the 59 amino acid insertion is too long to allow for the proper folding of the Fab and toxin domains.

The amino acid lysine (K) in the ext sequence of Fab-ext-PE38 is substituted with glutamine (Q) in the SWn sequence. Previously, a positively charged residue (Lys) or negatively charged residue (Glu) was attempted immediately after the Fd chain and before a connector (C3: ASGGPE) to increase refolding yield [13]. However, we noted no differences in refolding yield. The HindIII enzyme site (KAS: AAA GCT TCC, HindIII site is underlined) has been utilized for plasmid construction. When the Gln (Q) residue (QAS: CAA GCT TCC) was introduced, the HindIII site was conserved, and the Van der Waals volume of the residue was reduced from 135 Å to 114 Å. We assume that this permitted greater flexibility for the SWn sequence.

In our previous study, we utilized scFv-toxin [6,14–16,19] as a reference molecule for assessing the cytotoxicity of newly constructed divalent Fab-toxins, and we have attempted to construct a dimer that exceeds the cytotoxicity of scFv-toxin. In this study, we compared a divalent Fab-toxin to its monovalent form. It is clear that in order to see any effect from the divalency of the divalent molecule, it must be compared with its monovalent form. The structural formats of scFv and Fab differ significantly.

scFv consists of a V_L/V_H heterodimer connected by a flexible linker, and each domain can bind to antigens with large degrees of freedom in terms of orientation and distance of V_L/V_H domain. Fab consists of a light chain and a heavy chain, and these two chains are covalently linked by a disulfide bond. The profound hydrophobic affinity between C_{H1} and C_L not only provides good structural stability, but also limits the degrees of freedom of V_L/V_H domain for their binding as compared to scFv.

Although the scFv-toxin has a higher binding affinity toward Ag than Fab-toxin, its lower stability limits its clinical applicability. Fab has a lower affinity toward Ag, but this is compensated for by its stability [20]. In order to improve upon the low affinity of monovalent Fab-toxin, our group has generated divalent forms [6,14–16,19], In this study, we detected higher cytotoxicity for a dimer as compared to a monomer, which indicates that the divalent form may prove to be an excellent therapeutic agent with a wide array of applications.

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