Differential killing of CD56-expressing cells by drug-conjugated human antibodies targeting membrane-distal and membrane-proximal non-overlapping epitopes

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Abstract

CD56 (NCAM, neural cell adhesion molecule) is over-expressed in many tumor types, including neuroblastoma, multiple myeloma, small cell lung cancer, ovarian cancer, acute myeloid leukemia, NK-T lymphoma, neuroendocrine cancer and pancreatic cancer. Using phage display, we identified 2 high-affinity anti-CD56 human monoclonal antibodies (mAbs), m900 and m906, which bound to spatially separated non-overlapping epitopes with similar affinity (equilibrium dissociation constant 2.9 and 4.5 nM, respectively). m900 bound to the membrane proximal fibronectin type III-like domains, whereas m906 bound to the N-terminal IgG-like domains. m906 induced significant down-regulation of CD56 in 4 neuroblastoma cell lines tested, while m900-induced downregulation of CD56 was much lower. Antibody-drug conjugates (ADCs) made by conjugation with a highly potent pyrrolobenzodiazepine dimer (PBD) exhibited killing activity that correlated with CD56 down-regulation, and to some extent with in vivo binding ability of the antibodies. The m906PBD ADC was much more potent than m900PBD, likely due to higher CD56-mediated downregulation and stronger binding to cells. Treatment with m906PBD ADC resulted in very potent cytotoxicity (IC50: 0.05–1.7 pM). These results suggest a novel approach for targeting CD56-expressing neuroblastoma cells. Further studies in animal models and in humans are needed to find whether these antibodies and their drug conjugates are promising candidate therapeutics.

Introduction

Neuroblastoma is one of the most common pediatric solid tumors, particularly among young children. While prognosis for low and intermediate-risk neuroblastoma has improved in the past decade, there has been little progress for high-risk disease. Current treatment for high-risk disease includes multimodal chemo-, radio-, and immuno-therapeutic interventions in addition to surgery. In 2015, an antibody therapeutic targeting GD2, dinutuximab,1,2 was approved in the United States and European Union as a new treatment for the high-risk neuroblastoma. However, this product causes debilitating pain due to GD2 expression on normal peripheral nerves.3 Thus, alternative immunotherapeutic strategies to improve outcomes for patients with neuroblastoma are needed.

CD56 is a type I plasma membrane glycoprotein involved in cell-cell and cell-matrix adhesion.4 The extracellular domain has 5 IgG-like domains at the N-terminus and 2 fibronectin type III-like domains on the membrane proximal region. The two domains may have different roles in its function: the IgG-like domains participated in homophilic interaction with another CD56 molecule on the opposite cell or next molecules on the same cell,5 while the fibronectin type III-like domain may mediate signaling to downstream proteins. CD56 is over-expressed in almost all neuroblastoma,6 98% of small cell lung cancer6,7 and 78% of multiple myeloma8 patients. Some ovarian cancers, acute myeloid leukemia, and Wilms tumor9 patients also have elevated CD56,10 highlighting the widespread appeal of developing new therapies targeting CD56.

It must be noted, however, that CD56 is also expressed in several normal tissues, including natural killer (NK) cells, activated T cells, neurons, glial cells and skeletal muscle cells.4 There are a number of reasons that the CD56 expression on NK cells need not impede development and clinical use of a CD56-targeted therapy. First, NK cells turn over approximately every 2 weeks in the circulation, and lymphoid progenitor cells do not express CD56.11 Second, NK cells in the tumor microenvironment generally function to promote cell growth via the secretion of cytokines and lack effector function.12 Third, CD56 bright NK cells are in the minority in the peripheral blood, although they are the majority of NK cells in the secondary lymphoid tissues. NK cells with antibody-dependent cell-mediated cytotoxicity activity are usually CD56 dim or CD56−, which will be unlikely to be affected by CD56 targeted therapy.13 Thus, the major hurdle is to avoid neural toxicity. Antibody-drug conjugates (ADCs) combine the exquisite specificity of antibodies with the potent cytotoxic drugs. It takes a much lower dose of ADC than naked antibody to have clinical benefits. And only a small fraction (0.1–0.2%) of peripherally
administered antibodies permeates the blood-brain barrier. Therefore, the possibility of harming the normal CD56-positive neural cells is much reduced with ADC strategy.

As reported in the 1990s, several murine radio-immuno-therapies or bispecific anti-human CD56 antibodies have been tested in humans. However, further studies on these antibodies have not been published. Since then, the ADC lorvotuzumab mertansine (IMGN901) has been the only actively studied anti-CD56 antibody-based therapy. IMGN901 is composed of the humanized anti-CD56 antibody and a microtubule disrupting maytansinoid (DM1) linked through a cleavable disulfide cross-linker. IMGN901 showed some activity on CD56-positive neuroblastoma (42%) and rhabdomyosarcoma (28%) xenograft models, but resistance was observed in some xenografts with high CD56 expression. In early clinical trials, IMGN901 showed promising results in small cell lung cancer and multiple myeloma patients IMGN901 was tested in a Phase 2 clinical trial in combination with etoposide and carboplatin for small cell lung cancer. The trial was halted due to lack of increased efficacy over chemotherapy alone and an increased rate of infection; adverse events included one infection-related death. These results indicate that high levels of CD56 on cancer cells do not necessarily translate into response to IMGN901. A better understanding of CD56 biology may help in identifying the types of tumors that will respond well to CD56-targeting therapies.

We report here the identification and characterization of 2 human CD56 antibodies targeting 2 spatially separated epitopes: proximal or distal to the plasma membrane. By using several model neuroblastoma cell lines, we found that antibody-induced down-regulation of CD56 and high in vivo binding ability may be important factors in the response of CD56-positive cancer cells to these antibody treatments. We propose that high affinity antibodies capable of inducing CD56 downregulation (e.g., m906) are excellent candidates for developing ADCs. These two antibodies are also useful research reagents, e.g., for studying dimerization of CD56.

Results

Identification and characterization of CD56-specific antibodies

To our knowledge, fully human CD56 antibodies have not been previously reported. In this study we identified several CD56 antibodies from a human naïve Fab phage library through panning and screening using a recombinant ecto domain of CD56. Two identified clones, m900 and m906, are described in detail here. m900 and m906 were purified and were found to bind to distinct regions of CD56 molecule, as shown in Fig. 1B and 1C. While m900 bound to the membrane-proximal fibronectin III-like domains, m906 bound to the distal N terminal IgG-like domains. The two antibodies do not compete for binding to the ecto domain CD56 on ELISA, supporting the notion that they bind to different epitopes of CD56. m900 had a similar binding pattern to the commercially available mouse antibody BD 555514. Because a dual mouse/human CD56 binding antibody may be useful for toxicity studies in mouse models, we tested binding of m900 and m906 to mouse CD56 protein. By ELISA, IgG1 m906 recognized mouse CD56, whereas m900 did not, despite nearly 90% homology between mouse and human CD56 proteins. The BD mouse antibody also did not recognize mouse CD56 on ELISA.

Binding of the 2 antibodies to cell surface CD56 was measured with flow cytometry on neuroblastoma cell line IMR-05 cells (Fig. 2C). Both m900 and m906 bound to cell surface

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**Figure 1.** Two newly identified CD56 human monoclonal antibodies with different binding features on human and mouse CD56. (A) Gel image of purified CD56 recombinant proteins. Lane e, the whole ecto domain. Lane G, the N-terminal IgG-like domains. Lane F, the fibronectin type III domains. Fab m900 and m906 were also shown. (B) Binding of m900 and m906 Fabs to different regions of CD56 ecto domain with ELISA method. A mouse mAb from commercial source (BD PharMingen cat#555514) was used as the positive control (P control). G1-5: the 5 IgG-like domains. FN1-2: the 2 fibronectin-like domains. (C) Diagram of CD56 molecular structure and binding regions of the 2 antibodies. The ecto domain is divided into 2 parts, the 5 IgG-like domains and 2 fibronectin-like domains. TM, transmembrane domain.
CD56 on IMR-05. The addition of soluble recombinant CD56 ecto protein during the antibody/cell incubation reduced the binding intensity, confirming that CD56 is the binding target of the 2 antibodies. Due to the high avidity of surface-associated CD56 binding to the bivalent IgG1s, the soluble CD56 did not completely block the binding of the 2 antibodies.

By Biacore analysis, the 2 antibodies have similar binding affinity to CD56 (Fig. 3). The Fab format of the 2 antibodies have nanomolar dissociation rate constants (m900: $K_D = 2.9 \text{ nM}$ and m906: $K_D = 4.5 \text{ nM}$). By ELISA, both m900 and m906 IgGs have subnanomolar IC50 to human CD56. To estimate whether the 2 antibodies have similar abilities to bind surface CD56 on cells, they were incubated at concentrations ranging from 0.4 to 250 nM with the 4 neuroblastoma cell lines. Based on the mean fluorescence intensity value at each concentration, equilibrium dissociation constants were calculated using the PRISM software. Interestingly, m906 demonstrated 5 (in SK-N-FI cells) to 50 (in NGP cells) times lower $K_D$ values than m900 (Fig. 3C), although they had similar binding affinities to the recombinant CD56 in vitro. It is likely that on the cell surface m906 epitope, being distal to the membrane, is easier to access than the m900 epitope, which may be masked by other membrane-associated molecules.

It is reported that the 5 IgG-like domains and the 2 fibronectin type III-like domains of CD56 protein have different functions. The N-terminal IgG-like domains are thought to be involved in cell-cell adhesion through dimerization with IgG-like domains of CD56 on another cell/matrix or the same cell. The fibronectin type III domain might be involved in downstream signaling. Given the functional difference of the domains, we further investigated whether the 2 antibodies have different binding behavior on CD56-positive neuroblastoma cells.

**Surface levels and dynamics of CD56 in neuroblastoma cell lines**

Neuroblastoma cell lines, SK-N-FI, NGP, IMR-05 and SK-N-AS, were used to characterize the CD56-binding ability of m900 and m906. The CD56 surface levels were measured with the commercial mouse mAb from BD by flow cytometry in these cells (Fig. 4). Results indicated that the 4 cell lines have surface CD56 levels in the following order, SK-N-FI > NGP > IMR-05 > SN-N-AS. This binding pattern is similar to that of m906 (the Y-axis values in Figs. 3D–G.)

Many cell surface receptors (e.g., IGF-1R) undergo down-regulation upon bindings of their cognate ligands or antibodies.20 To test whether CD56 down-regulation was induced by the antibodies, SK-N-FI, NGP, IMR-05 and SK-N-AS cells were treated with a control IgG (m912),21 m900 or m906 IgG overnight, and stained with the mouse anti-hCD56 mAb to measure the surface levels of CD56 that still remained (Fig. 5). Overnight incubation of m906 had resulted in a reduction of surface CD56 in all cells tested, with SK-N-FI, NGP and IMR-05 having a higher percentage of reduction than SK-N-AS cells. In all cell lines tested, m900 did not measurably reduce the cell surface levels of CD56, similar to cells treated with a control IgG. A competition ELISA between the mouse anti-hCD56 mAb and m900 or m906 (Fab) was performed, and the mouse mAb did not compete with either m900 or m906. Therefore, the surface CD56 level measured with the mouse mAb reflected the actual CD56 protein on cell surface. These results indicate that antibody binding to the distal IgG-like domains induced down-regulation of CD56 in these neuroblastoma cell lines.

Some receptors or surface proteins (such as many G protein-coupled receptors) are quickly recycled back to the cell surface22 after they are internalized. Alternatively, some receptors are trafficked deeply into the lysosomes of cells.
where the receptor is degraded through proteolysis. The depth of internalization has implications when the binding antibody is being considered for development as a therapeutic. For example, ADCs rely on the release of drugs within the lysosomes to make the cytotoxic drugs available to intracellular organelles. To test whether antibody-induced internalization of CD56 reached the lysosomes, we conjugated the two antibodies with a pH sensitive dye, pHAb Thiol Reactive Dye, via the internal cysteines. The dye has very low fluorescence at physiological pH, but becomes fluorescent when it encounters acidic pH. The pH sensitivity of the conjugated antibody-Dye was confirmed first at pH 4.0 and 7.4 (Fig. 6A). Conjugation of the dye to m900 and m906 did not change the in vivo binding affinity. m900-Dye and m906-Dye were incubated at 25 nM with the 2 high CD56 expressing cell lines, SK-N-FI and NGP. After 4 hours incubation at 37°C, m906-Dye derived fluorescence was readily detected by flow cytometry (Figs. 6B & C) in both cell lines. Fluorescence emitted by m900-Dye was also detectible, but was not as strong as the signal from m906-Dye, indicating a greater amount of m906-Dye reached the acidic compartments of the cell such as the lysosomes.

Internalization of CD56 following antibody binding was further tested via hFab-ZAP. hFab-ZAP is an anti-human Fc Fab coupled with saporin, a very potent ribosomal-inactivating protein. When hFab-ZAP gains entry to cells through the primary antibody, in this case m900 or m906, saporin is released if the antibodies reach the lysosomes and are not recycled back to the cell surface. Saporin inhibits the protein synthesis, and in turn causes apoptosis.
SK-N-FI, IMR-05 and SK-N-AS cells were incubated with fixed concentrations of hFab-ZAP in combination with 0, 0.05 and 0.5 nM of m900 and m906 (Fig. 5F). After four days, the cell viability was assessed and cell death was observed from m906 and hFab-ZAP co-incubation in a dose-dependent fashion. Conversely, co-incubation of m900 with hFab-ZAP did not cause significant cell death. These results suggest that m906 may induce trafficking of CD56 via the lysosome pathway, and may thus be a good candidate for incorporation into ADCs.

Antibody-drug conjugate m906PBD induced cell death in CD56-positive neuroblastoma cell lines

Because m906 is a high affinity human antibody with the ability to induce CD56 downregulation, which may indicate internalization, we constructed an ADC by conjugating m906 with maleimidocaproyl-valine pyrrolobenzodiazepines dimers (MC-VC-PBD) through the internal cysteines (Fig. 7A). PBD is a highly cytotoxic small molecular drug that cross-links DNA by binding to the minor groove and causes DNA damage, which leads to apoptosis. One:four (antibody:PBD) molar ratio was maintained during the conjugation reaction. It is expected that the resulting ADC would have no more than 4 drug molecules per antibody. m900 and, as a negative control, m610, a human monoclonal antibody to IGF2, were conjugated to MC-VC-PBD with the same linker chemistry. Conjugation of PBD to m906 did not interfere with its ability to down-regulate CD56 (Fig. 7B). In IMR-05 cells, toxicity of m906PBD was diminished by adding non-conjugated m906 IgG (Fig. 7C), which competed for the binding of CD56 with the m906PBD. This further supports that the cytotoxicity of m906PBD is CD56-specific.

Treatment of non-conjugated m900 or m906 did not change cell growth rates. We then treated SK-N-FI, NGP, IMR-05 and SK-N-AS cells with all 3 ADCs at concentrations ranging from 0.01 pM to 10 nM. These cell lines have different levels of surface CD56, as shown in Fig. 4. Results indicated that m906PBD had potent killing effects on NGP and IMR-05 cells in 4 days (Fig. 8), while SK-N-FI and SN-N-AS cells responded to m906PBD in 6 days (Fig. 9). After 6 d of incubation with m906PBD, the survival rates of IMR-05 and NGP were very low, even at 0.01 pM m906PBD. The estimated IC50 for NGP and IMR-05 cells based on 4-day growth test were 0.05 pM and 1.7 pM, respectively. Based on the 6-day growth results, the IC50 for SK-N-FI and SK-N-AS cells were 0.16 pM and 0.19 pM, respectively. Even after 6 days, the survival rate of these 2 cell lines did not decrease to low percentages as the NGP and IM-05 cells did. On the contrary, m900PBD had little effect on the cell viability, similar to the negative control ADC, m610PBD. Concentrations as high as 100 pM m900PBD were required for detectible killing effect on these cells, which is in agreement with its relatively lower in vivo binding ability and its inefficiency in inducing CD56 down-regulation. All four cell lines were sensitive to free drug PBD, particularly IMR-05 and NGP. In SK-N-AS cells, m906PBD was not very potent, despite the internalization by m906. This might be due to the overall low levels of CD56 on the cell surface and the low down-regulation rate in SK-N-AS cells. Overall, the sensitivity of the tested cell lines to the cytotoxic effect of ADCs did not correlate with CD56 surface levels alone. In addition, the CD56 down-regulation and sensitivity to the free PBD also contributed to the response of the cells to ADCs.

Discussion

CD56 is an important cancer target in many tumors, including neuroblastoma, multiple myeloma, and small cell lung cancer. Antibody-guided target therapy for these diseases will be a welcome addition to the available treatment choices. The anti-CD56 ADC IMGN901, which incorporates a humanized antibody, showed clinical efficacy in small cell lung cancer. IMGN901 had
a relatively short half-life (18–24 hours) in humans (similar to its non-conjugated counterpart); this may be partially attributed to the uptake of IMGN901 in some normal tissues with low CD56 expressions. We hypothesized that a fully human anti-CD56 antibody may have a longer half-life. Here, we reported the identification and characterization of 2 anti-CD56 human antibodies that could be used as naked antibody or as drug conjugates for treatment of CD56-positive cancers.

High-risk neuroblastoma patients undergo a very aggressive treatment regimen consisting of surgery, chemotherapy, radiation, immunotherapy and autologous stem cell transplant, yet roughly half of these patients ultimately die. Neuroblastoma tumors are a collection of very heterogeneous neuroendocrine tumors, and cell lines derived from neuroblastomas reflect the heterogeneity of the tumors. A better understanding of CD56 biology in these cancer cells will help to select tumor types that respond to CD56-targeted therapies. We showed here that m906 bound to the distal IgG-like domains of CD56, and was able to induce downregulation of CD56 efficiently.
whereas the membrane proximal region-binding antibody m900 did not induce significant levels of CD56 down-regulation. Although the 2 antibodies have similar in vitro affinities to recombinant CD56 based on ELISA and Biacore analyses, m906 bound to cell surface CD56 with much higher avidity. This discrepancy could result from the epitope accessibility of CD56; the membrane proximal region of CD56 is more likely to be masked by other membrane-associated molecules than the distal domain. With the ability to bind cell surface CD56 and to induce down-regulation and possibly internalization, m906 could be used as a component of ADCs targeting CD56-positive tumors.

In the cell lines tested here, m906PBD showed efficient cell killing rapidly in NGP and IMR-05 cells, and it took a slightly longer time to show effects in SK-N-FI and SK-N-AS cells. An isotype control antibody-PBD conjugate only had detectable cytotoxicity at >1000x concentrations. m900PBD was much less potent than m906PBD. SK-N-FI and SK-N-AS cells did not respond to m906PBD as rapidly as NGP and IMR5 cells did. There could be several reasons for this result. Both IMR-05 and NGP cells have intact p53 function, whereas SK-N-FI cells bear a p53 loss-of-function mutation and SK-N-AS cells only have partial p53 function due to homozygous deletion of the C-terminal p53 gene. Complete and partial loss of p53

|               | m900-Dye | m906-Dye |
|---------------|----------|----------|
| pH 4.0        | 8412.0   | 656.9    |
| 100 nM        | 12402.4  | 674.5    |
| 10 nM         | 1706.8   | 576.9    |
| 1 nM          | 681.4    | 594.4    |
| 0             | 618.0    | 623.2    |

Figure 6. CD56 antibodies reached acidic compartments after internalization. (A) m900-Dye and m906-Dye was diluted into buffers at pH 4.0 and 7.4, fluorescence signals measured at Em/Ex=532/560 nm were listed to show pH-dependent nature of the dye-conjugated antibodies. SK-N-FI (B) or NGP (C) cells were treated with m900-Dye or m906-Dye at 25 nM for 4 hours at 37° C. Cells were directly examined with flow cytometry. m906-Dye had strong fluorescence signal in both cell lines due to its relatively high amount in acidic lysosomes.

Figure 7. Design of ADC and specificity of m906PBD. (A) Diagram of antibody-PBD. The PBD dimer was conjugated to internal cysteines through a cleavable linker, maleimodacryl valine citrulline. In the ADCs the drug-to-antibody ratio (in molar) is expected to be <4. (B) PBD conjugation to m906 did not change its ability to induce CD56 internalization. (C) Toxicity of m906PBD in IMR-05 cells was diminished by adding naked m906.
function makes these cells less sensitive to DNA damage induced by PBD. This notion is confirmed by the slower response of SK-N-AS and SK-N-FI cells in the cell survival test with the free drug PBD treatment. In addition, the doubling time for SK-N-FI cells is at least 2-fold that of IMR-05 and NGP cells. SK-N-AS cells have very low surface CD56 level, and PBD drug likely was slow to accumulate to a critical concentration inside cells. These may also be the reasons that the survival rate only decreased to around 40–50% in these 2 cell lines, and the growth curves of these 2 cells did not follow the typical sigmoid shape. Together, these results suggest that p53 function is an important factor to consider in addition to CD56 surface level/down-regulation, when therapy with CD56-targeting ADC is concerned. While the in vitro growth test is a good preliminary method to predict the efficacy of ADC, it lacks the 3 dimensional tissue structures or microenvironment and had short test durations. In addition, it does not provide enough information to determine doses. Therefore, further studies in animal models are necessary.

In the CD56 downregulation experiment, overnight treatment of m900 did not significantly reduce surface level of CD56 in the 4 cell lines tested, whereas in the 4 hours incubation of m900-Dye with SK-N-FI and NGP cells, there was some level of internalization. The difference could be the result of the more sensitive detection of the latter method. Furthermore, the presence of m900-Dye in the lysosomes was a snapshot of the internalization process, and the surface CD56 levels reflected the balance of internalization and recycling of CD56. It is possible that m906-induced internalization outpaced recycling of CD56, and the small amount of m900-induced internalization was equivalent to the recycled CD56. The two assays together provide a more complete picture of CD56 dynamics in cells treated with these antibodies.

It is noteworthy that antibodies which bind to different regions of erbB3 and erbB2 (or Her2/neu) have been reported to have similar internalization-inducing ability as we observed in the study. The erbB2 antibody pertuzumab, which binds to the domain II on the distal end, is a potent inducer of receptor down-regulation and disrupter of receptor dimerization, whereas trastuzumab, which binds to domain VI, is not.28,29 In the case of erbB3, the mAb A2 binds to the membrane proximal domain VI, and A3 to distal domain II of erbB3. As is the case with m900, A2 could not induceerbB3 internalization by itself, whereas A3 could.30 These results suggest that for CD56, erbB2 and erbB3, antibodies targeting the distal end of membrane proteins, particularly the dimerization region, have potential to be potent inhibitors, and could induce receptor downregulation. Antibodies targeting the membrane proximal region may have other characteristics. Whether this correlation applies broadly to other membrane proteins remains to be seen.

We chose PBD, a potent DNA-damaging drug as the small molecule moiety of the ADC because many childhood tumors, especially in patients with prior treatment history, are resist to microtubule drugs (MMAF and maytansinoid).31,32 It has been
reported that in some tumors with high multi-drug resistance (MDR) activity, PBD-conjugated ADCs can overcome the resistance mechanism and remain active in acute myeloid leukemia model. We tested m906MMAF initially and compared it with m906PBD (data not shown), and found that the latter was more potent in killing IMR-05 cells. For the current study, a more traditional conjugation method with cleavable linker and internal cysteines was used as a proof-of-concept. We realize that this method gives heterogeneous ADCs. Site-specific conjugation of m906 to PBD is in progress. Because of the toxicity of PBD, finding a good therapeutic window is crucial for further development of m906PBD. Animal studies with m906PBD are in progress.

In conclusion, we identified 2 human CD56 antibodies from a large Fab phage library. The two antibodies, m900 and m906, bound to the fibronectin type III domain and IgG-like domains of CD56, respectively. m906 was very efficient in binding to cell surface CD56, as well as inducing down-regulation of CD56 in neuroblastoma cell lines. m900 was less efficient in both features, although the 2 antibodies share similar affinities in vitro. Correspondingly, m906 when conjugated with the cytotoxic drug PBD could kill neuroblastoma cell lines with pM IC50. Therefore, m906PBD is a potential candidate drug to target CD56-positive tumors. The two antibodies could be used to study the dynamics of CD56 on different neuroblastoma and multiple myeloma cell lines.

Materials and methods
Identification of CD56 antibodies from a phage library
CD56 full-length cDNA (isoform 2) in pCMV vector was purchased from Origene, and was used as the source for constructing expression vectors of CD56 fragments. DNAs encoding the following CD56 fragments were amplified with BamHI and HindIII sites flanked: CD56-G1-5 (aa20–495), 475 aa; CD56-FN1, 2 (aa496–720), 224 aa; whole ecto domain: (aa 20–720), 700 aa. The amplified DNAs were ligated with the psectag2A vector that had been restricted with BamHI and HindIII. After the sequences of the expression vectors were confirmed, the recombinant proteins were expressed transiently in 293 FreeStyle cultures. Recombinant CD56 fragments were purified from the conditioned culture media with Ni-NTA column. Proteins were dialyzed into phosphate-buffered saline (PBS).

The whole ecto domain of CD56 was used for panning of the naïve human Fab phage library (5×10^10 unique clones). Briefly, CD56 ecto domain protein was coated on Maxisorp plate; phage was added to pre-absorbed surface with CD56. After washes with PBST (PBS with 0.05% Tween 20), the eluted phages were rescued by infecting exponentially growing TG1 cells. The final 2 clones, m900 and m906, with good affinity to CD56 were further investigated in this study. Both Fabs were converted into scFv and human IgG1 formats.
Expression and purification of antibodies

Expressions of Fabs and scFvs were performed in HB2151 bacterial culture according to the protocol in reference, and purified on Ni-NTA column. Recombinant Fabs and scFvs carried Flag and His tags on their C termini. Conversion and preparation of IgG1s are performed according to the standard protocol previously described. IgGs were expressed in 293 FreeStyle culture and purified with a protein G column. All antibody fragments and IgG1s were dialyzed into PBS.

Measurement of binding with ELISA

Protein was coated on half area ELISA plate wells at 50 ng/well in PBS at 4°C overnight. After blocking of unbound surface with 2% non-fat dried milk in PBST (0.05% Tween 20 in PBS), serially diluted antibody solutions were added, and incubated for 2 h at 37°C. The bound human antibodies (e.g., IgG1 m900 and m906) were detected with goat anti-human Fc specific IgG conjugated with horseradish peroxidase (HRP). Binding of Fab or scFv was detected with anti-Flag antibody coupled with HRP. The enzyme activity was measured with the subsequent addition of substrate ABTS and signal reading was carried out at OD 405 nm. For Fab and IgG competition ELISA, constant concentration of Fab (50 nM) was used, and the competing IgG was included at concentrations ranging from 0.00128 nM to 100 nM.

Measurement of antibody affinity with surface plasmon resonance

The binding affinity of the Fabs to the CD56 ecto domain was measured on a BIAcore X100 instrument (GE Healthcare). Purified human CD56-ecto was diluted in 10 mM sodium acetate buffer (pH 5.0) and immobilized on a CM5 biosensor chip using an amine coupling kit. The running buffer was HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20). The Fabs diluted in running buffer were allowed to flow through the cells, at concentrations ranging from 0.05 nM to 500 nM. After 10 min of dissociation, the chip was regenerated with 10 mM glycine, pH 3.0, 0.5 M NaCl. The data were fitted with 1:1 binding model and the dissociation rate constant was estimated with the BIAevaluation software.

Cell culture, growth and internalization test

Neuroblastoma cell lines, SK-N-FI, NGP, IMR-05 and SK-N-AS (from ATCC), were maintained in RPMI1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. For the growth test with ADCs, 2000 cells/well were seeded in 96-well clear bottom plates. Cells were allowed to attach to the plate overnight, and on the next day they were treated with the ADCs at designated final concentrations. Each concentration was repeated in triplicate. After 4 and 6 d of growth, the viability of cells were monitored by adding reagent from CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), and incubated for 1 hour at 37°C. The viability of cells was recorded as OD 490 nm.

For the measurement of CD56 downregulation via flow cytometry method, cells were seeded in a 6-well plate at the density of 0.6 million cells/well. After overnight culture, m900 or m906 IgG, was added to cells at 50 nM. Cells in control wells were treated with 50 nM of isotype control human antibody, m912. After overnight incubation, cells were detached with Cell Dissociation Buffer (Invitrogen) and washed in PBS. The CD56 surface level was measured by incubation of cells with a commercially available anti-human CD56 mouse antibody (Clone 555514, BD Bioscience) on ice for 1 h. An anti-caspase 9 mouse antibody (Santa Cruz) was used as a negative control for the staining. The mouse antibody was detected with a goat anti-mouse Fc polyclonal Ab coupled with FITC. Cells were processed in FACSCalibur. Geometric of each histogram was used to calculate the percent of down-regulation in relation to untreated cells.

To determine whether the antibodies reach acidic lysosomes, antibodies were conjugated with a pH sensitive dye, pHAb Thiol Reactive Dye (Promega) via the internal cysteines according to the manufacturer’s instruction. Briefly, antibody was bound to protein G resin and reduced with TCEP (Tris (2-carboxyethyl) phosphine hydrochloride) in PBS/10 mM EDTA. For every 100 μg antibody, 0.6 μl of reconstituted pHAb Thiol Reactive Dye was added. After 60 minutes reaction at RT, the non-reacted dye was washed off, and the antibody-Dye was eluted from protein G resin with high pH buffer. The final conjugated antibody was dialyzed into PBS. To check the pH sensitivity of the antibody-Dye, each antibody-Dye preparation was diluted into solutions of pH 4 and 7.4, and measured in a fluorimeter with Ex/Em = 532/560 nm. To test localization of antibody-Dye in the lysosomes, cells were treated with antibody-Dye at 25 nM for 4 hours at 37°C. Cells were detached and directly examined with FACSCalibur to measure any fluorescence signal.

For the internalization test with the combination of primary antibody (m900 and m906) and hFab-ZAP (Advanced Targeting Systems), the cells were set up in 96-well plates similarly as in the growth test. Cells were treated with 5 nM of hFab-ZAP with 0, 0.05 and 0.5 nM of the primary antibody. An isotype-controlled antibody was included at 5 nM with 5 nM hFab-ZAP as a negative control. The growth of cells was monitored as above.

Preparation of antibody-drug conjugates

m900 or m906 IgG1 was conjugated with PBD-MC (pyrrolodiazepine dimer with maleimidocaproyl-valine-citrulline) with a simplified protocol from reference. PBD-MC was purchased from The Chemistry Resource Solution, LLC, PA. Briefly, the IgGs were bound to protein G resin, and reduced in 10 mM TCEP/PBS/1 mM EDTA for 1 h at RT. The excess TCEP was washed off, and the IgG-bound protein G resin was suspended in 45% propylene glycol (PPG) in PBS/EDTA. The drug PBD-MC was reconstituted in DMSO/PPG (1:1 in v:v). For each 10 mg IgG in 5 mL PPG/PBS, 300 μg PBD-MC in ~300 μL DMSO/PPG was added. This buffer condition kept both IgG and the drug soluble. The antibody: drug ratio is at 1:4 (in molar) assuming 100% conjugation efficiency. The conjugation reaction was performed at RT for 2 h. The resin slurry
was washed with PPG/PBS buffers with gradually decreasing PPG concentrations, and finally in PBS. The conjugated ADCs were eluted with acidic buffer and dialyzed into PBS. The final ADCs were resolved in a Superdex200 10/300GL size-exclusion column.

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References
1. Siebert N, Seidel D, Eger C, Jutttner M, Lode HN. Functional bioassays for immune monitoring of high-risk neuroblastoma patients treated with ch14.18/CHO-antti-GD2 antibody. PLoS One 2014; 9:e107692; PMID:25226154; http://dx.doi.org/10.1371/journal.pone.0107692
2. Siebert N, Eger C, Seidel D, Jutttner M, Lode HN. Validated detection of human anti-chimeric immune responses in serum of neuroblastoma patients treated with ch14.18/CHO. J Immunol Methods 2014; 407:108-15; PMID:24727144; http://dx.doi.org/10.1016/j.jim.2014.04.001
3. Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen Y, Nersom M, Maloney JA, Meilandt WJ, et al. Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates. Sci Transl Med 2014; 6:261ra154; PMID:25378646; http://dx.doi.org/10.1126/scitranslmed.3009835
4. Jensen M, Berthold F. Targeting the neural cell adhesion molecule in neuroblastoma. N Engl J Med 2010; 363:1324-34; PMID:20797981; http://dx.doi.org/10.1056/NEJMoa1011123
5. Wachowiak R, Metzger R, Quaas A, Fiegel H, K Litofsky, Houghton NJ, Houghton PJ, Smith MA. Initial testing (Stage 1) of the antibody-maytansinoid conjugate, IMGN901 in patients treated with ch14.18/CHO anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. N Engl J Med 2014; 367:281-90; PMID:23635388; http://dx.doi.org/10.1159/000346394
6. Miller SC. Production and renewal of murine natural killer cells in the spleen and bone marrow. J Immunol 1982; 129:2282-6; PMID:7119447
7. Carrega P, Morandi B, Costa R, Frumento G, Forte G, Altavilla G, Ratto GB, Mingari MC, Moretta L, Ferlazzo G. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells. Cancer 2008; 112:863-75; PMID:18203207; http://dx.doi.org/10.1002/cncr.23239
8. Lutz RJ, Whiteman KR. Antibody-maytansinoid conjugates for the treatment of myeloma. MAbs 2009; 1:548-51; PMID:20068397; http://dx.doi.org/10.4161/mabs.1.6.10029
9. Vesaei M, Moch H, Mousavi A, Kajbafzadeh AM, Sauter G. Immunohistochemical profiling of Wilms tumor: a tissue microarray study. Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry and Molecular Morphology 2008; 16:128-34.
10. Ohishi Y KT, Oya M, Kobayashi H, Wake N, Tsureyoshi M. CD56 expression in ovarian granulosa cells tumors, and its diagnostic utility and pitfalls. Gynecol Oncol 2007; 107:30-8; PMID:17583777; http://dx.doi.org/10.1016/j.ygyno.2007.05.020
11. Miller SC. Production and renewal of murine natural killer cells in the spleen and bone marrow. J Immunol 1982; 129:2282-6; PMID:7119447
12. Carrega P, Morandi B, Costa R, Frumento G, Forte G, Altavilla G, Ratto GB, Mingari MC, Moretta L, Ferlazzo G. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells. Cancer 2008; 112:863-75; PMID:18203207; http://dx.doi.org/10.1002/cncr.23239
13. Poli A, Michel T, Thereseins M, Andres E, Hengtes F, Zimmer J. CD56 bright natural killer (NK) cells: an important NK cell subset. Immunology 2009; 126:458-65; PMID:19278419; http://dx.doi.org/10.1111/j.1365-2567.2008.03027.x
14. Yu YI, Watts RJ. Developing therapeutic antibodies for neurodegenerative disease. Neurotherapeutics 2013; 10:459-72; PMID:23549647; http://dx.doi.org/10.1007/s11065-013-0187-4
15. Yu YI, Atwal JK, Zhang Y, Tong RK, Wildsmith KR, Tan C, Bien-Ly N, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology 2009; 126:458-65; PMID:19278419; http://dx.doi.org/10.1111/j.1365-2567.2008.03027.x
16. Nitta T, Sato K, Yokura K, Ishii S. Preliminary trial of specific targeting therapy against malignant glioma. Lancet 1990; 335:368-71; PMID:1968115; http://dx.doi.org/10.1016/0140-6736(90)90205-J
17. Papanastassiou V, Pizer BL, Coakham HB, Bullimore J, Zananiri T, Kemshead JT. Treatment of recurrent and cystic malignant gliomas by a single intracavitary injection of 131I monoclonal antibody: feasibility, pharmacokinetics and dosimetry. British journal of cancer 1993; 67:144-51; PMID:8427774; http://dx.doi.org/10.1038/bjc.1993.25
18. Hopkins K, Chandler C, Bullimore J, Sandeman D, Coakham H, Kemshead JT. A pilot study of the treatment of patients with recurrent malignant gliomas with intratumoral yttrium-90 radioimmunoconjugates. Radiother Oncol 1995; 34:121-31; PMID:7597210; http://dx.doi.org/10.1016/0167-8140(95)01514-H
19. Wang L, Amphlett G, Blattler WA, Lambert JM, Zhang W. Structural characterization of the maytansinoid-monoclonal antibody immunocoujugate, huN901-Dmi1, by mass spectrometry. Protein Sci 2005; 14:2436-46; PMID:16081651; http://dx.doi.org/10.1110/ps.051478705
20. Jackson-Booth PG, Terry C, Lackey B, Lopaczynska M, Nissley P. Inhibition of the biologic response to insulin-like growth factor I in MCF-7 breast cancer cells by a new monoclonal antibody to the insulin-like growth factor-I receptor. The importance of receptor down-regulation. Horm Metab Res 2003; 35:850-6; PMID:14710368; http://dx.doi.org/10.1055/s-2004-814144
21. Feng Y, Xiao X, Zhu Z, Streaker E, Ho M, Pastan I, Dimitrov DS. A novel human monoclonal antibody that binds with high affinity to mesothelin-expressing cells and kills them by antibody-dependent cell-mediated cytotoxicity. Mol Cancer Ther 2009; 8:1113-8; PMID:19417159; http://dx.doi.org/10.1158/1535-7163.MCT-08-0945
22. Roman-Vendrell C, Yu YI, Yudowski GA. Fast modulation of mu-opioid receptor (MOR) recycling is mediated by receptor agonists. J Biol Chem 2012; 287:14782-91; PMID:22378794; http://dx.doi.org/10.1074/jbc.M111.319615
23. Kohls MD, Lappi DA. MAB-ZAP: a tool for evaluating antibody efficacy for use in an immunotoxin. Biotechniques 2000 Jan; 28(1):162-5; PMID:10743381; http://dx.doi.org/10.1055/s-2004-814144
24. Thiele C. Neuroblastoma cell lines. Lancaster: Kluwer Academic Publishers, 1998.
25. Wood AC MJ, Gorlick R, Kolb EA, Keir ST, Reynolds CP, Kang MH, Wu J, Kurmasheva RT, Whiteam K, Houghton PJ, Smith MA. Initial testing (Stage 1) of the antibody-maytansinoid conjugate, IMGN901 (Lorvotuzumab mertansine), by the pediatric preclinical testing program. Pediatr Blood Cancer 2013; 60:1860-7; PMID:23798344; http://dx.doi.org/10.1002/pbc.24647
26. Van Maerken T, Rihani A, Dreixda D, De Clercq S, Yigit N, Marine JC, Westermann F, De Paepe A, Vandesompele J, Speleman F.
Functional analysis of the p53 pathway in neuroblastoma cells using the small-molecule MDM2 antagonist nutlin-3. Mol Cancer Ther 2011; 10:983-93; PMID:21460101; http://dx.doi.org/10.1158/1535-7163.MCT-10-1090

27. Cheng J, Fan YH, Xu X, Zhang H, Dou J, Tang Y, Zhong X, Rojas Y, Yu Y, Zhao Y, et al. A small-molecule inhibitor of UBE2N induces neuroblastoma cell death via activation of p53 and JNK pathways. Cell Death Dis 2014; 5:e1079; PMID:24556694; http://dx.doi.org/10.1038/cddis.2014.54

28. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Slawkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell 2004; 5:317-28; PMID:15093539; http://dx.doi.org/10.1016/S1535-6108(04)00083-2

29. Cho HS, Leahy DJ. Structure of the extracellular region of HER3 reveals an interdomain tether. Science 2002; 297:1330-3; PMID:12154198; http://dx.doi.org/10.1126/science.1074611

30. Belleudi F, Marra E, Mazzetta F, Fattore L, Giovagnoli MR, Mancini R, Aurisicchio L, Torrisi MR, Ciliberto G. Monoclonal antibody-induced ErbB3 receptor internalization and degradation inhibits growth and migration of human melanoma cells. Cell Cycle 2012; 11:1455-67; PMID:22421160; http://dx.doi.org/10.4161/cc.19861

31. Loganzo F, Tan X, Sung M, Jin G, Myers JS, Melamud E, Wang F, Diesl V, Follette MT, Musto S, et al. Tumor cells chronically treated with a trastuzumab-maytansinoid antibody-drug conjugate develop varied resistance mechanisms but respond to alternate treatments. Mol Cancer Ther 2015; 14:952-63; PMID:25646013; http://dx.doi.org/10.1158/1535-7163.MCT-14-0862

32. Yu SF, Zheng B, Go M, Lau J, Spencer S, Raab H, Soriano R, Jhunjhunwala S, Cohen R, Caruso M, et al. A Novel Anti-CD22 Anthracycline-Based Antibody-Drug Conjugate (ADC) That Overcomes Resistance to Auristatin-Based ADCs. Clin Cancer Res 2015; 21:3298-306; PMID:25840969; http://dx.doi.org/10.1158/1078-0432.CCR-14-2035

33. Kung Sutherland MS, Walter RB, Jeffrey SC, Burke PJ, Yu C, Kostner H, Stone I, Ryan MC, Sussman D, Lyon RP, et al. SGN-CD33A: a novel CD33-targeting antibody-drug conjugate using a pyrrolobenzodiazepine dimer is active in models of drug-resistant AML. Blood 2013; 122:1455-63; PMID:23770776; http://dx.doi.org/10.1182/blood-2013-03-491506

34. Feng Y, Zhu Z, Xiao X, Choudhry V, Barrett JC, Dimitrov DS. Novel human monoclonal antibodies to insulin-like growth factor (IGF)-II that potently inhibit the IGF receptor type I signal transduction function. Mol Cancer Ther 2006; 5:114-20; PMID:16432169; http://dx.doi.org/10.1158/1535-7163.MCT-05-0252

35. Lyon RP MD, Setter JR, Senter PD. Conjugation of anticancer drugs through endogenous monoclonal antibody cysteine residues. Methods Enzymol 2012; 502:123-38; PMID:22208984; http://dx.doi.org/10.1016/B978-0-12-416039-2.00006-9