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A Human Single-Chain Antibody Specific for Integrin $\alpha_3\beta_1$ Capable of Cell Internalization and Delivery of Antitumor Agents

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Summary

Selective antitumor chemotherapy can be achieved by using antibody-drug conjugates that recognize surface proteins upregulated in cancer cells. One such receptor is integrin $\alpha_3\beta_1$, which is overexpressed on malignant melanoma, prostate carcinoma, and glioma cells. We previously identified a human single-chain Fv antibody (scFv), denoted Pan10, specific for integrin $\alpha_3\beta_1$ that is internalized by human pancreatic cancer cells. Herein, we describe the chemical introduction of reactive thiol groups onto Pan10, the specific conjugation of the modified scFv to maleimide-derivatized analogs of the potent cytotoxic agent duocarmycin SA, and the properties of the resultant conjugates. Our findings provide evidence that Pan10-drug conjugates maintain the internalizing capacity of the parent scFv and are cytotoxic at nanomolar concentrations. Our Pan10-drug conjugates may be promising candidates for targeted chemotherapy of malignant diseases associated with overexpression of integrin $\alpha_3\beta_1$.

Introduction

Targeted treatment of tumors has advanced considerably in the last two decades, primarily due to the establishment of monoclonal antibody (mAb) technology [1]. An early fundamental application was the development of radiolabeled mAbs, some of which have attained clinical use for imaging and cancer therapy [2, 3]. Significantly, mAb-drug conjugates are another potential class of anticancer agents that have been extensively investigated [4–6]. However, although isolated examples of success have been reported, considerable advances are necessary in order to address the complex issue of cancer treatment.

A central goal in our laboratory as well as other groups [7, 8] has been the search for human mAbs or peptides that can be specifically internalized by tumor cells upon binding to overexpressed cell surface receptors or ligands [9, 10]. This line of research presents opportunities to use protein vectors to deliver drug payloads that can increase the efficacy and lessen side effects of cancer chemotherapy. One demonstration of the clinical potential for such a strategy invoked the cell internalizing anti-CD33 antibody P67.6 conjugated to calicheamicin for use against acute myeloid leukemia that has resulted in the FDA approved drug Mylotarg [11].

Integrin $\alpha_3\beta_1$, also known as the VLA-3 membrane receptor, is expressed by both fetal and adult tissues mediating adhesive, migratory, and invasive cell interactions with the extracellular matrix [12]. Elevated expression of $\alpha_3\beta_1$ has been observed in several types of metastatic cancer types and has been associated with increased migration and invasion. Notably, expression of this integrin is upregulated in malignant melanoma and correlates well with the degree of migration and dermal invasiveness [13–16]. The $\alpha_3\beta_1$ integrin is also expressed by invasive clones of human PC-3 prostate carcinoma cells, but not by the noninvasive parent cell population [17, 18]. Similarly, the invasive properties of different squamous cell cancers have been correlated to overexpression of several integrins including $\alpha_3\beta_1$ [19, 20]. It has also been shown that functional inhibition of $\alpha_3\beta_1$ in malignant glioma cells can block their invasive ability [21]. The $\alpha_3\beta_1$ is also associated with mammary carcinoma cell metastasis, invasion, and collagen degradation activity [22]. Finally, expression of $\alpha_3\beta_1$ in murine hepatocellular carcinoma (HCC) has been associated with the occurrence of intrahepatic metastasis, which is considered to be a major modality in recurrence [23]. Given the often distinct levels of expression between malignant cancer cells and normal cells, $\alpha_3\beta_1$ can be considered a viable target for a specific antibody-based antineoplastic treatment designed to kill cancer cells and control metastatic dissemination.

Selective control of metastasis by targeting $\alpha_3\beta_1$ has been shown to be successful in the treatment of intrahepatic metastasis of HCC using an RGD (Arginine-Glycine-Aspartate) pseudopeptide [23]. Also, squamous cell carcinoma of the head and neck has been treated by selective gene delivery via an $\alpha_3\beta_1$ integrin-targeted adenoviral vector [24]. Several murine mAbs are known to target either the α_3 or β_1 subunits of $\alpha_3\beta_1$ [25–27]; however, none are known to be internalized by tumor cells nor have they ever been used as anticancer therapeutics. Significantly, the typical murine origin of most mAbs is a detriment for human clinical application [28–31]. In addition, another barrier can be the effective use of a mAb as whole immunoglobulin G (IgG), generally attributed to the high molecular weight, which hinders efficient penetration of solid tumors. For instance, studies have indicated that less than 1% of an infused radiolabeled IgG can reach its target tumor mass [32, 33]. One method to circumvent this problem is the use of a mAb in the scFv format. Compared to whole IgG and the fragments Fab and $F(ab')_2$, scFvs have been shown to permeate more rapidly and deeper into tumors in addition to demonstrating very rapid plasma and body

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clearance (<30 min) [34–38]. Therefore, in many cases, a preferred therapeutic strategy may be the use of a human scFv conjugated with an anticancer agent.

CC-1065 [39] and duocarmycin [40, 41] are two antitumor antibiotics [42] possessing sequence-selective DNA alkylation properties [43]. The development of these anticancer molecules for single-agent therapies has not been pursued because of delayed toxicities that limit the therapeutic dose range for treatment. For instance, despite its high potency and broad spectrum of antitumor activity, CC-1065 can not be used in humans because it has been shown to cause delayed death in experimental animals [39]. However, these drugs may be well suited for antibody-targeted chemotherapy, where restricted antigen expression makes the potency of the cytotoxic agent crucial and targeting can avert some toxic effects [44, 45]. Great efforts have been made to specifically target the high cytotoxicity of these compounds to the tumor mass sparing normal healthy cells. Investigations have included tumor-activated prodrug (TAP) [46, 47] and antibody-directed enzyme prodrug therapy (ADEPT) [48–50] approaches. Both methods are intended to reduce the cytotoxicity of CC-1065 or duocarmycin analogs by conjugating these molecules to substrates of enzymes at the tumor site. In the first study, the targeted enzyme was naturally present in the tumor environment, while in the second study, the enzyme was brought to the tumor site upon conjugation to a tumor-specific antibody. Despite their elegance, the main drawbacks of these approaches are the residual cytotoxicity of the prodrugs and the release of the free drug outside the tumor cell. To date, no attempts to deliver duocarmycin analogs specifically into tumor cells by conjugating this drug to antibody fragments have been reported.

We previously described the biopanning of a human scFv-phage display library based on the selection requirement of internalization by the SW1990 human pancreatic adenocarcinoma cell line [9]. Our efforts produced a scFv, denoted Pan10, which upon immunoprecipitation, mass spectrometric analysis, and database searching was found to target membrane receptor integrin $\alpha_3\beta_1$. Because of the specific Pan10 interaction with $\alpha_3\beta_1$ and the internalization capability, we viewed this scFv as a vector for conjugation with potent duocarmycin-SA analogs 3-(5-acetylindole-2-carbonyl)-1-(*S*)-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole [51] (compound **1**, Figure 1) and 3-[5-(1-(3-aminopropyl)indole-2-carbonyl)aminoindole-2-carbonyl]-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*] indole (compound **2**, Figure 1) to promote the destruction of malignant tumor cells overexpressing integrin $\alpha_3\beta_1$. Hence, our initial efforts focused upon a number of challenging tasks, including: (1) the conjugation of antitumor drug(s) to scFv Pan10 without compromising target affinity and internalization properties; (2) the design of linkers promoting efficient attachment of the drug(s) to the scFv without compromising the cytotoxic activity of the drug(s); and (3) the search for a reliable cell-based assay designed to evaluate the biological activity of Pan10-drug conjugates. We believe that the results herein are an important step toward the therapeutic application of scFv-mediated, tumor-targeted delivery of anticancer compounds.

Results and Discussion

Pan10 Expression, Purification, and Site-Directed Mutagenesis

To use Pan10 as a tool for the delivery of duocarmycin analogs to malignant cancer cells, phage-free Pan10 was expressed as a scFv of 27,868 kDa (Table 1) and purified to homogeneity (Figure 2, lane 4). Since typical V_L and V_H domains each possess a buried single disulfide linkage [52], but no free cysteines, we investigated several strategies intended to make available free thiol groups on the surface of Pan10 and to conjugate the modified scFv to maleimide-derivatized drugs.

Our initial approach was aimed at single site-specific conjugation using a cysteine incorporated into the wild-type Pan10 sequence by site-directed mutagenesis. In an attempt to preserve the scFv binding affinity, we first looked at introducing the cysteine residue into the linker region of Pan10. Additionally, a commercially available maleimide-derivatized fluorescein (FM) was used as a sensitive reagent to optimize and quantify conjugation protocols. When any one of the linker residues S131, G130, G128, or G127 (see Supplemental Figure S1) were mutated to cysteine, the efficiency of conjugation of the mutants with FM was only similar to the wild-type Pan10. We attributed this result to the linker region and cysteine residue being sequestered within the Pan10 structure. Therefore, we turned our attention to several other residues, which according to a web antibody modelling (WAM c/o University of Bath at Swindon, Oakfield Campus, Marlowe Avenue, Walcot Swindon Wilts, United Kingdom) theoretical structure of Pan10 appeared to be surface exposed. To preserve the tumor cell binding and internalizing ability of Pan10, only framework residues were considered. Improved FM conjugation was achieved when the more exposed residues S73 or S197 were mutated, confirming our rationale about the inaccessibility of the linker residues. Nevertheless, the efficiency of conjugation achieved was at best 68% (Supplemental Table S1), which we hypothesize was due to oxidation or dimerization of the introduced cysteines.

Chemical Modification of scFv Pan10

Insertion of free cysteines by site-directed mutagenesis, while elegant, presents several drawbacks. First, if the mutated residue is solvent accessible it will likely undergo oxidation or induce dimerization. Thus, an additional reduction-purification step is needed, and the subsequent reactions must be carried out in an inert atmosphere as previously reported [53]. To solve this dilemma, we examined the chemical addition of thiol groups onto Pan10. In our initial approach, the thiolation and the conjugation to maleimide-derivatized molecules were performed in two separate steps. In the first step, the free thiol groups were introduced by reacting Pan10 with 2-iminothiolane (Traut's reagent), an amine scavenger that reacts with lysine residues. We were aware of the fact that the presence of twelve lysines in the Pan10 sequence might have led to a massive and potentially harmful modification. However, we were encouraged by knowing that ten of those lysines were in the framework regions (Supplemental Figure S1), and therefore, their

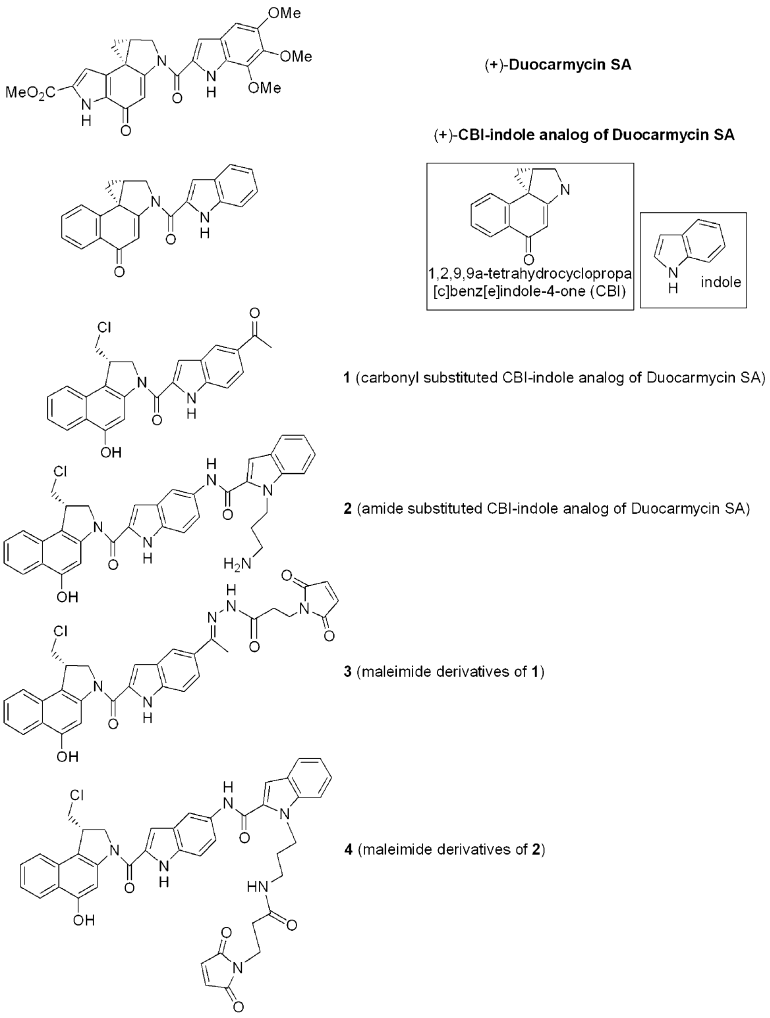


Figure 1. Duocarmycin SA, CBI Indole Analogs, and Maleimide Derivatives

modification would unlikely affect Pan10 binding to integrin $\alpha_3\beta_1$. Our expectations were confirmed, as whole-cell enzyme-linked immunosorbent assay (ELISA) revealed that binding of wild-type Pan10 and thiolated Pan10 were virtually indistinguishable. However, nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the thiolated Pan10 (Figure 2) revealed time-dependent formation of dimers and higher polymers (Figure 2A, lane 2), which caused a progressive reduction of the number of free thiol groups available for drug conjugation. In order to avoid this problem, we attempted to perform the subsequent drug conjugation step immediately after thiolation. This strategy improved the coupling efficiency and reduced dimerization. A further improvement was obtained with the use of a one-step procedure, where thiolation and conjugation occurred in one pot. SDS-PAGE analysis of Pan10 modified using this method revealed only a negligible formation of dimers (Figure 2A, lane 3). We anticipate this procedure to be of general use with other scFvs (vide infra).

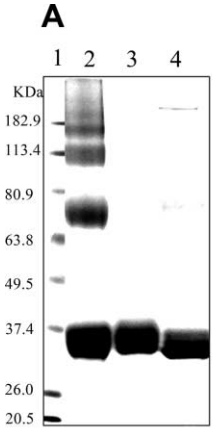
Maleimide-Derivatized Drugs

The maleimide moiety was attached to duocarmycin SA analog **1** via an acid-labile hydrazone linkage to give the maleimide derivative **3** (Figure 1). The hydrazone linkage method is widely utilized in antibody-drug conjugates as a way of controlled release of the cytotoxic drug

Table 1. Results of Mass Spectrometry Analysis

Analyte	Calculated MW (g/mol)	Experimental Mass (m/z)	Molar Ratios[a] (Pan10:FM/drug-maleimide)
Pan10	27610	27868	–
Pan10-FM	28216	28388	0.997
Pan10-3	28297	28634	1.003
Pan10-4	28456	28545	0.994

[a] Measured from MR = $(MW_{conjugate} - MW_{Pan10})/MW_{FM/\ drug\text{-}maleimide}$ using MALDI-measured MWs.



B

Lane	Band	R_f	Area	%
2	1	0.04	0	0
2	2	0.18	65281	2.9
2	3	0.29	376783	16.7
2	4	0.52	796174	35.2
2	5	0.95	1024566	45.3
3	1	0.7	62538	5.2
3	2	0.62	253587	21.2
3	3	0.95	879062	73.6
4	1	0.27	113534	9.8
4	2	0.44	206074	17.7
4	3	0.86	844530	72.5

Figure 2. SDS-PAGE Analysis of Purified Pan10 and Pan10 Conjugates

(A) SDS-PAGE gel slab. Lane 1, Invitrogen prestained markers; lane 2, Pan10 after separate thiolation and conjugation; lane 3, Pan10 after one-pot thiolation and conjugation; lane 4, unmodified Pan10. (B) Bands density analysis (AlphaEaseFC StandAlone Software). The shaded boxes contain data relative to the band corresponding to the monomeric scFv in each lane.

upon internalization into lysosomes where the pH value is slightly lower (pH = 5.0–5.5) than in the cytosol [54]. This strategy has proven to be clinically effective in many instances, such as in the development of BR96-DOX by Bristol-Myers Squibb and the design of Mylotarg by Wyeth. As a comparison, we derivatized the Boc-protected duocarmycin analog **2** (compound **10**, Figure 5) through a pH-insensitive amide bond linkage, producing the maleimide derivative **4** (Figure 1).

Pan10 Conjugation to Maleimide-Derivatized Molecules

As noted above, thiolated Pan10 was initially conjugated to FM to give Pan10-FM in order to test and directly visualize internalization by pancreatic cancer cells. Subsequently, we conjugated the thiolated Pan10 to maleimide derivatives **3** and **4** obtaining conjugates Pan10-3 and Pan10-4, respectively. The ratio of either fluorescein (conjugation efficiency measured by UV/Vis spectrometry and matrix-assisted laser desorption/ionization mass spectrometry [MALDI-MS]) **3** or **4** (conjugation efficiency measured by MALDI-MS) to Pan10 was found to be approximately 1:1 using our two-step coupling procedure (Table 1). On the other hand, when conjugation and thiolation were performed in a single step, the ratio of fluorescein to Pan10 was 2:1 (conjugation efficiency measured by UV/Vis spectrometry only). We believe that this difference in conjugation efficiency is due to the polymerization of the thiolated Pan10 in the absence of thiol-quenching small molecules. Indeed, several additional higher molecular weight species were detected by SDS-PAGE (Figure 2) and size-exclusion chromatography (Supplemental Figure S2) of Pan10-drug conjugates obtained in two separate steps.

Our one-pot scFv conjugation method has been tested on other scFvs ($\alpha_v\beta_3$-specific antibodies Bc-12 and Bc-15 [B.F.-H. et al., submitted] and cocaine-specific antibody 92H2 [55]), affording a maximum ratio of fluorescein:protein of 3:1 without loss of antigen binding activity (data not shown). Therefore, we believe that such a conjugation method might be applicable to a vast array of scFvs.

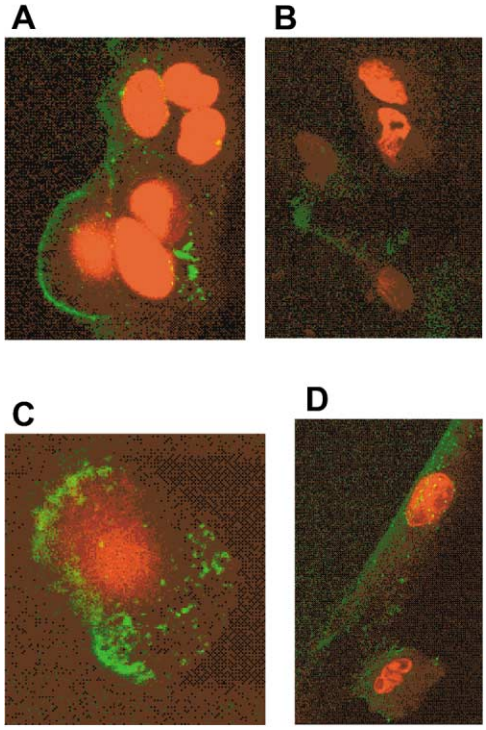


Figure 3. Confocal Microscopy, Overlaid 488 nm and 568 nm Images of SW1990 and HdFa Cells Treated with Pan10-FM

(A) SW1990 cells after 2 hr incubation, (B) HdFa cells after 2 hr, (C) SW1990 cells after 3 hr, and (D) HdFa cells after 3 hr.

Biological Activity of Pan10 Conjugates

Several methods were employed to explore the biological activity of our Pan10 conjugates. Confocal microscopy analysis was used to investigate the specificity of the interaction of the Pan10-FM with SW1990 cells versus the normal human dermal fibroblast cell line (HdFa). Our results showed that Pan10-FM was internalized by SW1990 cells in a time-dependent fashion (Figure 3). Moreover, after the second hour of incubation, internalization in these cancerous cells was much more pronounced than in noncancerous HdFa. These findings confirm that the Pan10-FM conjugate retains the wild-type activity of Pan10 and provide evidence that in pancreatic cancer cells the overexpression of integrin $\alpha_3\beta_1$ allows some selectivity versus HdFa used as a model for a noncancer cell type.

SW1990 cells treated with Pan10, Pan10-FM, Pan10-3, or Pan10-4 were examined by inverted microscopy for a qualitative determination of the effect of the drug conjugates on the cell viability. After 7 days in culture, the cells treated with Pan10 or Pan10-FM had expanded into healthy colonies, whereas the cells treated with Pan10-drug conjugates had either died or showed excessive vacuolization, indicating advanced apoptosis

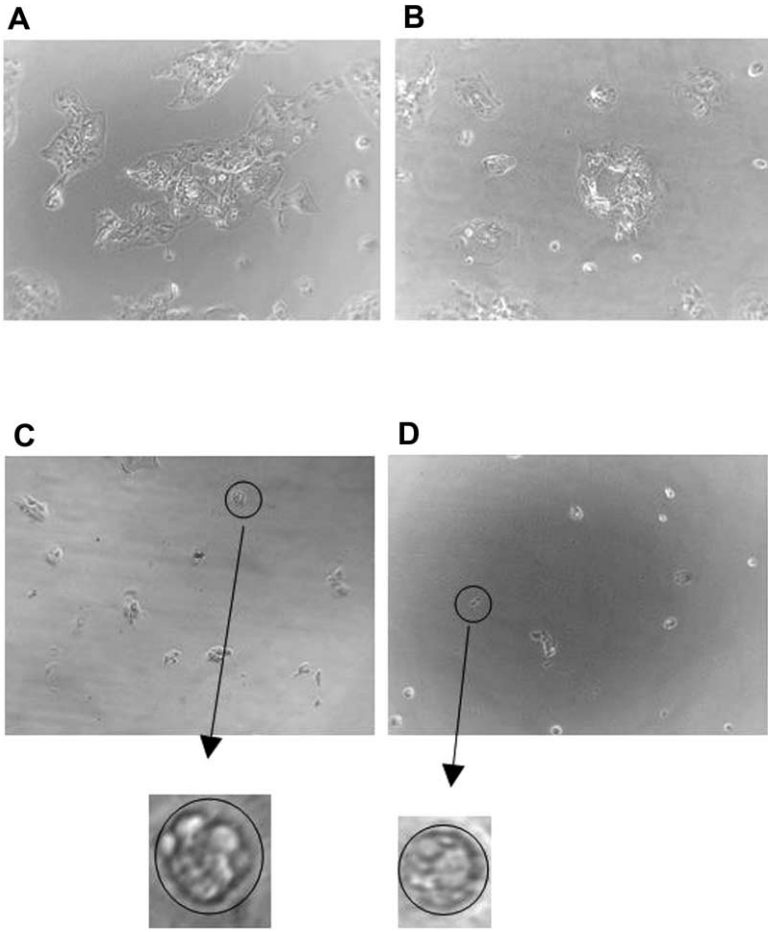


Figure 4. Inverted Microscope Images of SW1990

(A) Untreated cells. (B) Cells treated with Pan10-FM. (C) Cells treated with Pan10-4. (D) Cells treated with Pan10-3. The enlarged images of two of the cells treated with scFv-drug conjugates show extensive vacuolization.

(Figure 4). The cytotoxic effect of the Pan10-drug conjugates in comparison with the toxicity of the free drugs was then quantified by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide) cell proliferation assay [56–58]. SW1990 pancreatic carcinoma cells were seeded and allowed to attach in growth medium overnight. The cultures were then treated for either 3 or 12 hr with increasing concentrations of free drugs or Pan10-drug conjugates. After 7 days, the number of viable cells indicated a clear cytostatic/cytotoxic effect of Pan10-drug conjugates, especially after the 12 hr drug exposure time (Table 2). The inhibitory concentration 50% (IC_{50}) values measured for the free drugs (Table 2) were two to three orders of magnitude higher than the previously obtained values (**2** = 30 pM [51] and **1** = 2 pM [obtained as for **2**]). This inconsistency is probably due to a difference in the cell line used, duration of drug exposure, and cytotoxicity assay chosen. In our study, the free drugs had a more potent cytotoxic effect than the corresponding Pan10-drug conjugates, especially after a short exposure time. We attribute this effect to a more immediate availability of the free drug in the nucleus, where DNA is the site of action, upon diffusion through the plasma and nuclear membranes. Further evidence in support of this hypothesis came from the observation that the difference in efficacy between free drug and Pan10-drug conjugate was significantly reduced when the incubation time was extended. In particular, after a 12 hr incubation period, the Pan10-4 conjugate was as effective as the free compound **2**.

Table 2. Results of MTT Assay on SW1990 Cells

Cytotoxic Agent	IC_{50} (nM)	IC_{50} (nM)[d]
1	1.4 ± 0.2[b]	0.43 ± 0.17
Pan10-3 (1:1)[a]	94.3 ± 3.6[c]	2.7 ± 0.3
Pan10-3 (1:2)[a]	251.3 ± 75.8[b]	22.6 ± 3.8
2	32.1 ± 13.1[b]	4.3 ± 0.2
Pan10-4 (1:1)[a]	97.9 ± 38.6[c]	4.4 ± 0.7
Pan10-4 (1:2)[a]	1528 ± 369.4[b]	180.8 ± 30.6

[a] Ratio scFv to drug.
[b] Average of four experiments. Three hour incubation with drug.
[c] Average of two experiments. Three hour incubation with drug.
[d] Average of two experiments. Twelve hour incubation with drug.

Interestingly, Pan10-3 (scFv:drug = 1:1) displayed a cytotoxicity similar to Pan10-4 (scFv:drug = 1:1). This result, together with the lower cytotoxicity observed for conjugates carrying two drug molecules per molecule of scFv, suggests that in our case there is no advantage in derivatizing the drug through the hydrazone linkage. The results also imply that the mechanism of endocytosis of the Pan10 conjugates may not involve transfer into a low-pH environment and that upon cell internalization the drug remains linked either to the intact Pan10

or to peptides derived from the intracellular proteolysis of Pan10. The residual activity of such hypothetical complexes would not be surprising, since the tether we used between the scFv and the drug is probably long enough to allow for interaction with the DNA target and preservation of cytotoxicity. Indeed, a conjugate in which drug release from the scFv/scFv-derived peptides is not required for cytotoxic action could be advantageous, particularly within the context of a cell internalization mechanism. In this way, the scFv/peptide-drug compared to free drug might be trapped more effectively within the cell through reduced passive (diffusional) and active efflux processes. Overall, this mechanism would lead to the time-dependent accumulation of high intracellular concentrations of drug, affording the potential for efficient cancer cell killing, an excellent therapeutic index, and a decreased likelihood of acquiring drug resistance.

Finally, in testing the Pan10-3 and the Pan10-4 conjugates on the normal HdFa cells, cytotoxicity was observed with an IC_{50} ~3- and 5-fold higher, respectively, than those against the SW1990 cancer cells, whereas the free **1** had roughly the same IC_{50} values against both cells lines (data not shown). We had perhaps anticipated a greater cytotoxic specificity with the Pan10-drug conjugates. Yet, there may be a correlation between the result and a measurement by fluorescence-activated cell sorting (FACS) that showed a 5-fold greater level of α_3 integrin expression on SW1990 cell compared to HdFa cells (Supplemental Table S2). However, we emphasize that the value of such comparisons and correlations is difficult to assess, especially given the differences between the two cell types and the in vitro conditions of cell growth and analysis. Moreover, the cell-killing experiments were primarily intended to assess maintenance of internalization and drug activity of the Pan10-drug conjugates as evidenced by cytotoxicity, and not intended to be indicative of what might transpire in vivo. In particular, even the incubation conditions and the use of IC_{50} values can be considered artificial parameters to demonstrate efficacy. A scFv-drug would be cleared rapidly from the body, and IC_{50} is a thermodynamic parameter under essentially equilibrium conditions. Drug administration and activity in vivo will be a more kinetically controlled and dynamic process in which infusion conditions and dosing can be manipulated to better exploit the internalization and accumulation of the scFv-drug conjugate in tumor cells. Hence, it might not be necessary to even approach administered or systemic IC_{50} concentrations observed in vitro, which would allow enhanced tumor specificity and reduced side effects. In the current example, a more valid test for our Pan10-drug conjugates will be in subsequent animal models.

Significance

We have shown that chemically modified anti-integrin $\alpha_3\beta_1$ scFv Pan10 containing free thiols can be conveniently conjugated to maleimide-derivatized analogs of the potent cytotoxic agent duocarmycin SA. Our Pan10 conjugates conserve their ability to penetrate cells expressing integrin $\alpha_3\beta_1$. In particular Pan10-drug conjugates show excellent cytotoxic effects on pancreatic carcinoma cells in vitro. This first step is of critical importance considering the unique advantage of the scFv conjugates compared to the free drugs described herein, which are extremely potent but not clinically viable anticancer agents. The conjugates can deliver these drug molecules more specifically to the interior of cancer cells overexpressing integrin $\alpha_3\beta_1$, which should allow for reduced therapeutic drug exposure and enhanced efficacy. Using such a strategy, experiments continue in our laboratory to further elaborate the potential for scFv-drug designs in cancer treatment.

Experimental Procedures

Expression and Purification of Pan10

E. coli B834(DE3) (Novagen, Madison, WI) was selected as the expression host for transformation with plasmids pETflag-Pan10 [59]. *E. coli* B834(DE3)/pETflag-Pan10 were grown in SB medium (30% peptone, 20% yeast extract, 10% MOPS) supplemented with 100 μM carbenicillin (RPI Corp., Mount Prospect, IL) at 37°C to mid-log phase (OD_{600} 0.65). Protein expression was induced by addition of 0.5 mM IPTG (RPI Corp.). The cultures were incubated for an additional 1 hr at 37°C and for 15 hr at 26°C. A 4 L culture of IPTG-induced *E. coli* B834/pETflag-Pan10 was harvested by centrifugation. The resultant cell pellet was lysed using BugBuster Protein Extraction Reagent (Novagen) according to the vendor's instructions, while the supernatant was concentrated to ~200 ml (EasyLoad, Masterflex from Millipore, Bedford, MA). Upon filtration through a 0.2 μM filter (Nalgene, Rochester, NY), the cell-free lysate (~100 mL) or the concentrated supernatant was loaded at a flow rate of 1 mL/min onto an Anti-Flag M2 affinity column (1.7 × 5 cm from Sigma, St. Louis, MO) previously equilibrated with phosphate-buffered saline (PBS). After washing with 100 ml of PBS, the flag-tagged Pan10 was eluted from the column with ~20 ml of glycine buffer (0.1 M glycine [pH 2.5]) at a flow rate of 3 mL/min. The eluate was neutralized with ~1 mL 1 M Tris Base. The level of purity was assessed by SDS-PAGE (10% Bis-Tris from Bio-Rad, Hercules, CA). A 4 L culture of IPTG-induced *E. coli* B834(DE3)/pETflag-Pan10 usually afforded 3.5–5 mg of purified protein, 60% of which was derived from the cell pellet.

Cell Lines

The human pancreatic adenocarcinoma cell line SW1990 (ATCC, Manassas, VA) was grown in Leibovitz's L-15 medium supplemented with 10% fetal calf serum (FCS). The normal human dermal fibroblasts (HdFa) from adult skin (Cascade Biologics, Portland, OR) were grown in Medium 106 supplemented with low-serum growth supplement.

SW1990 Binding Assay by Whole-Cell ELISA

SW1990 cells were trypsinized and resuspended in PBS to a concentration of 10^6 cells/mL. Aliquots (150 μL) were poured in the wells of a 96-well ELISA plate (tissue culture treated, flat bottom from Corning Incorporated, Canton, NY) and incubated at 37°C to complete evaporation (note that two rows of wells contained medium only). The plate was then washed four times with 0.025% Tween 20 (Sigma, St. Louis, MO) in PBS, blocked with 1% bovine serum albumin (BSA from Sigma) in PBS, washed once with deionized water, and pat-dried. Aliquots (100 μL) of serially diluted Pan10 (0.1–0 mg/mL, free or conjugated) in 1% BSA/PBS were added to the plate. One of the cell-free rows was incubated with Pan10 while the other lacked Pan10. The plate was then incubated for 1 hr at 37°C and subsequently washed ten times with distilled water. Aliquots (30 μL) of M2 anti-flag/HRP (1.1 μg/mL, Sigma) in 1% BSA/PBS were added to all the wells, and the plate was incubated for 1 hr at 37°C. Finally, following extensive washing with distilled water, the plate was developed in the presence of TMB and H_2O_2 (Pierce, Rockford, IL) and read at 450 nm with a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA).

Pan10 Mutation

Mutants Pan10S73C and Pan10S131C were generated by site-directed mutagenesis on template pETflag-Pan10 using standard PCR techniques. The primers used (Qiagen, Valencia, CA, mutated bases in italics) were the following. Forward (5′-end) primers: *Pan10S73C*, 5′-AAGGGCAGGGCCACACTG*T*GTGTAGA-3′; *Pan10S131C*, 5′-GGA GGTGGC*T*GCGGCGGTGGC-3′. Reverse (3′-end) primers: *Pan-10S73C*, 5′-CTACAC*A*CAGTGTGGCCCTGCCCTT-3′; *Pan10S131C*, 5′-GCCACCGCCGC*A*GCCACCTCC-3′. The mutagenesis procedure consisted of three stages. In the first, one end-primer (either forward *ARAHF*, 5′-GCCTACGGCAGCCGCTGGATTGTTATTACT-3′, or reverse *FLAG*, 5′-CTGGCAAGCTTATTATTTGTCATCGTCATCTTTGTA GTC-3′) and either one of the afore mentioned mutation primers were combined to introduce the mutation (temperature program [Mastercycler, Epperdorf, Hamburg, Germany]: denaturation at 95°C for 10 min; 30 cycles of amplification; extension 2 min, 72°C; denaturation, 95°C, 30 s; annealing 60°C, 1 min and polishing, 72°C, 7 min). In the second stage, the two halves of the mutated genes were overlapped (temperature program: denaturation at 95°C for 10 min; 20 cycles of amplification; extension 2 min, 72°C; denaturation, 95°C, 30 s; annealing 50°C, 1 min and polishing, 72°C, 7 min). Finally, in the third stage, the product of overlap PCR was amplified using the two end primers (temperature program: denaturation at 95°C for 10 min; 30 cycles of amplification; extension 2 min, 72°C; denaturation, 95°C, 30 s; annealing 55°C, 1 min and polishing, 72°C, 7 min). The amplified products were purified with PCR purification kit (Qiagen), digested with SfiI (New England BioLabs, Beverly, MA), purified, and ligated (T4 DNA ligase, New England BioLabs) to SfiI-digested and purified pETflag. The sequence the *Pan10* mutants was confirmed by full-length DNA sequencing (The Protein and Nucleic Acids Core Facility at The Scripps Research Institute, La Jolla, CA) using the end-primers.

Pan10 Thiolation

Pan10 (4 mg/mL) in 50 mM triethanolamine, 1 mM EDTA, and 150 mM NaCl (pH 8.7) was incubated in the presence of a 10-fold stoichiometric excess of Traut's reagent (Pierce) for 5 hr at 4°C, under constant agitation. The resultant mixture was desalted using PD-10 columns (Pharmacia, Peapack, NJ), eluted with 50 mM HEPES (pH 8) and concentrated by centrifugal ultrafiltration (YM 10,000 filter, Millipore). The concentration of free thiol in the desalted scFv solution was determined by Ellman's assay.

Ellman's Assay

A 75% methanol solution of ~30 μM thiolated scFv or standard dithiotreitol (DTT, ICN, Costa Mesa, CA) and 600 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, Sigma) was centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to a 96-well ELISA plate (Fisher, Ottawa, Ontario), and the Abs_{412} was read in a Spectra Max 25 plate reader (Molecular Devices). The concentration of free thiols was extrapolated from a standard curve obtained by plotting known concentrations of DTT versus the corresponding Abs_{412}.

Synthesis of the Analogs of Duocarmycin SA

All the chemicals utilized were purchased from Aldrich (St. Louis, MO). Note that the characterization of all the synthetic compounds is included as Supplemental Data. The synthesis of 3-(5-acetylindole-2-carbonyl)-1-(*S*)-(chloromethyl)-5-hydroxy-1,2-dihydro-3H-benz[*e*]indole (**1**) has been previously reported [51]. The synthesis of the Boc-protected 3-[5-(1-(3-aminopropyl)indole-2-carbonyl) aminoindole-2-carbonyl]-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole (**10**) is as follows (refer to Figure 5).

Methyl 1-(3-Phthalimidopropyl)indole-2-carboxylate (**5**). A solution of methyl indole-2-carboxylate (550 mg, 3.14 mmol) in dimethylformamide (31 mL) at 0°C was treated with sodium hydride (60% suspension in mineral oil, 167 mg, 4.18 mmol) and allowed to warm at 25°C over 30 min. The reaction mixture was cooled to 0°C and treated with *N*-(3-bromopropyl)phthalimide (1.26 g, 4.71 mmol). The mixture was allowed to warm at 25°C over 30 min and warmed at 55°C for 30 min before being cooled and quenched with the addition of H_2O (30 mL). The reaction mixture was extracted with ethyl acetate (2 × 40 mL), and the combined organic layers were washed with water (40 mL), dried (Na_2SO_4), and concentrated in vacuo. Flash chromatography (silica gel, 0%–50% ethyl acetate/hexane) afforded **5** in 62% yield.

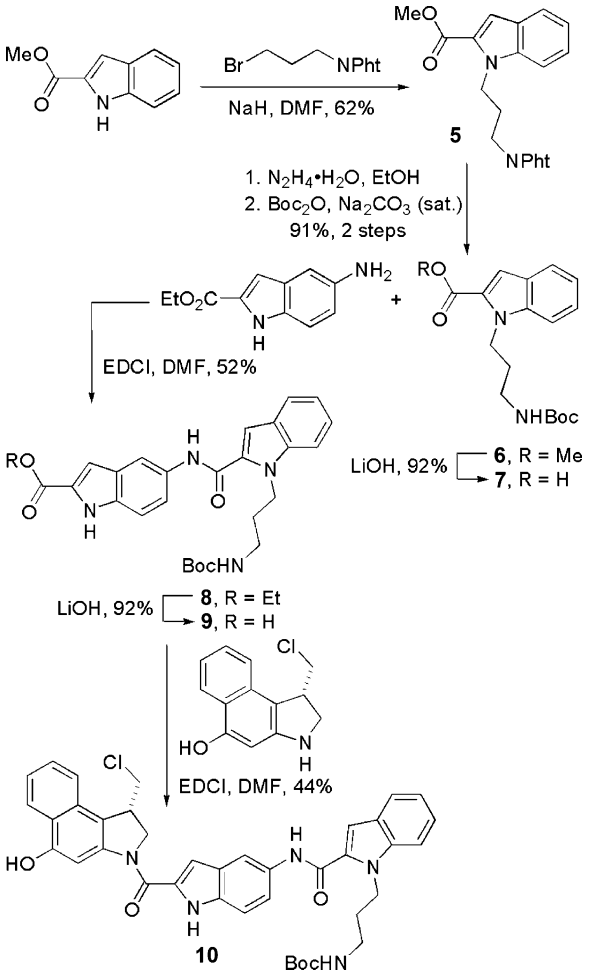


Figure 5. Schematic for the Synthesis of Boc-Protected **2**

Methyl 1-[3-(t-Butyloxycarbonyl)aminopropyl]indole-2-carboxylate (**6**). A suspension of **5** (500 mg, 1.39 mmol) in ethanol (14 mL) at 0°C was treated with hydrazine (200 μL, 4.14 mmol). The reaction mixture was stirred at 0°C for 1 hr and then allowed to warm to 25°C over 3 hr before being concentrated in vacuo. The residue dissolved in chloroform (10 mL) was treated with t-butoxycarbonyl anhydride (602 mg, 2.76 mmol) and saturated aqueous sodium carbonate (10 mL). The reaction mixture was stirred at 25°C for 12 hr before being extracted with chloroform (3 × 100 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo. Flash chromatography (silica gel, 10%–30% ethyl acetate/hexane) afforded **6** in 91% yield.

Ethyl 5-(1-{3-[N-(t-Butyloxycarbonyl)amino]propyl}indole-2-carbonyl)-aminoindole-2-carboxylate (**8**). A solution of **6** (332 mg, 1.0 mmol) in 10 ml dioxane/H_2O (4:1) was treated with 4 N LiOH (1 mL), and the mixture was stirred at 25°C for 15 hr. Aqueous HCl (1 N) (10 mL) was added, and the mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried (Na_2SO_4), and concentrated in vacuo to give **7** in 92% yield.

A solution of **7** (63.6 mg, 0.2 mmol) and ethyl 5-aminoindole-2-carboxylate (61.3 mg, 0.3 mmol) in dimethylformamide (4 mL) was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (115 mg, 0.6 mmol). The reaction mixture was stirred at 25°C for 18 hr and quenched with the addition of 15% aqueous citric acid (10 mL). The reaction mixture was extracted with ethyl acetate (75 ml and 2 × 25 mL), the combined organic layers were

washed with saturated aqueous NaCl (3 × 10 mL), dried (Na_2SO_4), and concentrated in vacuo. Flash chromatography (silica gel, 33% ethyl acetate/hexane) afforded **8** in 52% yield.

5-(1-{3-[N-(t-Butyloxycarbonyl)amino]propyl}indole-2-carbonyl)-indole-2-carboxylic Acid (**9**). A solution of **8** (50.5 mg, 0.1 mmol) in 2 ml dioxane/H_2O (4:1) was treated with 4 N LiOH (200 μL), and the mixture was stirred at 25°C for 18 hr. Aqueous HCl (0.5 N) (5 mL) was added, and the mixture was extracted with ethyl acetate (2 × 30 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo. Crystallization from tetrahydrofuran/hexane afforded **9** in 92% yield.

3-[5-(1-{3-[N-(t-Butyloxycarbonyl)amino]propyl}indole-2-carbonyl) aminoindole-2-carbonyl]-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3H-benz[e]indole (**10**). A solution of (–)-*seco*-*N*-Boc-CBI [60] (25 mg, 75 μmol, natural enantiomer) in 10 ml 4 N HCl (ethyl acetate) was stirred for 1 hr at 25°C before the solvent was removed under a stream of N_2. The residue was dried under high vacuum for 3 hr and **9** (39.5 mg, 83 μmol) was added. A solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (43 mg, 225 μmol) in dimethylformamide (2 mL) was added, and the reaction mixture was stirred for 14 hr at 25°C before the reaction mixture was concentrated in vacuo. Flash chromatography (silica gel, 20% tetrahydrofuran/hexane) afforded **10** in 44% yield.

Synthesis of 3

A mixture of **1** (3.0 mg, 7.2 μmol), maleimidopropionic acid hydrazide tetrahydrofuran salt (6 mg, 20 μmol), tetrahydrofuran (1 μL), and crushed 3 Å molecular sieves in 0.2 ml dimethylformamide was stirred overnight. Upon solvent evaporation the residue was dissolved in dichloromethane and purified by silica gel thin layer chromatography. **3** was obtained in 47% yield.

Synthesis of 4

Compound **10** (5 mg, 7.2 μmol) was treated with 50% trifluoroacetic acid in dichloromethane for 30 min. Upon trifluoroacetic acid evaporation, the crude free amine was dissolved in 0.1 ml dimethylformamide and added to a dimethylformamide solution containing maleimidopropionic acid (2.0 mg, 12 μmol), *O*-benzotriazol-1-yl-N, N, N′, N′-tetramethyluronium hexafluoro-phosphate (4.2 mg, 11 mmol), and N-methylmorpholine (3.2 μL, 29 μmol). The mixture was stirred for 2 hr, and the solvent was evaporated. The residue was purified by silica gel thin layer chromatography. **4** was obtained in 57% yield.

Conjugation of Thiolated Pan10

Aliquots (1 μl, 20 mM) of either fluorescein maleimide (Molecular Probes, Eugene, OR), **3**, or **4** in DMSO were added to 50 μl of Pan10 (~4 mg/mL in 50 mM HEPES) at intervals of 2 min. The resultant reaction mixture was incubated on a shaker for 10 hr at 4°C. Free dye or free drug were separated from the mixture of conjugated Pan10 and free Pan10 by size-exclusion chromatography (PD-10 column, Pharmacia). The percentage yield of Pan10 conjugation to fluorescein was calculated by fitting the Abs_{492nm} and Abs_{280nm} (Ultrospec 2000, Pharmacia) of the desalted mixture into Equation 1:

$$\%\ \text{conjugation to FM} = Abs_{492}/59880^{a} \times 100/[Abs_{280} - (0.2^{b} \times Abs_{492})]/1.35^{c}/\text{scFv-FM MW} \quad (1)$$

[a]ϵ_{492} experimentally determined for FM; [b]Abs_{280}/Abs_{492} experimentally determined for FM; [c]antibody ϵ_{280}.

The ratio of **3** or **4** to scFv was indirectly determined by calculating the amount of residual free scFv after the drug conjugation step. The mixture of Pan10-drug conjugate and free Pan10 was reacted with fluorescein-maleimide, and the amount of fluorescein-Pan10 (determined as described above) was assumed to correspond to the entire amount of Pan10 not bound to the drug. The percentage yield of Pan10 conjugation to **3** or **4** was calculated by fitting the $Abs_{492\ nm}$ and Abs_{280nm} of the desalted mixture obtained after the conjugation of scFv-drug + free scFv to the maleimide derivative of fluorescein into Equation 2:

$$\%\ \text{conjugation to drug} = 100 - \{Abs_{492}/59880 \times 100/[Abs_{280} - (0.2 \times Abs_{492})]/1.35/\text{scFv-FM MW}\} \quad (2)$$

Mass Spectrometry

MALDI-MS was performed on a Voyager DE Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA) in the linear mode using a nitrogen laser (337 nm) and sinapinic acid (Sigma) as matrix. Matrix solutions were prepared fresh daily as saturated solutions in a 1:1 mixture of acetonitrile and 0.1% aqueous trifluoroacetic acid. Samples were prepared for MALDI-MS analysis by diluting the desalted protein solution 1:10 with matrix and depositing 0.7 μl of the resulting suspension directly onto a stainless steel MALDI target well. The obtained masses were calibrated using two-point external calibration with equine cytochrome C and rabbit muscle aldolase (Sigma). All spectra were collected in positive ion mode with 140 ns delayed extraction and summed over approximately 50 laser shots.

One-Pot Antibody Conjugation

Pan10 (4 mg/mL) in 50 mM triethanolamine, 1 mM EDTA, and 150 mM NaCl (pH 8.7) was incubated 8 hr at 4°C, under constant agitation, in the presence of a 10-fold stoichiometric excess of Traut's reagent (Pierce) and an equal excess of either fluorescein maleimide (Molecular Probes), **3**, or **4**. The resultant mixture was desalted using PD-10 columns, eluted with PBS, and concentrated by centrifugal ultrafiltration (YM 10,000 filter, Millipore) to ~4 mg/mL.

Confocal Microscopy

SW1990 or HdFa cells were trypsinized, resuspended in PBS, and counted. Cells (10^4–10^5) were seeded into the wells of a chamber slide (Nunc, Naperville, IL) and allowed to attach for 24 hr at 37°C. Upon changing the medium (500 μL/well), 10 μl of ~3 mg/mL concentrated Pan10-fluorescein or 92H2-fluorescein (negative control) was added, and the cells were incubated for 30 min, 1, 2, or 3 hr at 37°C. The cells were then washed ten times with their respective medium and once with PBS, then they were fixed and permeabilized with 95% ethanol for 5 min, washed once with PBS, stained with propidium iodine (Sigma, 1:50 diluted in PBS) for 1 min, washed five times with PBS, and sealed with a coverslip upon addition of antifade solution (Slow Fade, Molecular Probes). The slides were observed with a laser scanning confocal microscope (MRC1024, Bio-Rad).

FACS Analysis

SW1990 or HdFa cells were trypsinized, washed in cold PBS, and aliquoted (~5×10^5 cells/tube). The primary antibody (either W6/32, Novus Biologicals, Littleton, CO; P1B5, Chemicon, Temecula, CA; or P5D2, Chemicon) was then added (final concentration 10 μg/mL), and the incubation was carried on for 45 min on ice. The cells were then washed with cold PBS and incubated in the presence of FITC-labeled goat anti-mouse Ab (Pierce, Rockford, IL) on ice for 45 min. A final wash with cold PBS was followed by PI counterstain and analysis (FACScan, Becton Dickinson, Franklin Lakes, NJ).

Inverted Microscopy

SW1990 cells were trypsinized, resuspended in PBS, and counted. Cells (10^4–10^5) in 500 μl of growth medium were seeded into the wells of a chamber slide (Nunc) and allowed to attach for 24 hr at 37°C. The old medium was than replaced by medium containing 400 nM of either Pan10-3, Pan10-4, Pan10-FM, or wt-Pan10. Cells were then observed with an inverted microscope (Zeiss Imm, Thornwood, NY) every day for 7 days.

Cell Proliferation Assay

The cytotoxicity of scFv-drug or free drug was quantified by using the Vybrant MTT cell proliferation assay kit (Molecular Probes). Assays were performed using 48-well microtiter plates containing 2×10^4 SW1990(HdFa) cells/well in 300 μl of phenol-free growth medium. Cells were allowed to attach to the wells for 12 hr. For determination of IC_{50}, cells were incubated for 3 or 12 hr at 37°C with various concentrations of Pan10-drug conjugates, maleimide derivatives, or free drugs. Then the incubation was continued in conjugate/drug-free medium, and the MTT assay was performed at

the end of the seventh day. Medium was replaced with 100 μl of fresh medium containing 1.2 mM MTT and the incubation continued for 3 more hours. The cells were then lysed by adding 100 μl of a 10 mM solution of HCl containing SDS (100 mg/mL). The cell lysis was allowed to proceed for a period of 8 hr at the end of which the plate was centrifuged at 3000 rpm for 3 min and the supernatant transferred in a 96-well plate and read at 570 nM. Each assay included a negative control of cells treated with free Pan10 and a positive control lacking cells. All assays were performed at least twice. A set of eight data points was obtained with various concentration of cytotoxicity agent. In order to obtain the IC_{50} values, data points from each set were fit to the sigmoidal dose-response curve defined by Equation 3 using Grafit5 (Leatherbarrow, R.J. 2003. Grafit version 5.08, Erithieus Software Ltd, Staines, England).

$$y = y^{min} + \{(y^{max} - y^{min})/[1 + (IC_{50}/x)^{slope}]\} \quad (3)$$

y = percent of live cells; x = concentration of drug (drug-scFv).

Data points which were outliers (typically one to two per experiment) were discarded.

Supplemental Data

Supplemental Data including characterization of synthetic compounds, amino acid sequence of Pan10, chromatographic analysis of Pan10-drug conjugates, efficiency of drug conjugation to genetically modified Pan10, and FACS analysis results can be found at http://www.chembiol.com/cgi/content/full/11/7/897/DC1.

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