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Novel immunoconjugates comprised of streptonigrin and 17-amino-geldanamycin attached via a dipeptide-*p*-aminobenzyl-amine linker system

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ABSTRACT

Cytotoxic agents streptonigrin and 17-amino-geldanamycin were linked to monoclonal antibodies (mAbs), forming antibody–drug conjugates (ADCs) for antigen-mediated targeting to cancer cells. The drugs were conjugated with a linker construct that is labile to lysosomal proteases and incorporates a valine-alanine-*p*-aminobenzyl (PAB)-amino linkage for direct attachment to the electron-deficient amine functional groups present in both drugs. The resulting ADCs release drug following internalization into antigen-positive cancer cells. The drug linkers were conjugated to mAbs cAC10 (anti-CD30) and h1F6 (anti-CD70) via alkylation of reduced interchain disulfides to give ADCs loaded with 4 drugs/mAb. The streptonigrin ADCs were potent and immunologically specific on a panel of cancer cell lines in vitro and in a Hodgkin lymphoma xenograft model. We conclude that streptonigrin ADCs are candidates for further research, and that the novel linker system used to make them is well-suited for the conjugation of cytotoxic agents containing electron-deficient amine functional groups.

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There is a great deal of interest in the use of mAbs that bind specifically to antigens overexpressed on cancer cell populations for the targeted delivery of cytotoxic agents.[1,2] The strategy seeks to combine the exquisite targeting specificity of mAbs with the potent cytotoxicity of small-molecule antitumor agents. In contrast to small molecule drugs, which are often rapidly cleared from systemic circulation, mAb therapeutics typically remain in circulation for many days to weeks.[1,2] Therefore, in addition to tumor targeting, ADCs benefit from enhanced exposure relative to free drugs. Early work has elucidated the importance of drug linker circulation stability, efficient on-target drug release, antitumor agent potency, and a clean antigen expression profile as important parameters for the success of the ADC strategy.[2,3] Various different drug linkers have been used for conjugation to mAbs, including hydrazones, disulfides, and peptides, which are cleaved to release drug under acidic, reducing, and proteolytic conditions, respectively.[1,3] Our experience with the auristatin drug class has led us toward amine-containing drugs, which are amenable to conjugation via dipeptide-PAB-carbamate linkages.[4] We have also shown that phenolic drugs can be stably attached to mAbs through a dipeptide-PAB-ether linker and that drug is efficiently released upon proteolysis.[5] Monoclonal antibodies empowered with drugs attached through dipeptide linkers have been characterized by a high degree of circulation stability.[4,6]

We are interested in all classes of cytotoxic drugs that may be suitable for empowering mAbs. In an effort to expand our ADC platform, we sought to evaluate streptonigrin and 17-amino-geldanamycin as potential ADC chemotypes (Scheme 1A). Streptonigrin (**1**) is a quinoline quinone antitumor agent originally isolated in 1959 from *Streptomyces flocculus*.[7] The cytotoxic mechanism is thought to involve metal chelation and redox cycling leading to the production of reactive oxygen species and subsequent DNA strand breaks, culminating in cell death.[8,9] The

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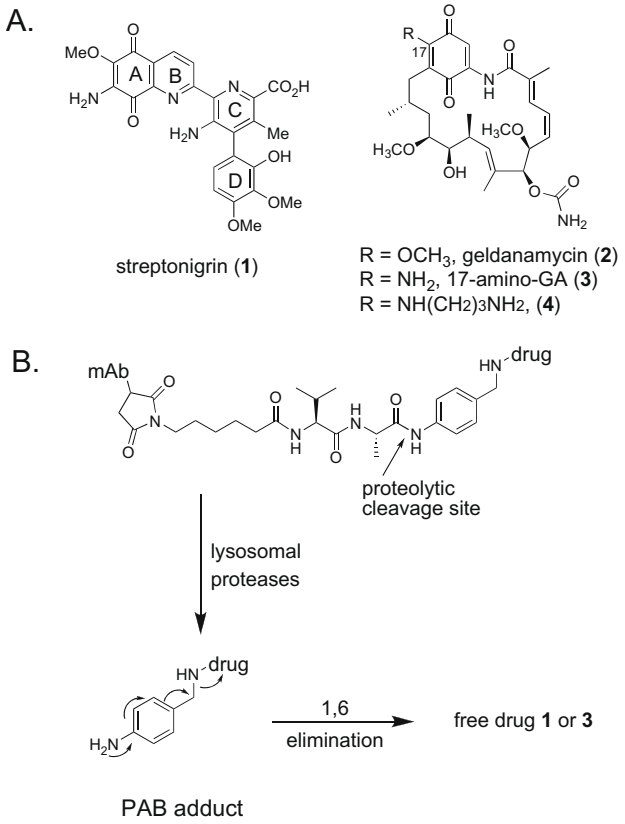


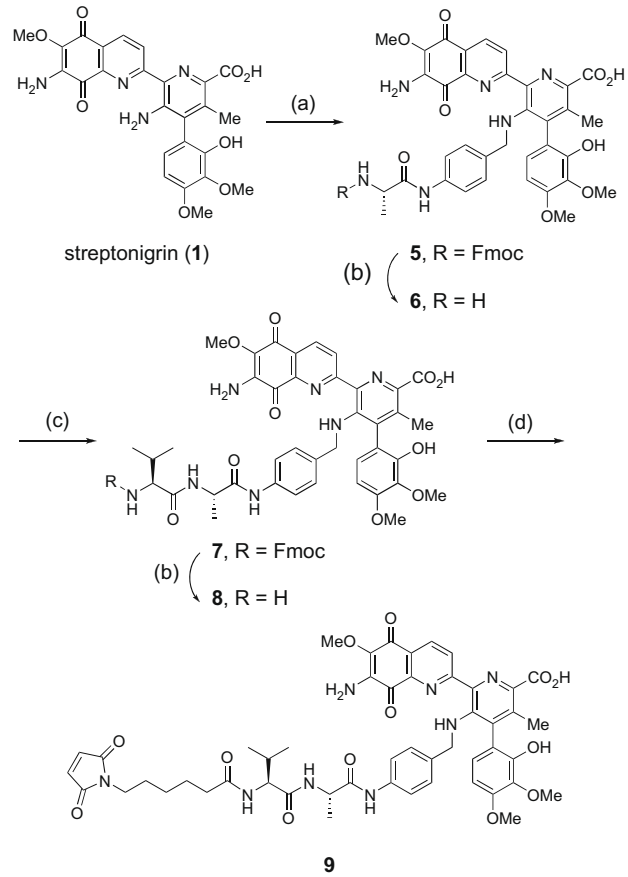
Scheme 1. (A) Structure of cytotoxic agents, (B) design of conditionally-stable streptonigrin and 17-amino-geldanamycin immunoconjugates.

compound was found to be highly potent in vitro and efficacious in vivo for the treatment of various lymphomas and carcinomas. However, in 1977 the NCI discontinued clinical investigations due to severe and unpredictable toxicities,[9] perhaps making this drug a suitable candidate for targeted delivery. Geldanamycin (GA), a benzoquinoid ansamycin produced by actinomycete *Streptomyces hydroscopicus*, exerts cytotoxic activity through binding to hsp90, leading to the accelerated degradation of signal-transducing protein tyrosine kinases and, ultimately, cell death.[10,11] Although a highly potent antitumor agent, severe toxicity and formulation challenges have precluded the clinical development of GA.[12] Geldanamycin (**2**) lacks a suitable functional group, such as an amine, phenol, or thiol, with which to devise a circulation-stable conjugation strategy. However, structure activity relationships have established that 17-amino-GA (**3**) retains the cellular potency of GA.[12,13]

Analogs of STN and GA have been used to prepare ADCs previously. The C-ring NHS ester of streptonigrin was conjugated to solvent-exposed antibody lysines to form an ADC.[14,15] The conjugate was shown to be active against a hepatocarcinoma cell line[15] and Ehrlich carcinoma cells[14] in vitro. To the best of our knowledge, in vivo data was never reported for this conjugate. One potential liability with this approach is that the conjugation strategy includes no provision for the release of the free drug. A Herceptin–GA immunoconjugate has also been described.[12,16] The drug payload in this case was 17-(3-aminopropyl)-GA (**4**) attached directly to Herceptin with a putatively non-cleavable maleimidobutyryl linker. Despite the fact that 17-amino-GA (**3**) is more potent than 17-(3-aminopropyl)-GA (**4**), analog **4** was employed because the electron deficient amino group (a vinylogous amide) in **3** was deemed not suitable for conjugation.[12] The Herceptin-GA conjugate was moderately active in vitro, with IC_{50} = 40 μg ADC/mL or 580 nM in terms of GA,[12] and displayed enhanced efficacy relative to Herceptin in a mouse xenograft.[17] Potential liabilities with this conjugation strategy include the lack of a mechanism for drug release and the use of an analog (**4**) with inferior potency relative to the parent compound (**2**).

In contrast to the conjugation strategies described above, our approach involves the incorporation of a conditionally-stable dipeptide drug linker to achieve release of the free drug upon internalization of ADC into the lysosomes of the targeted cells (Scheme 1B). The Val-Ala-PAB drug linker system is designed to provide circulation stability and efficient drug release in the presence of lysosomal proteases.[18] One unique aspect of this drug linker approach is that electron-deficient, aryl amine-containing drugs are linked through a benzyl amine functional group to the mAb, instead of the commonly used carbamate moiety.

The streptonigrin drug linker was synthesized as shown in Scheme 2. Commercially available streptonigrin (**1**) was alkylated with Fmoc-Ala-*para*-aminobenzyl bromide in the presence of Hunig's base to provide **5** in 44% yield. Chemoselectivity of alkylation was established by NOESY, in which an NOE was observed between the benzyl methylene and a D-ring aromatic proton. This would not be observed if alkylation had occurred at the D-ring phenol or the A-ring vinylogous amide. Deprotection of the Fmoc group, followed by coupling with Fmoc-Val-OSu, afforded dipeptide intermediate **7**. Another round of Fmoc deprotection and coupling with the NHS ester of 6-maleimidocaproic acid (MC-OSu) provided drug linker **9**. It is worth noting that attempts to make the analogous PAB-carbamate linked constructs were unsuccessful, presumably due to the low nucleophilicity of the electron deficient aryl amine in the C-ring. This is in contrast to drugs containing electron-rich, primary or secondary aliphatic amines that readily react with



Scheme 2. Synthesis of a streptonigrin drug linker. Reagents and conditions: (a) Fmoc-Ala-PAB-Br, DIPEA, DMF, rt, 18 h, 44%; (b) DMF:piperidine (4:1), quant.; (c) Fmoc-Val-OSu, DIPEA, DMF, rt, 88%; (d) MC-OSu, DIPEA, DMF, rt, 86%.

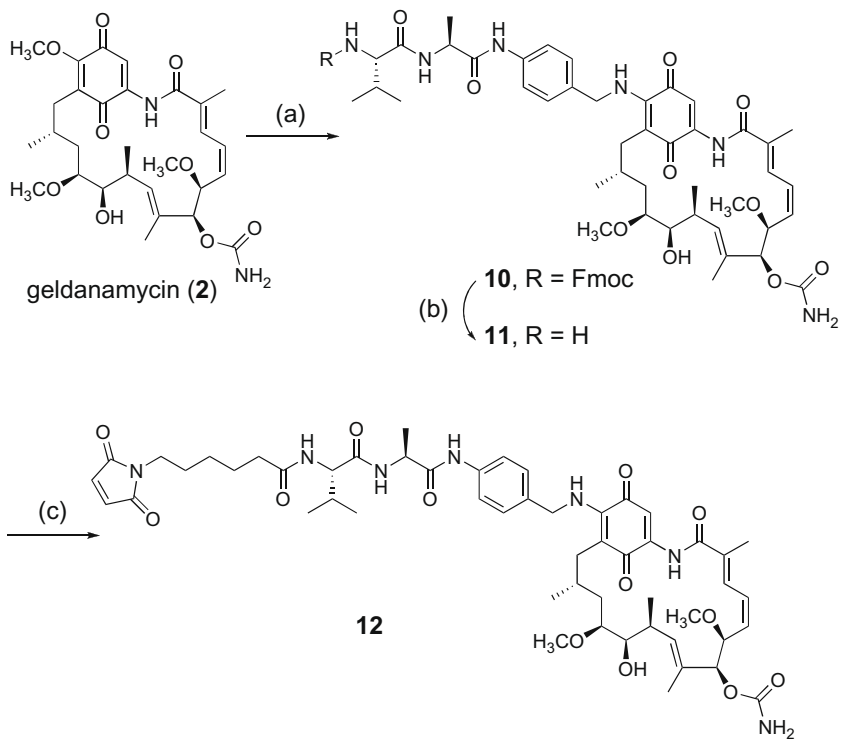
para-nitrophenyl carbonate reagents to form PAB-carbamate based drug linkers.[18,19]

The preparation of a 17-amino-GA drug linker was achieved as shown in Scheme 3. The 17-methoxy group in GA was exchanged[20] via addition/elimination with Fmoc-Val-Ala-*para*-aminobenzylamine in the presence of Hunig's base, providing intermediate **10** in 50% yield. Subsequent Fmoc deprotection provided free amine **11**, which was then subjected to diethyl cyanophosphonate-mediated coupling with 6-maleimidocaproic acid (MC–OH) to give drug linker **12** in 33% yield over two steps.

Prior to the preparation of ADCs, it was desirable to ensure that the 1,6-elimination of the PAB adducts (Scheme 1B) would occur following protease cleavage at the dipeptide. Drug linkers **9** and **12** were quenched at the maleimide and subjected to incubation with papain, a general cysteine protease, or cathepsin B, a lysosomal protease, to effect quantitative cleavage of the Ala-PAB amide bond. The reactions[21] were followed by LC–MS which showed that in both cases the quenched drug linkers had been quantitatively converted to free drugs **1** and **3** within 30 minutes following treatment with papain and cathepsin B, respectively. No intermediate PAB-STN or PAB-17-amino-GA adducts were observed.

The STN drug linker (**9**) was conjugated to chimeric AC10,[22] specific for CD30 on hematologic malignancies, and to humanized 1F6,[23] which recognizes the CD70 antigen on hematologic tumors and renal cell carcinoma. The GA drug linker (**12**) was conjugated to h1F6. Drug linkers were conjugated through reduction of mAb interchain disulfides and subsequent alkylation of the cysteine thiols with the drug linker maleimide.[4,24] The mAbs were alkylated at a level of 4 drugs/mAb to minimize aggregation, as determined by size exclusion chromatography. SEC analysis indicated that cAC10-**9** (3.8 drugs/mAb) and h1F6-**9** (4.0 drugs/mAb) were 89% and 96% monomeric, respectively. h1F6-**12** (4.0 drugs/mAb) was 95% monomeric. Excess drug linker was removed by purification of the conjugates via gel filtration.

The ADCs were assayed for in vitro cytotoxic activity and specificity (Table 1). The STN conjugates displayed potency and immunologic specificity on Karpas 299 (anaplastic large cell lymphoma), L540cy (Hodgkin lymphoma), and Caki-1 (renal cell carcinoma) cell lines. Karpas 299 and L540cy are CD30 positive and CD70 negative, while Caki-1 is CD30 negative and CD70 positive. Therefore, h1F6-**9** serves as an IgG specificity control conjugate for Karpas 299 and L540cy, while cAC10-**9** serves as the specificity control for Caki-1. The STN ADCs displayed antigen-specific potencies across the panel with the IC_{50} values ranging from 2.1 to 17 nM (in terms of the drug component). Immunological specificity was assessed with non-binding conjugates, which had a small fraction of the activity compared to binding conjugates. The 17-amino-GA ADC was inactive (IC_{50} in terms of drug component >240 nM) on



Scheme 3. Synthesis of a 17-amino-geldanamycin drug linker. Reagents and conditions: (a) Fmoc-Val-Ala-PAB–NH_2, DIPEA, pyridine, 50 °C, 7 h, 50%; (b) CH_2Cl_2:piperidine (4:1); (c) MC–OH, DEPC, DIPEA, CH_2Cl_2, rt, 33% (two steps).

Table 1
Characterization and in vitro potency of the STN conjugates

Test article	Antigen	Monomer (%)	Drugs/mAb	IC_{50} (nM)		
				Karpas 299[a] $CD30^+/CD70^-$	L540cy[a] $CD30^+/CD70^-$	Caki-1[a] $CD30^-/CD70^+$
cAC10-**9**	CD30	89	3.8	3.3	2.1	>270[b]
h1F6-**9**	CD70	96	4.0	>270[b]	>270[b]	17
1	–	–	–	0.4	0.8	0.4

[a] Cells were treated with test articles for 96 h and cell viability was assessed by resazurin conversion. The IC_{50} values represent the concentrations (nM) of the drug component of the antibody–drug conjugates.
[b] IC_{50} values are greater than the highest concentration tested.

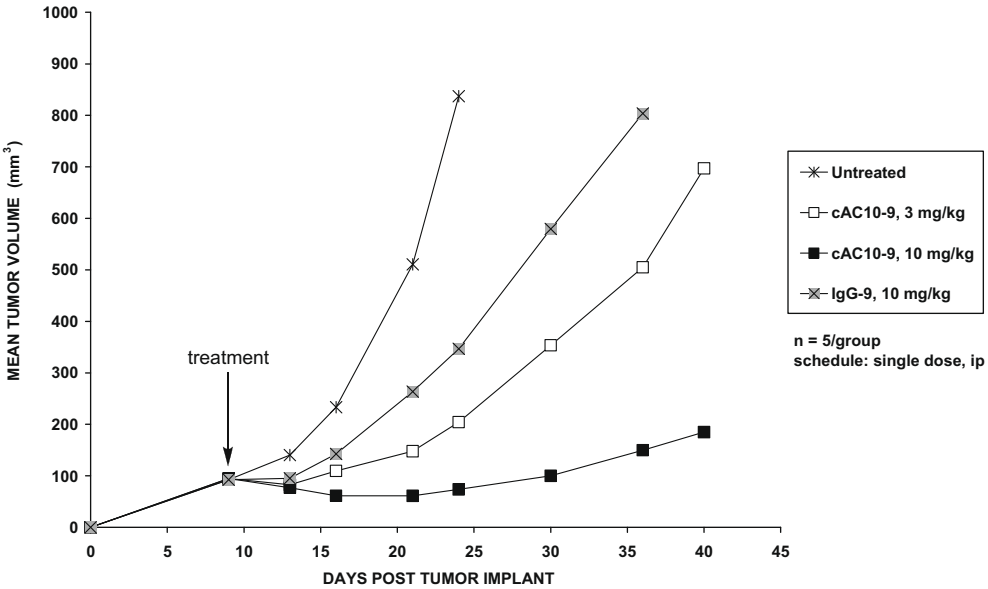


Figure 1. Single dose efficacy of 4-loaded streptonigrin immunoconjugate in a subcutaneous L540cy Hodgkin lymphoma xenograft model.

a panel of CD70 positive cell lines (data not shown) and was excluded from further evaluation. While free 17-amino-GA (**3**) is a potent small molecule, the lack of potency of the ADC may be due to many reasons including drug metabolism in lysosomes and altered intracellular trafficking.

In light of the encouraging in vitro data for the STN conjugates, in vivo experiments were performed in an L540cy mouse xenograft model.[25] SCID mice were implanted with L540cy cells and treated with test articles on day 9 when the tumors had reached nominally 100 mm^3. Mice were treated with one injection (delivered ip) of cAC10-**9** at 3 mg/kg or 10 mg/kg. Controls consisted of an untreated group and non-binding IgG control group treated with h1F6-**9** at 10 mg/kg. As seen in Figure 1, an immunologically specific, dose-dependent response was observed. Mice treated with 3 and 10 mg/kg cAC10-**9** experienced delay in tumor outgrowth, while the non-binding ADC was less active. The observed activity of the non-binding IgG control is perhaps a consequence of non-specific conjugate uptake. Both doses were tolerated (<20% weight loss and no outward signs of toxicity), but 50 mg/kg of cAC10-**9** exceeded the maximum tolerated dose. Thus, the STN ADC described here is not as well-tolerated as an analogous cAC10-auristatin immunoconjugate that utilizes a dipeptide benzylcarbamate linker.[26]

In summary, ADCs were prepared with potent cytotoxic agents utilizing a dipeptide-PAB-amine linker system. Upon protease-mediated dipeptide hydrolysis, the linker efficiently releases drugs such as STN (**1**) and 17-amino-GA (**3**) in chemically unmodified form, suggesting that the 1,6-elimination is rapid. Applications for this linker technology were demonstrated with STN (**1**) ADCs, which were mainly monomeric ($\geqslant$89%) with 4 drugs/mAb, and were active and immunologically specific in vitro and in vivo. Thus, the dipeptide-PAB-amine linker is well-suited for mAb conjugation and efficient protease-mediated drug release.

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