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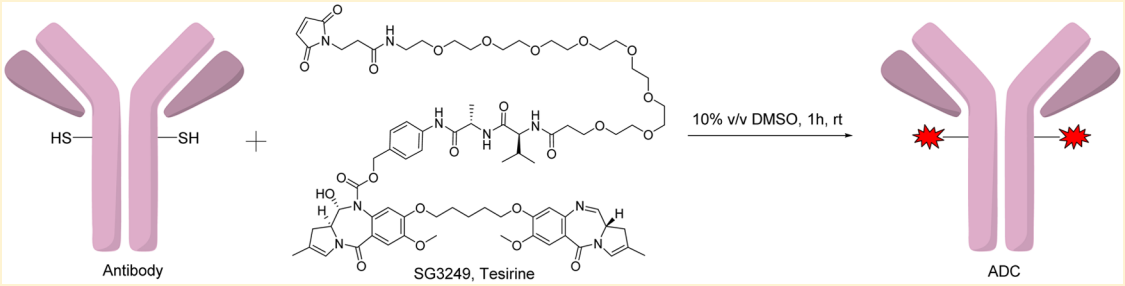
Design and Synthesis of Tesirine, a Clinical Antibody–Drug Conjugate Pyrrolobenzodiazepine Dimer Payload

Arnaud C. Tiberghien,* Jean-Noel Levy,† Luke A. Masterson, Neki V. Patel, Lauren R. Adams, Simon Corbett, David G. Williams, John A. Hartley, and Philip W. Howard*

QMB Innovation Centre, Spirogen, 42 New Road, E1 2AX London, U.K.

Supporting Information



ABSTRACT: Pyrrolobenzodiazepine dimers are an emerging class of warhead in the field of antibody–drug conjugates (ADCs). Tesirine (SG3249) was designed to combine potent antitumor activity with desirable physicochemical properties such as favorable hydrophobicity and improved conjugation characteristics. One of the reactive imines was capped with a cathepsin B-cleavable valine-alanine linker. A robust synthetic route was developed to allow the production of tesirine on clinical scale, employing a flexible, convergent strategy. Tesirine was evaluated *in vitro* both in stochastic and engineered ADC constructs and was confirmed as a potent and versatile payload. The conjugation of tesirine to anti-DLL3 rovalpituzumab has resulted in rovalpituzumab-tesirine (Rova-T), currently under evaluation for the treatment of small cell lung cancer.

KEYWORDS: *Tesirine, antibody–drug conjugates, talirine, SG3249, Rova-T*

Since their discovery in 1963,[1,2] pyrrolobenzodiazepines (PBDs) have been studied for their antitumor properties. Both naturally occurring monomer anthramycin, and synthetic dimer SG2000 have undergone clinical evaluation as stand-alone agents.[3] Until recently,[4] however, this more potent class of molecule remained under-exploited as a source of warhead for antibody–drug conjugates (ADCs).

Antibody–drug conjugates combine the potency of a cytotoxic warhead with the selectivity of an antibody to enable targeted killing of tumor cells. Most ADCs currently in clinical evaluation are based on antimitotic warheads such as auristatin and maytansine.[5]

Given their potency and mode of action as sequence-selective cross-linking agents, we hypothesized the ADC field would benefit from the employment of PBDs as a new class of warhead. A collaboration between Spirogen and Seattle Genetics led to the development of SGN-CD33A, an ADC under clinical investigation for the treatment of acute myeloid leukemia, featuring the PBD payload SGD-1910 or talirine.[6,7] We aimed to improve on the design of SGD-1910 by lowering hydrophobicity while maintaining potency (Figure 1).

We also investigated trapping one of the reactive imine moieties in its carbinolamine form by incorporating the N10 nitrogen as part of a cathepsin B-cleavable valine-alanine carbamate prodrug linker.[8] Finally, a discrete PEG_8 spacer was inserted to further enhance the solubility properties of the

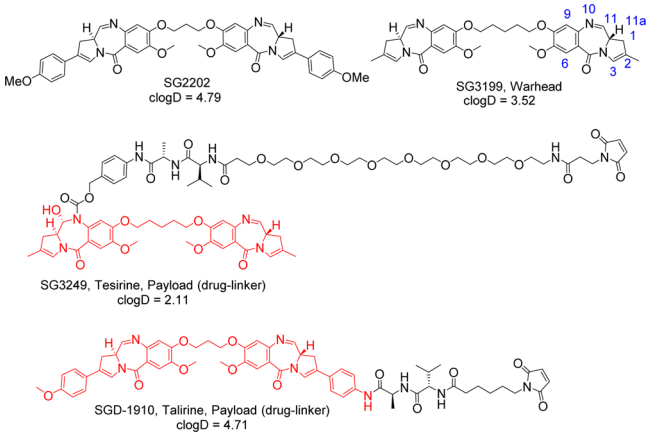


Figure 1. Structures of SG2202 and ADC warhead SG3199. LogD values calculated at pH 7.4. Structures of payloads (drug-linker), SG3249 (tesirine), and SGD-1910 (talirine). Red coding denotes released warhead. Conventional ring numbering shown on SG3199.

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molecule. This design resulted in the payload SG3249 (tesirine).

The warhead component of tesirine, SG3199 (Figure 1), was based on rational design principles. The C2 aryls, responsible both for the high potency and poor aqueous solubility of SG2202 and analogues,[9,10] were replaced with simpler methyl groups. The expected drop in potency was offset by the introduction of a 5-carbon tether between the two monomers. Both 3-carbon and 5-carbon tether linked PBDs are isohelical with the DNA helix, but the 5-carbon tether is more flexible and has greater opportunities for contact with the minor groove.[11,12] SG3199 was tested in a panel of cancer cell lines and retained picomolar activity *in vitro* (Table 1).

Table 1. *In Vitro* Activity of Warhead SG3199

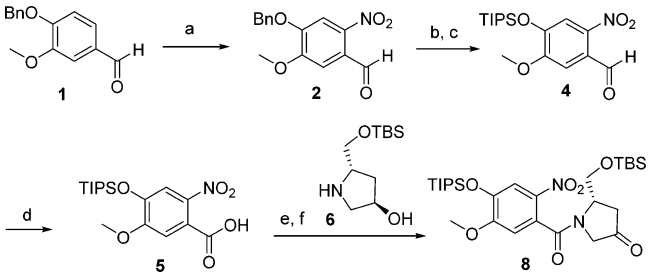
	cell line IC_{50}[a]			
compd	K562	NCIN87	BT474	SKBR3
SG3199	150 pM	20 pM	1 nM	320 pM

[a]IC_{50} values (mean of three independent experiments) were determined using CellTiter96 (MTS) following 96 h incubation.

Rather than following a linear route as used in the synthesis of SG2202 or SG2000 (SJG-136),[13] we decided to follow a convergent synthetic route, allowing both the left-hand (linker) and right-hand (imine) sides of the unsymmetrical dimer to be made in parallel, followed by late-stage dimerization. Monomeric routes have been developed previously by Howard et al.[12] and Kamal et al.[14] Recently, Kolakowski et al.[15] have reported the application of a convergent strategy to the synthesis of an unsymmetrical C2-aryl substituted PBD. In contrast to these other strategies, we selected *N*-alloc and *O*-TBS as the N10-imine and C11-hydroxy protecting groups for mild and efficient deprotection in the final stages of the synthesis. Both groups are resistant to base and mild acid but can be orthogonally removed when required.

Controlled nitration of benzylvanillin afforded intermediate **2** (Scheme 1).[16] This was followed by an exchange of protecting

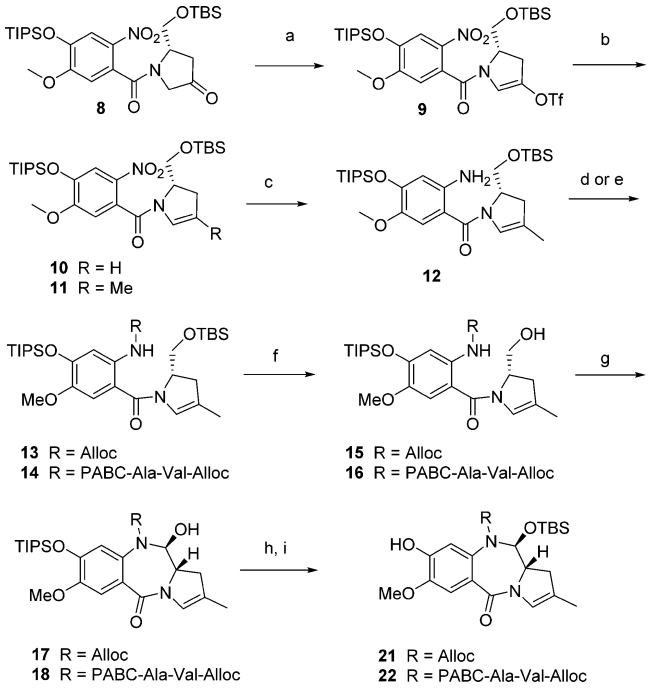
Scheme 1. Synthesis and Coupling of A and C Rings[a]



[a]Reagents and conditions: (a) HNO_3, 12 °C, 95%; (b) TFA, 85 °C, 50%; (c) TIPSCl, imidazole, 100 °C, 88%; (d) $NaClO_2$, NaH_2PO_4, H_2O_2, THF, −78 °C to rt, 100%; (e) DCC, HOBt, Et_3N, DCM, −10 °C to rt, 90%; (f) TEMPO, TCCA, DCM, 100%.

groups at the phenolic position. TIPS was selected for its relatively high resistance to acid and base, with the option of selective phenolic silyl deprotection in the presence of aliphatic silyl ethers. Clean and efficient Pinnick oxidation of benzaldehyde **4** gave acid **5**. Amide bond formation with hydroxyproline derivative **6**[11] gave alcohol **7** (see Supporting Information), which was oxidized using the reactive TEMPO/TCCA combination. Thermodynamic treatment of compound **8**[17,18] with triflic anhydride gave triflate **9** in 78% yield (Scheme 2). This was followed by a sp^2−sp^3 Suzuki coupling to install a

Scheme 2. Synthesis of Monomeric Phenol 20 and 21[a]



[a]Reagents and conditions: (a) Tf_2O, 2,6-lutidine, DCM, −45 °C, 78%; (b) $MeB(OH)_2$, Ag_2O, $AsPh_3$, $Pd(PhCN)_2Cl_2$, 70 °C, 70%; (c) Zn (30 equiv), HCOOH/EtOH 5/95, 30 °C, 80%; (d) Alloc-Cl, pyridine, DCM, −78 °C to rt, 100%; (e) triphosgene, Et_3N, THF, 5 °C, then Alloc-Val-Ala-PAB-OH, Et_3N, THF, 40 °C, 50%; (f) AcOH/MeOH/THF/water 7/1/1/2, rt, 71%, **15**, 80%, **16**; (g) DMSO, $(COCl)_2$, Et_3N, DCM, −78 °C to rt, 66%, **17**, 60%, **18**; (h) TBS-OTf, 2,6-lutidine, DCM, 0 °C, 85%, **19**, 65%, **20**; (i) LiOAc, DMF/water, 95/5, rt, 100%, **21**, 100%, **22**.

methyl group at the C2 position. Activated conditions developed by Mu and Gibbs,[19] employing silver oxide and triphenylarsine, were required for efficient coupling. Optimization of the reaction conditions was necessary to avoid competing triflate reduction; the resulting impurity **10** having similar chromatographic retention properties to the desired compound **11**. When the same conditions were investigated with homologous ethyl boronic acid, a much larger proportion of reduced material **10** was found to contaminate the mixture. The nitro group was reduced with zinc and dilute formic acid in ethanol. These conditions were mild enough to leave both the primary TBS ether and the internal unsaturation intact.

At this key juncture in the synthesis, carbamates **13** and **14** were formed from **12**, either by treatment with alloc chloroformate or through the initial formation of an isocyanate[20] followed by addition of alloc-Val-Ala-*para*-amino-benzylalcohol.[21] The two resulting monomeric intermediates **13** and **14** were subjected to the same sequence of reactions beginning with mild acid hydrolysis of the primary TBS and ring-closing oxidation under Swern conditions. Protection of the 11-hydroxy group is critical at this point to allow further chemistry to proceed under basic or acidic conditions while preserving the **11*a*** stereochemistry.[22] The use of highly reactive TBS triflate[23] was necessary to achieve efficient secondary

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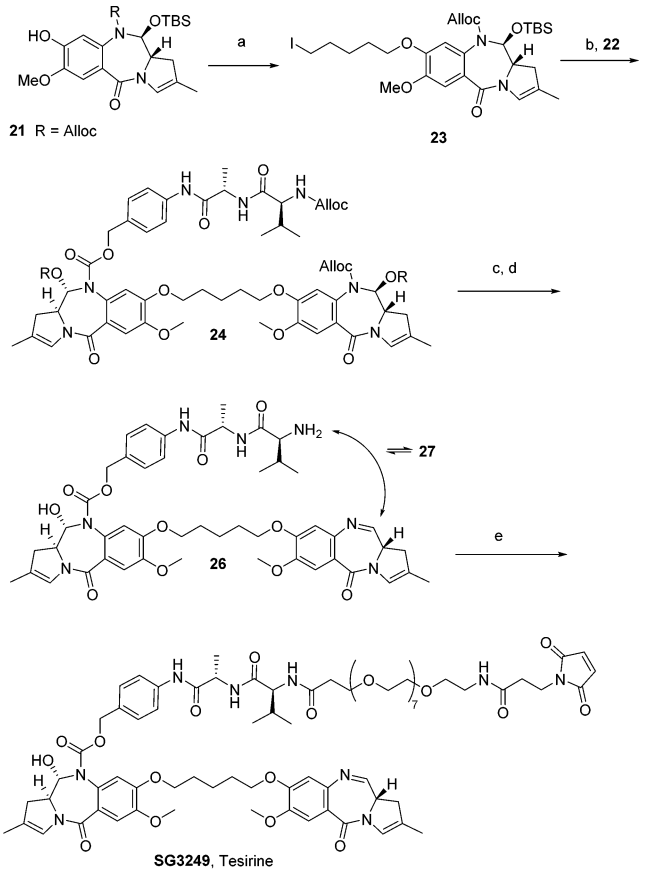
alcohol protection. Finally, mild and orthogonal deprotection of the phenolic TIPS in the presence of aliphatic TBS was achieved with LiOAc in wet DMF[24] to provide both the right-hand and left-hand side monomers **21** and **22**.

Williamson ether chemistry, proceeding via iodopentane derivatization of alloc-protected monomer **21** and subsequent reaction with monomer **22**, provided dimer **24** in 86% yield (Scheme 3). Removal of 11-hydroxy TBS with TBAF resulted

Scheme 3. Final Steps, Synthesis of SG3249 (Tesirine)[a]



[a]Reagents and conditions: (a) 1,5-diiodopentane, K_2CO_3, acetone, 60 °C, 90%; (b) **22**, K_2CO_3, acetone, 65 °C, 86%; (c) TBAF/AcOH, THF, rt, 80%; (d) $Pd(PPh_3)_4$, pyrrolidine, DCM, rt, 100%; (e) Mal-$dPEG_8$-acid, EDCI, DCM, rt, 73%.

in partial racemization at C11a (detectable by LC and optical rotation analyses of SG3249: Figure 2, Table 2). Buffering the fluoride solution with acetic acid was found to prevent racemization.[25,26]

Both alloc groups were rapidly and efficiently removed with Pd(0) and pyrrolidine as an allyl scavenger,[27] in a Tsuji–Trost reaction.[28] The absence of pyrrolidine scavenging during the deprotection resulted in the formation of N10-allylated impurities. The resulting compound **26** was immediately used in the next step without further purification. Traces of palladium were effectively removed, both by chromatography of the payload SG3249 and purification of the final ADC. Interestingly, reversible macrocycle **27** was found to form upon standing in solution with the valine amino group adding across the PBD imine of **26** (NMR and structure in Supporting Information). However, we found that both **26** and **27** could be fully consumed under final coupling conditions with EDCI and

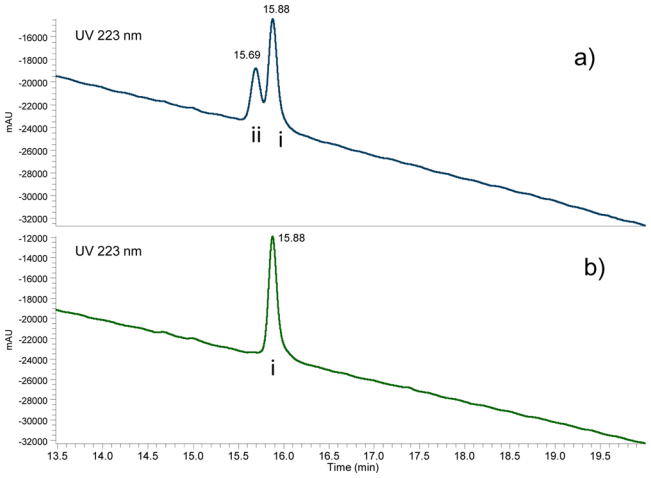


Figure 2. UPLC of SG3249: (a) unbuffered TBAF used in Scheme 3, step c; (b) buffered TBAF. Ace Excel 2 C18-AR (2 μm, 3.0 mm × 100 mm), 40 °C, 20 mM ammonium formate (pH = 4)/acetonitrile, 25/75 to 55/45 over 30 min, 0.6 mL/min. Peaks i and ii in (a) diastereoisomers of SG3249. Peak i in (b) pure SG3249.

Table 2. SG3249 Optical Rotation[a]

SG3249 batch	optical rotation	TBS cleavage
unbuffered	+118° (c = 0.43)	TBAF
buffered	+262° (c = 0.56)	TBAF/AcOH 1/1

[a]SG3249 batches made with either an unbuffered or buffered TBS cleavage step (Scheme 3, step c). Measurement in chloroform at 20 °C.

Mal-$dPEG_8$-Acid to form SG3249 in 73% yield. The overall yield for the synthesis was 0.54% from benzylvanillin over 30 steps.

Despite its length, the synthetic route described has shown a number of advantages such as robustness on scale, modularity of monomeric building blocks, and mild and efficient final steps. The same route was used to provide clinical grade material and is continuously being optimized.

To explore its potential as a payload, SG3249 was conjugated to trastuzumab (Herceptin) in a stochastic fashion and to an engineered version featuring two reactive cysteine positions (site-specific Herceptin-SG3249) (Table 3). Finally, SG3249 was conjugated to a control, nonbinding, engineered antibody.

SG3249 conjugation to all antibodies was highly efficient, as illustrated by a readily tunable stochastic ADC average drug-to-antibody ratio (DAR of 2.5 in this instance; the DAR can be tuned by varying the amount of TCEP reducing agent) and site-specific ADC DAR of 1.8 (90% conjugation efficiency). SG3249 was found readily soluble in the 10% DMSO aqueous

Table 3. ADC Conjugation Properties and GI_{50} in SKBR3[a]

ADC	DAR	yield (%)	process aggregation (% dimer)	GI_{50} (ng/mL)
Her-SG3249	2.51	86	2.81	1.74
site-specific Her-SG3249	1.81	89	3.93	2.62
IgG control mAb-SG3249	1.79	87	3.11	1101

[a]DAR assessed by RP-HPLC. Process aggregation assessed by SEC. GI_{50} in SKBR3 (Figure 3). Yields calculated based on concentration measurements.

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conjugation buffer (50 mg/L, see Supporting Information, solubility limit not determined). In contrast, SGD-1910, bearing C2-aryls and devoid of PEG linker, had to be conjugated in 50% propylene glycol.[6] SG3249 low hydrophobicity ($logD_{7.4}$ = 2.11) resulted in low levels of aggregation (usually <5%). As a result, the conjugation process reproducibly delivered high monomeric purity ADCs in high yields, on microgram to gram scale.

Gratifyingly, both the stochastic and site-specific Herceptin-SG3249 ADCs were active in the ng/mL range against HER2-expressing human breast cancer cell line SKBR3 (Figure 3, Table 3). Both HER2-targeted ADCs were greater than 2 orders of magnitude more potent than the control non-HER2-binding ADC, indicating target specificity. The residual activity of the nontargeted control ADC is likely due to nonspecific uptake (such as clathrin-mediated endocytosis) at high assay concentrations. Linker stability studies will be undertaken to further address this point and will appear in subsequent publications. Preliminary evidence tends to show good linker stability when SG3249 is conjugated at buried positions within the antibody structure.

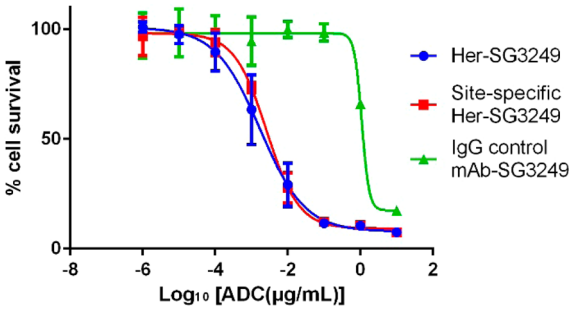

Figure 3. Growth inhibition curves in human SKBR3 cells for SG3249 ADCs.

In conclusion, SG3249 possesses an attractive set of properties: potency, synthetic route scalability, low hydrophobicity contributing to low aggregation, cathepsin B-cleavable linker, and efficient maleimide conjugation.

The *in vivo* evaluation of SG3249 ADCs will be described elsewhere. SG3249 has been conjugated with rovalpituzumab. The resulting ADC has recently successfully completed a phase I clinical trial for the treatment of small cell lung cancer.[29,30] Initial data from rovalpituzumab-tesirine (Rova-T) is encouraging[31] and should pave the way for a phase II trial. In addition, SG3249 forms the payload component of ADCT-301, which is currently in a phase I trial for CD25-positive hematological malignancies.[32,33]

The pyrrolobenzodiazepine dimer class of warheads is finding increasing utility in the ADC arena based on their versatility and complementary mode of action to antimitotic tubulin binders.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00062.

Detailed experimental procedures with analytical data for all intermediates. Graphical ^{1}H NMR and ^{13}C NMR for compounds of interest. Interpreted, graphical ^{1}H NMR for SG3249 and macrocycle **27** (PDF)

Preparation of SG3249 antibody−drug conjugates (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: tiberghiena@medimmune.com.

*E-mail: philip.howard@spirogen.com.

Present Address

†Stemcentrx, Inc., 450 East Jamie Court, South San Francisco, California 94080, United States.

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Notes

The authors declare the following competing financial interest(s): A.T., L.M., N.P., L.A., D.W., J.H., and P.H. are Spirogen employees. J.L. is a Stemcentrx employee.

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■ ABBREVIATIONS

ADC, antibody−drug conjugate; PBD, pyrrolobenzodiazepine; DLL3, delta-like 3; Her, herceptin; HER2, human epidermal growth factor receptor 2; PABC, *para*-aminobenzylcarbamate; DAR, drug-to-antibody ratio; TBAF, tetrabutylammonium fluoride; DCM, dichloromethane; Mal, maleimide; dPEG, discrete poly ethylene glycol; EDCI, *N*-(3-(dimethylamino)-propyl)-*N*′-ethylcarbodiimide hydrochloride; TFA, trifluoroacetic acid; TIPS, triisopropyl silyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl; TCCA, trichloroisocyanuric acid; TBS, *tert*-butyldimethylsilyl; alloc, allyloxycarbonyl

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