Design and Synthesis of Isoquinolidinobenzodiazepine Dimers, a Novel Class of Antibody–Drug Conjugate Payload

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Supporting Information

ABSTRACT: Antibody–drug conjugates (ADCs) represent an important class of emerging cancer therapeutics. Recent ADC development efforts highlighted the use of pyrrolobenzodiazepine (PBD) dimer payload for the treatment of several cancers. We identified the isoquinolidinobenzodiazepine (IQB) payload (D211), a new class of PBD dimer family of DNA damaging payloads. We have successfully synthesized all three IQB stereoisomers, experimentally showed that the purified (S,S)-D211 isomer is functionally more active than (R,R)-D221 and (S,R)-D231 isomers by >50,000-fold and ~200-fold, respectively. We also synthesized a linker-payload (D212) that uses (S,S)-D211 payload with a cathepsin cleavable linker, a hydrophilic PEG8 spacer, and a thiol reactive maleimide. In addition, homogeneous ADCs generated using D212 linker-payload exhibited ideal physicochemical properties, and anti-CD33 ADC displayed a robust target-specific potency on AML cell lines. These results demonstrate that D212 linker-payload described here can be utilized for developing novel ADC therapeutics for targeted cancer therapy.

KEYWORDS: Antibody–drug conjugates, ADCs, isoquinolidinobenzodiazepine dimers, IQB dimers, CD33, acute myeloid leukemia, AML

Targeted delivery of potent chemotherapeutic drugs in the form of antibody–drug conjugates (ADCs) has been going through an intensive investigation in oncology therapeutics.¹ Currently, there are over 60 ADCs² in the clinical investigation by using several different classes of cytotoxic payloads such as maytansines,³ auristatins,⁴ calicheamycins,⁵ duocarmycins,⁶ tubulysins,^{7,8} and pyrrolobenzodiazepines (PBDs).⁹ In recent years, there has been increasing interest in the development and application of the PBD class of cytotoxic payloads as ADC therapeutics. They are attractive as a class of cell-killing agents because, unlike tubulin binding compounds (maytansines and auristatins), their mechanism of action (DNA intercalation/alkylation) is effective at killing both dividing and nondividing cells.^{10,11} PBD dimers bind in the minor groove and cross-link specific repeat sequences in the DNA through the C11 position of both monomers. Two of the PBD ADCs that are or have been in clinical development, SGN-CD33A and Rova-T, use PBD linker-payloads SGD-1910 and SG3249, respectively.^{12,13}

In order to further expand this potent DNA-intercalating payload family and their use as an ADC therapeutic, we synthesized isoquinolidinobenzodiazepine (IQB) dimer (±)-D201 and evaluated its activity *in vitro*. We have observed the potency of D201 dimer to be in the picomolar range in AML cell lines, and it performed similarly to PBD (SGD-1882), albeit with slightly decreased *in vitro* potency values (Figure 1 and Table 1). The requisite (S,S) stereochemistry at the C11a

position is well established for pyrrolobenzodiazepine (monomers and dimers), as being necessary for high affinity minor groove binding.¹⁴ Given that SGD-1882 was an isolated (S,S) isomer, we therefore devoted additional effort to systematic preparation, and evaluation, of the complete set of possible stereoisomers for D201 dimers. A structurally similar class of DNA intercalators (indolinobenzodiazepine dimers and heterodimers (IGNs)) has recently been disclosed.¹⁵ In the interest of thoroughness and given that the IQB motif has a somewhat more extended unsaturated ring system, we felt it best to determine the identity of the best isomer empirically.

As can be observed from Figure 2 and Table 1, our results clearly demonstrate that (S,S) stereochemistry at the C6a positions are critical for optimum IQB activity. While D211 displayed a comparable profile to PBD in a selection of cell lines, it demonstrated even higher activity when applied to solid tumor cell lines (HEPG2, A704).

In an attempt to maximize minor groove intercalation and gain additional potency, we thought it was important to evaluate a five-carbon-length spacer analog. As can be garnered from the results in Table 1, increasing the distance between the two

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Figure 1. Structures of IQB derivatives and SGD-1882, along with ring numbering convention(s).

Table 1. *In Vitro* Potency Data of IQB Class of Payloads (IC_{50} Values Described Are in Picomolar)^a

cell line	PBD	D201	D211	D221	D231	D241
AML2	0.22	0.61	0.21	14001	40	0.31
AML3	0.25	1.14	0.31	13925	49	0.47
AML5	0.14	0.91	0.18	13925	116	0.85
HL60	1.58	3.19	1.57	91005	230	2.28
SHI	0.28	1.25	0.61	150480	685	1.14
HEPG2	121	NA	18.88	NA	NA	11.72
A704	9.85	NA	2.59	NA	NA	3.67

^aCancer cell lines were incubated with PBD or IQB dimer payload for 72 h. IC_{50} values were determined by quantitating viable cells using a CellTiter-Glo luminescent cell viability assay.

Figure 2. *In vitro* potency data of IQB payload in AML2 cell line.

binding units (e.g., (S,S)-D241) did not have a particularly dramatic impact on the activity of the compound across an array of cell lines; at the same time, there were no advantages of this modification that were immediately clear. The entire set of IQBs illustrated in Figure 1 can be prepared following a convergent strategy that allows for the dimers to be prepared via a mix-and-match pairing of monomers late in the syntheses. One can get a clear picture of the preparation of this class of IQBs examining the synthesis of (S,S)-D211 (Scheme 1). Its preparation commences with the conversion of benzoic acid 1, via a HATU-mediated amide coupling with methyl (S)-1,2,3,4-tetrahydroisoquinoline-3-carboxylate 2, to amide 3 in a moderate 57% yield. The tetracyclic precursor to (S,S)-D211 was then produced via a zinc-mediated reduction of the nitro group, performed under aqueous conditions, which accomplished the generation of an aniline capable of cyclizing intramolecularly to form the seven-membered ring, characteristic of this class of

Scheme 1. Synthesis of IQB Dimer (S,S)-D211^a

^aReagents and conditions: (a) HATU, DIEA, 2, DCM, 57%; (b) Zn, NH_4Cl , THF/ H_2O , 92%; (c) NaH, SEM-Cl, THF, 88%; (d) H_2 , Pd/C, MeOH, 99%; (e) 1,3-propanediol, PPh_3 , DIAD, THF, 37%; (f) superhydride, THF, $-78^\circ C$ to rt, 55%.

benzodiazepines, in exceptional yield. The N5 of the dilactam can then be protected in a straightforward fashion: treatment with the kinetic base NaH and addition of SEM-Cl nets a compound primed for imine generation at a later stage of the synthesis. The benzyl protecting group is removed rapidly under standard hydrogenolysis conditions to produce the phenolic monomer 5 in nearly quantitative yield. The final target can then be produced in a two-step sequence: generation of the dimer via a double Mitsunobu reaction with 1,3-propanediol is followed by a reduction of the SEM-protected lactam¹⁶ to the N5-imine (via treatment with superhydride at low temperature) to smoothly produce the desired IQB, (S,S)-D211. One advantage of this synthetic strategy is that a wide number of variants to (S,S)-D211 can be accessed rapidly through judicious selection of the amine precursor in step one of this sequence and the diol applied during the Mitsunobu step.

As D211 showed comparable *in vitro* potency to that of PBD payload, we endeavored to prepare a linker-payload construct, suitable for conjugation to an antibody. Not wanting to sacrifice any of the observed cell-killing potency of this new payload, we sought to attach a traceless/cleavable linker to ensure the released payload would be unmodified D211. The use and success of the cathepsin-cleavable linker for ADCs are well-established as phenomena in the clinic; we therefore decided to go with a cathepsin cleavable Val-Ala linker that was attached via N5 by way of a *para*-amino-benzyl carbamate. A PEG8-containing spacer was included in the design to help lower the construct's overall hydrophobicity. This same strategy, reported recently, was successfully employed with the ADC Rova-T.¹³ With the earlier convergent syntheses of the free-IQBs (Scheme

Scheme 2. Synthesis of D212 Linker-Payload^a

^aReagents and conditions: (a) NaOH, THF, 99%; (b) HATU, DIEA, 7, DCM, 86%; (c) Zn, NH₄Cl, THF/H₂O, 96%; (d) alloc-Cl, pyridine, DCM, 21%; (e) TBS-Cl, imidazole, DMF, 79%; (f) 0.5 equiv of **10**, THF, 6 days, 90%; (g) TBAF, THF, 85%; (h) IBX, DMSO, 99%; (i) Pd(PPh₃)₄, pyrrolidine, DCM, 88%; (j) TFA, DCM/H₂O, 20%; (k) **13**, DIEA, DCM, 84%.

Scheme 3. Generation of Undesired Benzyl Ether/Alkylation Product

1), not immediately amenable to the attachment of the linker, we turned our attention to the monofunctionalization approach shown in [Scheme 2](#). The bis-nitro compound **6** is readily available in three steps from vanillic acid. Hydrolysis of the methyl esters was performed, in analogous fashion to the monomers, followed by a HATU-mediated amide coupling with (S)-1,2,3,4-tetrahydroisoquinolin-3-ylmethanol to produce diol **8** in excellent yield. Using Zn/NH₄Cl under the same conditions that were successful for the monomers, a symmetrical bis-aniline can be generated cleanly. It is at this stage in the preparation of D212 that we addressed the need for monofunctionalization, by first reacting the aniline with 1 equiv of allyl chloroformate (Alloc-Cl), followed by treatment with TBS-Cl in DMF, with imidazole as a base to produce the triprotected monoaniline **9**. After numerous unsuccessful attempts to attach the linker via trapping of an intermediate

isocyanate with a benzyl alcohol,¹⁷ and yet further efforts directed at preparation of a chloroformate-functionalized linker, we were quite pleased to find successful appendage of the linker could be achieved when aniline **9** was mixed, in THF, in the presence of the pentafluorocarbonate **10** (see [Supporting Information](#) for the synthesis), in the absence of any supplementary base. Initial successes were obtained when the process was performed at 50 °C, but it was soon noted that, at elevated temperature (or in the presence of a base), **10** can decompose to the pentafluorobenzyl ether, a compound also capable of alkylating NS, but found to produce a construct incapable of the facile release of D211 (see [Scheme 3](#)).¹⁸

When the linker attachment was performed at room temperature, the reaction was particularly clean but required 6 days to reach completion. Treatment of **9** under standard desilylation conditions readily produced diol **11** in good yield.

The next required hurdle to clear en route to the final payload-linker construct was the sequential oxidation/cyclization transformations that are required to produce the hallmark benzodiazepines. Evaluation of numerous means to accomplish this feat (e.g., PCC mediated-, Swern-,¹⁷ Dess Martin-,¹⁹ and Ley-oxidation) ultimately led to a highly efficient process through the judicious application of IBX²⁰ (2.0 equiv per alcohol functionality for fast reaction yet limited overoxidation side products). With **12** in hand, all that remained was the removal of the Alloc- and Boc-protecting groups under standard conditions, followed by installation of the maleimide spacer via a simple amide coupling to the terminus of the PEGylated linker to produce the D212 linker-payload construct in 15% yield over the last three steps.

Prior to the preparation of an ADC, we thought it appropriate to evaluate the efficiency of payload release via the performance of an *in vitro* enzymatic cathepsin B cleavage assay. Upon treatment of the D212 linker-payload with bovine spleen derived cathepsin B under standard conditions,²¹ we were pleased to observe rapid and competent release of the “free-drug” D211. Figure 3 illustrates the time-course and profile of release, both of

Figure 3. Results of D212 linker-payload cathepsin B cleavage assay; reaction progress was monitored by RP-HPLC.

which were quantified using a RP-HPLC-based method, connected to an inline mass spectrometer. Beginning with the pure D212 linker-payload construct (Figure 3A), the cleavage reaction was determined to be at 59% conversion after 30 min (Figure 3B) and reached over 80% conversion after overnight incubation (Figure 3C).

With the successful preparation of D212 (and the encouraging results of the cathepsin B cleavage assay), we performed its conjugation anti-CD33 with two engineered cysteine residues (S239C) per antibody as described earlier.¹² Applying a global reduction (using DTT) and reoxidation (using dhAA) strategy as described earlier,²² the engineered cysteines were unmasked and then reacted with the maleimide-functionalized linker-payload construct to produce anti-CD33-D212 ADC (DAR of 1.7) with both high efficiency and high monomer content, circumventing the need for chromatographic purification. A nonbinding isotype control ADC (IgG1-D212 with a DAR of 1.8) was also produced (see full details within the Supporting Information).

The anti-CD33-D212 ADC and IgG1-D212 ADC were evaluated via incubation with AML cell lines (AML2 and HL60) at 37 °C for 5 days. The cell viability was tested using Promega CellTiter-Glo assay. These data demonstrate excellent target-specific potency (>200–500 fold) of the anti-CD33-ADC compared to a nonbinding control ADC (Figure 4).

AML2

HL60

Cell line	anti-CD33-D212 DAR 1.7	IgG1-D212 DAR 1.8
AML2	14.1	2424
HL60	12.3	8582

Figure 4. *In vitro* potency data of D212 ADCs in AML2 and HL60 cell lines. IC₅₀ values for both ADCs reported are in picomolar with respect to their payload concentration (normalized to DAR values).

In conclusion, D212 exhibits ideal ADC linker-payload characteristics: its conserved mechanism of action of DNA alkylation is similar to the well-known PBD dimer class of payloads (extensively used for several ADC programs that are in clinical development),²³ it contains a clinically proven cleavable linker (Val-Ala-PAB),⁴ inclusion of a PEG8 spacer contributes to the favorable solubility and hydrophilicity properties of the linker-payload, and last, conjugation can be carried out via well-established cysteine-maleimide chemistry. Preliminary *in vitro* characterization of anti-CD33 ADC using D212 linker-payload suggested that D212 linker-payload exhibited similar potency as a well-characterized PBD linker-payload, as shown earlier.^{12,13} Thus, D212 could be valuable linker-payload asset to the growing family of diverse linker-payloads that are being used in developing novel ADC therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.7b00436.

Experimental procedures and tabulated and scanned spectra of key compounds, including conjugates (PDF)

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Author Contributions

V.J. and J.R.J. conceived the idea. S.W.S., D.B., and S.J.D. established synthesis protocols and synthesized all chemical compounds described in this manuscript. J.Z., J.L., J.H., and Y.-P.J. tested potency of payloads and ADCs. S.W.S., D.B., and J.R.J. wrote the manuscript, and J.R.J. managed and led the program.

Notes

The authors declare the following competing financial interest(s): All authors from TCRS (now Abzena) worked on this project under Cellerant Therapeutics as part of a fee-service agreement. All authors are/were either full-time employees or contractors of Cellerant Therapeutics or from The Chemistry Research Solutions (now Abzena).

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ABBREVIATIONS

ADC, antibody–drug conjugate; AML, acute myeloid leukemia; DCM, dichloromethane; DIEA, diisopropyl ethyl amine; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate; HL, Hodgkin's lymphoma; PBD, pyrrolbenzodiazepines; IQB, isoquinolidinobenzodiazepine; TBAF, tetrabutylammonium fluoride; THF, tetrahydrofuran

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