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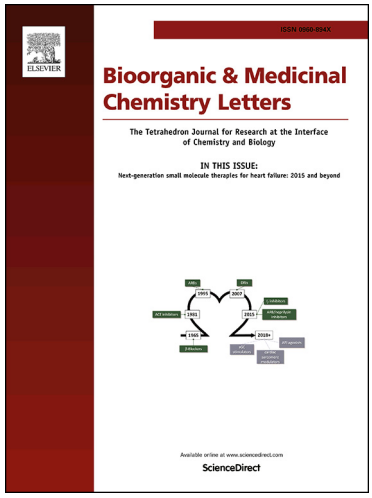

Design, synthesis and evaluation of novel, potent DNA alkylating agents and their antibody-drug conjugates (ADCs)

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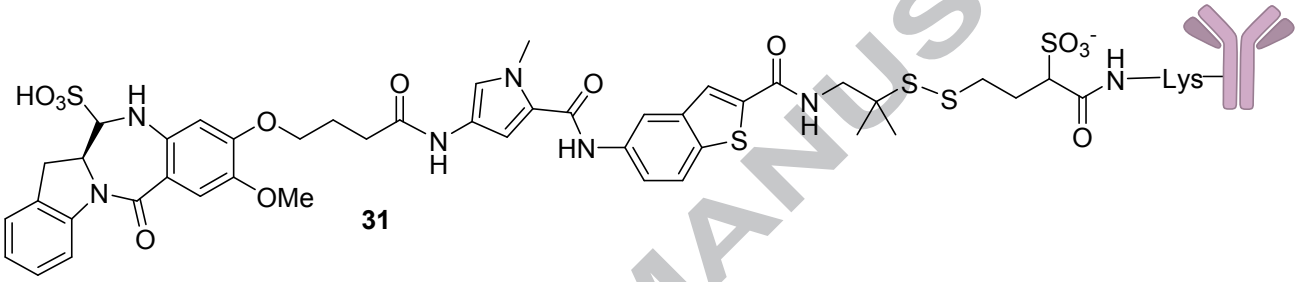

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ABSTRACT

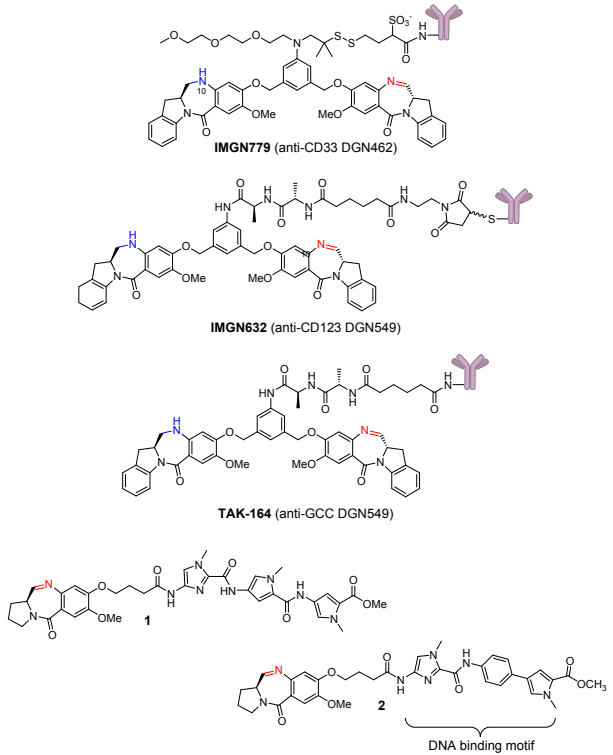
Antibody-drug conjugates (ADCs) incorporating potent indolinobenzodiazepine (IGN) DNA alkylators as the cytotoxic payload are currently undergoing clinical evaluation. The optimized design of these payloads consists of an unsymmetrical dimer possessing both an imine and an amine effectively eliminating DNA crosslinking and demonstrating improved tolerability in mice. Here we present an alternate approach to generating DNA alkylating ADCs by linking the IGN monomer with a biaryl system which has a high DNA binding affinity to potentially enhance tolerability. These BIA ADCs were found to be highly cytotoxic *in vitro* and demonstrated potent antitumor activity *in vivo*.

Antibody-drug conjugates (ADCs) link highly cytotoxic payloads to a tumor-selective monoclonal antibody in an effort to increase targeted delivery of the payload and improve its therapeutic index (TI).[1-4] The majority of clinical-stage ADCs have used tubulin-interacting agents, the maytansinoids and auristatins,[5-6] but more recently payloads with alternative mechanisms of action have emerged. These include topoisomerase 1 inhibitors, such as the camptothecins, and DNA crosslinkers, such as the pyrrolobenzodiazepine (PBD) dimers.[7-9] The anti-tumor activity of ADCs of the latter class has been limited, since clinically achievable doses have been quite low due to cumulative toxic side effects.[10-11]

We have previously reported on a potent new class of DNA alkylating indolinobenzodizepine (termed IGN) pseudodimers purposely designed to eliminate DNA crosslinking.[12-13] The controlled reduction of one of the two imine moieties present in the dimer effectively changes the mechanism of action from DNA crosslinking to DNA alkylation. These IGN ADCs show improved tolerability, with only a slight loss in potency, but were free of the delayed toxicity in mice observed for their DNA crosslinking counterparts. As a result of these efforts, multiple IGN ADCs (IMGN779, IMGN632, and TAK-164) have recently been advanced into clinical evaluation.

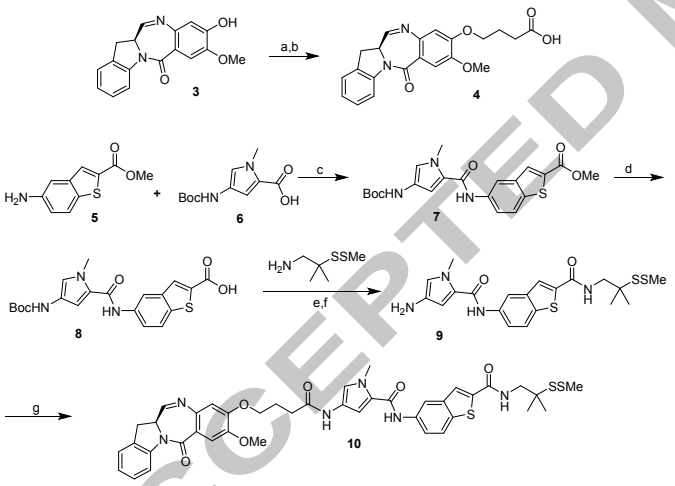
Fig. 1. Structures of DNA Alkylating IGN ADCs and representative structure of previously reported PBD-biaryl compound.

To further explore the structure-activity relationship (SAR) of IGNs and develop new IGN ADCs with a greater therapeutic index (TI), we considered replacing the IGN monomer subunit bearing the N-10 amine of our pseudodimer with a DNA binding motif.



Thurston et.al have previously described PBD monomers to which a DNA binding unit (e.g. either a polyamide (**1**) or biaryl (**2**) system) was attached. The enhanced DNA-binding affinity of these compounds resulted in PBDs which demonstrated significant *in vitro* cytotoxicity improvements in tumor cell lines versus the PBD monomer itself.[14-15] We rationalized that replacing the N-10 amine containing IGN monomer with a DNA binding motif may lead to increased DNA minor-groove interactions, greater potency, and potentially improved tolerability as an ADC over that of a DNA alkylating IGN dimer. Additionally, the removal of the amine significantly decreases the synthetic complexity associated with the unsymmetrical IGN pseudodimer. Here, we report on the design, synthesis, and preclinical evaluation of these IGN monomers linked with a DNA binding moiety (termed BIAs).

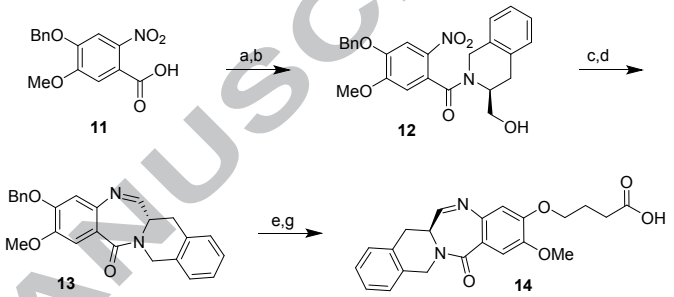
The synthesis of a typical BIA (**10**) suitable for conjugation to an antibody requires a modified IGN monomer (**4**) and a DNA binding group containing a linker handle (**9**) (Scheme 1). Carboxylic acid **4** was prepared via coupling IGN monomer **3** with ethyl 4-bromobutanoate in the presence of potassium carbonate, followed by hydrolysis of the resulting methyl ester with LiOH. The synthesis of DNA binding group **9** began with the coupling of methyl 5-aminobenzo[b]thiophene-2-carboxylate (**5**) with methyl-pyrrole carboxylic acid **6** using EDC and DMAP. Hydrolysis of methyl ester **7** to the corresponding carboxylic acid **8** followed by condensation with 2-methyl-2-methyldithio-propan-1-amine, and Boc deprotection with HCl gave the desired DNA binder **9**. Finally, carboxylic acid **4** and amine **9** were coupled in the presence of EDC and DMAP to generate BIA **10** containing a methyl disulfide in high yield.



Scheme 1. Synthesis reagents and conditions. a) ethyl 4-bromobutanoate, K_2CO_3, DMF, 100%; (b) LiOH, CH_3OH, water, 64%; (c) EDC, DMAP, DMF, 72%; (d) NaOH, CH_3OH, 96%; (e) 2-methyl-2-methyldithio-propan-1-amine**,** EDC, DMAP, DMF, 96%; (f) HCl, dioxane, 100%; (g) **4**, EDC, DMAP, DCM, 83%.

Our previous internal studies with the IGN pseudodimer series had shown that replacing the IGN monomer with a tetrahydroisoquinoline (THIQ) unit led to improved *in vitro* potency. Thus, we wanted to determine if a similar enhanced potency would be achieved in the BIA series. The synthesis of a THIQ monomer suitable for attachment of a DNA binding unit is shown in Scheme 2. Carboxylic acid **11** was converted to its corresponding acid chloride with oxalyl chloride and then coupled with (S)-tetrahydroisoquinolin-3-yl)methanol to give the nitro alcohol **12**. Reduction of the nitro group to the amine with iron powder, followed by oxidation of the primary alcohol with Dess-Martin periodinane led to spontaneous cyclization to give the protected THIQ monomer **13.** Hydrogenolysis of **13** with Pd/C followed by coupling with ethyl 4-bromobutanoate and subsequent hydrolysis of the methyl ester gave the THIQ monomer **14** containing a carboxylic acid.

Using the modified IGN monomer **4** and THIQ monomer **14** we synthesized a series of BIAs incorporating a variety of aryl subunits as shown in Figure 2. The modified monomer was coupled to a biaryl system containing a 5 membered heteroaryl (i.e., methylpyrrole, methylimidazole, thiazole) ring in place of unit 1 and a phenyl containing substituent (i.e., benzothiophene, benzofuran, methyl-3-phenylpyrrole) in place of unit 2. At the terminal end of the BIA we incorporated a side chain bearing a variety of functional groups, some of which could be converted to a moeity used for conjugation to an antibody, such as an N-hydroxysuccinimide (NHS) ester (**A**,**D**) or thiol (**B**-**C**). (See supporting information for synthesis and structures of BIAs **15-28**)



Scheme 2. Synthesis reagents and conditions. a) Oxalyl chloride, DMF, 100%; (b) (S)-tetrahydroisoquinolin-3-yl)methanol, DCM, TEA, 98%; (c) Dess-Martin Periodinane, DCM, 91%; (d) Iron powder, THF, MeOH, water, NH_4Cl, 84%; (e) Pd/C, 1,4-cyclohexadiene, EtOH, 44%; (f) ethyl 4-bromobutanoate , K_2CO_3, DMF, 100%; (g) LiOH, MeOH, 53%.

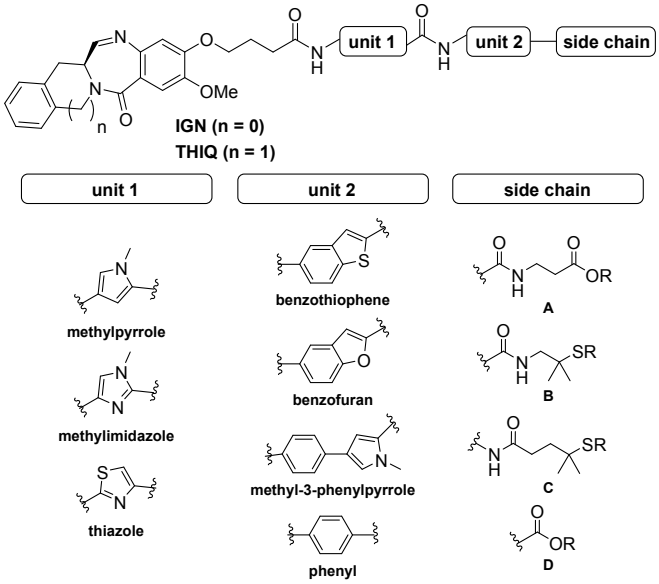


Fig. 2. Representative structures of synthesized BIAs.

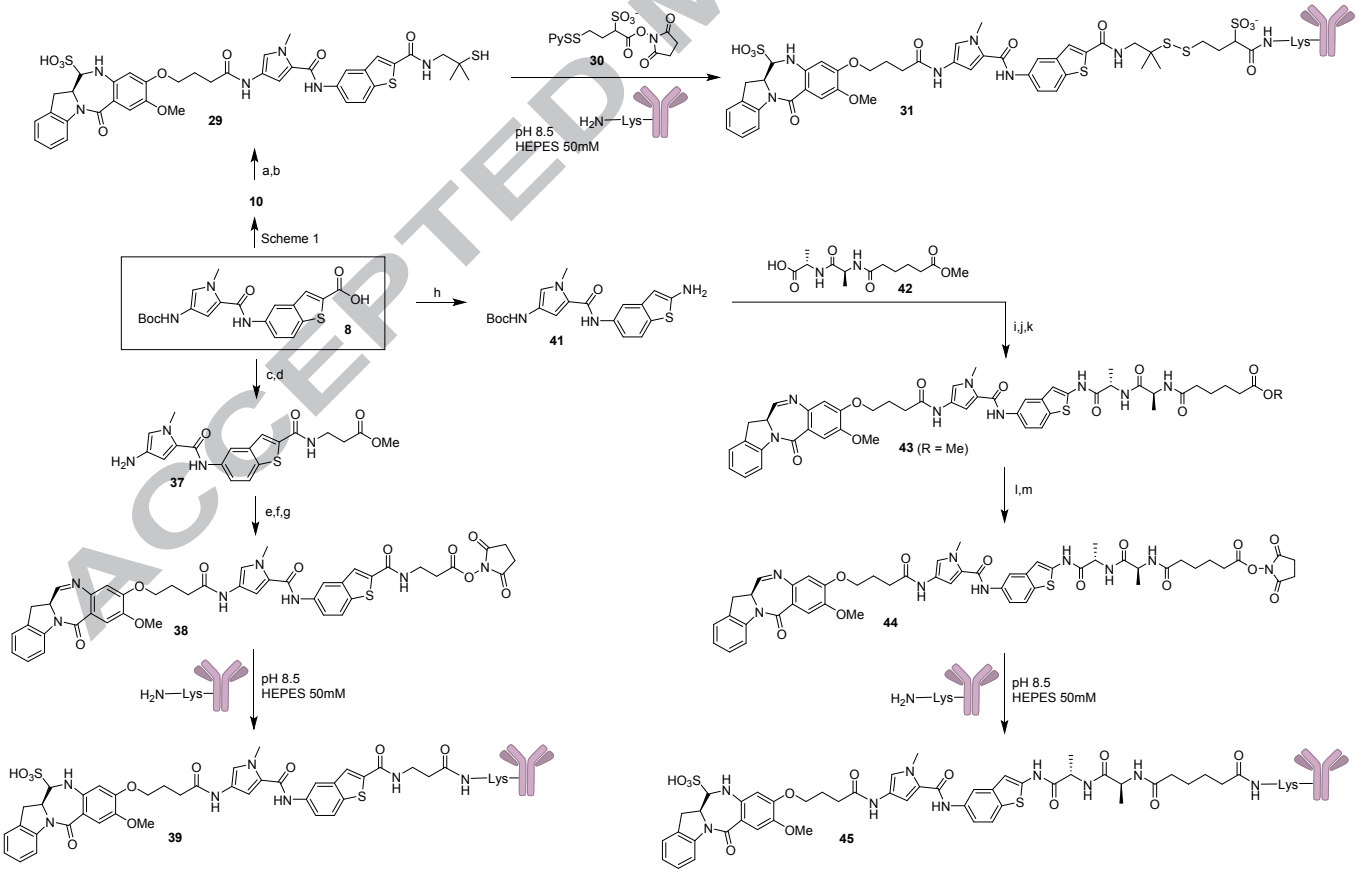
The *in vitro* potency for a representative set of BIAs against three different cell lines (KB, NCI-H2110, and Namalwa) is shown in Table 1. All BIAs (**10**, **15-28**) were found to be highly potent towards the cell lines tested. In general, potency of the BIAs was primarily due to the chosen biaryl systems (unit 1 and 2) and did not vary greatly based on the side chain that was incorporated. For example, similar potency was observed for BIA **19**, which had no side chain substituent on the terminal heterocycle, and those with the same biaryl unit but containing side chains. Incorporation of the THIQ monomer significantly increased (5-10 fold) *in vitro*

activity (compare **27** and **28**) presumably as a result of a stronger interaction with the DNA.

Table 1. Structure and *in vitro* potency of BIAs

BIA	Monomer	Unit 1[a]	Unit 2[a]	Side Chain[a]	IC_{50} pM[b]		
					KB	NCI-H2110	Namalwa
10	IGN	methylpyrrole	benzothiophene	B (R = SMe)	80	300	10
15	IGN	methylpyrrole	benzothiophene	D (R = Me)	10	50	20
16	IGN	methylpyrrole	benzothiophene	A (R = Me)	40	200	30
17	IGN	methylpyrrole	benzofuran	A (R = Me)	200	500	60
18	THIQ	methylpyrrole	benzothiophene	B (R = SMe)	5	30	2
19	IGN	methylimadazole	benzothiophene	-H	40	80	20
20	IGN	methylimadazole	benzothiophene	A (R = Me)	--	--	10
21	IGN	methylimadazole	benzothiophene	B (R = SMe)	--	--	30
22	IGN	methylimadazole	benzofuran	A (R = Me)	80	500	60
23	IGN	methylimadazole	phenyl	$-NH_2$	200	400	60
24	IGN	methylimadazole	phenyl	C (R = SMe)	30	400	30
25	IGN	thiazole	phenyl	C (R = SMe)	100	600	30
26	IGN	methylpyrrole	Methyl-3-phenylpyrrole	B (R = SMe)	10	100	30
27	IGN	methylimadazole	Methyl-3-phenylpyrrole	B (R = SMe)	40	60	10
28	THIQ	methylimadazole	Methyl-3-phenylpyrrole	B (R = SMe)	6	10	1

[a]See Figure 2 for definition. [b]Cancer cell lines were incubated with BIAs for 5 days at 37°C. IC_{50} values were determined using a WST-based cell viability assay.



Scheme 3. Synthesis reagents and conditions. a) TCEP, 100%; (b) $Na_2S_2O_5$, isopropanol, water, 13%; (c) β-alanine-OMe, EDC, DMAP, DMF, 73%; (d) HCl, dioxane, 94%; (e) **4**, EDC, DMAP, DCM, 65%; (f) Me_3SnOH, DCE, 100%; (g) N-hydroxysuccinimide, EDC, DMAP, DMF, 28%; (h) DPPA, TEA, DMF, 49%; (i) **42**, HATU, DIEA, DMF, 59%; (j) HCl, dioxane, 100%; (k) **4**, EDC, DMAP, DCM, 24%; (l) LiOH, water, THF, 100%; (m) N-hydroxysuccinimide, EDC, DMAP, DMF, 30%.

We selected DNA binding motif **8** for evaluating BIA ADCs with different linker types (disulfide, non-cleavable amide, and dipeptide**)**. Methyl disulfide **10**, prepared from **8**, was treated with TCEP to generate the free thiol followed by reversible sulfonation

of the imine (**29**) using sodium metabisulfite. The introduction of the sulfonate was found to enhance the aqueous solubility of the BIA and facilitate conjugation via lysine residues. Conjugation was performed through *in situ* treatment of **29** with sulfo-SPDB (**30**) and addition to an anti-FRα antibody resulting in BIA anti-FRα ADC **31**. Disulfide linked anti-FRα ADCs **32-36** were prepared in a similar fashion.

A second conjugation approach involved converting the free acid of intermediate **8** to an NHS ester. This was accomplished by treating **8** with the methyl ester of β-alanine under EDC/DMAP conditions, followed by Boc deprotection with HCl to give the free amine **37**. Coupling of **37** with the modified IGN monomer **4**, followed by hydrolysis of the terminal methyl ester and treatment with N-hydroxysuccinimide, in the presence of EDC/DMAP, generated the desired NHS ester **38**. *In situ* sulfonation of the imine in **38**, using sodium metabisulfite, followed by conjugation with an anti-FRα antibody via lysine residues as described previously gave the direct amide linked anti-FRα ADC **39.** Anti-FRα ADC **40** was prepared in a similar fashion.

A third method of conjugation was via a cleavable dipeptide bond generating an amine catabolite with the potential to have bystander activity. A Curtius rearrangement of intermediate **8** with diphenylphosphoryl azide (DPPA) gave amino benzothiophene **41** in good yield. Coupling of **41** with Ala-Ala methyladipate (**42**) in the presence of HATU, followed by HCl deprotection of the Boc group and coupling to IGN monomer **4** gave BIA methyl ester **43**. Hydrolysis with LiOH and conversion to the desired NHS ester **44** allowed for conjugation to an anti-FRα antibody, after *in situ* sulfonation with sodium metabisulfite, to give anti-FRα ADC **45**. Anti-FRα ADC **46** was prepared in a similar fashion. All BIA ADCs, regardless of conjugation method, were found to have an average of 2.3 – 3.1 BIAs per antibody, were >97% monomeric by SEC and contained <0.5% unconjugated BIA (free drug).

Table 2. *In vitro* potency of BIA ADCs

ADC Anti-FRα	Linked BIA	Linker	DAR	IC_{50} pM KB (3,000k)[a]	IC_{50} pM T47D (100k)[a]
31	**10**	B	2.8	40	200
32	**18**	B	2.9	4	30
33	**21**	B	2.5	30	100
34	**26**	B	2.7	30	2000
35	**27**	B	2.6	100	1000
36	**28**	B	2.3	7	60
39	**16**	A	3.1	20	30
40	**20**	A	2.9	10	10
45	**37**	peptide	3.1	5	400
46	**23**	peptide	2.5	20	>3000

[a]Number of antibody molecules bound per cell.

The anti-FRα ADCs **31-36**, **39-40** and **45-46** were evaluated *in vitro* against two cell lines which expressed either a high (KB) or low (T47D) number of folate receptors. As shown in Table 2, all anti-FRα ADCs were found to be highly potent towards the higher expressing KB cells. There was a much wider range of activity towards the lower expressing T47D cells, as a few anti-FRα ADCs (**34**, **35**, **46**) were considerably less active though there is no clear reason to account for this observation. As with the free BIAs (Table 1) the anti-FRα ADCs possessing a THIQ monomer were found to be more potent (~10-fold) compared with their IGN monomer containing counterparts (i.e., **31** vs **32**). Interestingly, in the anti-FRα ADCs containing a methyl-3-phenylpyrrole group in unit 2 (**34-36**) the THIQ monomer was found to have a significant impact on potency towards the lower antigen expressing T47D cells line, being up to 32-fold more potent as compared with anti-FRα ADC **31**.

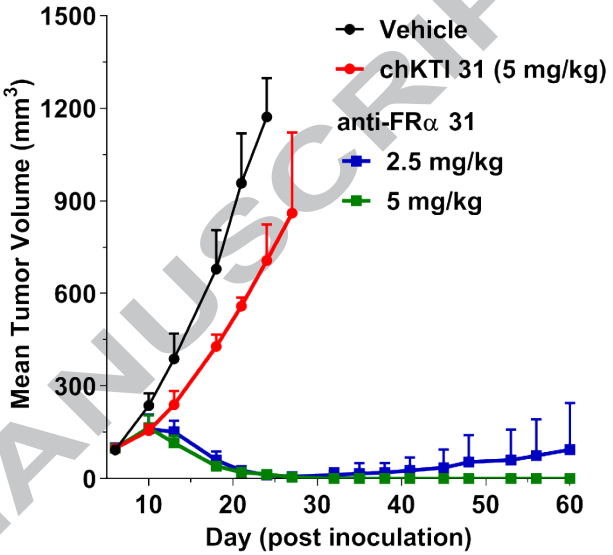


Fig. 3. *In vivo* antitumor activity of anti-FRα ADC **31** and chKTI-ADC **31** in a cervical KB xenograft in SCID mice.

Encouraged by the potency of a number of FRα BIA ADCs, we selected anti-FRα ADC **31** for *in vivo* evaluation. Treatment of SCID mice bearing subcutaneous KB cervical tumor xenografts with a single i.v. dose of 2.5 mg/kg ADC (equivalent to 50 μg/kg linked BIA) resulted in significant tumor growth delay resulting in 6/6 PRs and 4/6 CRs, while a dose of 5 mg/kg ADC produced complete tumor regressions in all mice lasting more than 60 days (duration of the experiment). In addition, the non-targeting chKTI ADC **31** was inactive even at the higher dose of 5 mg/kg indicating the antitumor activity was antigen specific (Figure 3).

In conclusion, we have prepared a series of highly potent BIAs containing either an IGN or THIQ modified monomer, with a variety of biaryl units that demonstrate a high binding affinity for DNA. A subset of these BIAs were converted and conjugated, *via* antibody lysine residues, to an anti-FRα antibody. All of these anti-FRα BIA ADCs were found to be highly potent *in vitro* on the high expressing KB cell line whereas a wider range of activity was observed for the lower expressing T47D cell line. Anti-FRα ADC **31** demonstrated antigen-specific highly potent antitumor activity at the lowest dose tested of 2.5 mg/kg. These data confirm that potency and antitumor activity can be achieved through the replacement of the N-10 amine of an IGN dimer with a biaryl DNA binding moiety. We are continuing to explore these BIAs in an effort to further expand the TI and utility of this class of molecules.

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Supplementary Material

Supplementary data (synthetic details, experimental details, and conjugation methods) associated with this artcle can be found, in an online version, at .