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Novel PIKK inhibitor antibody-drug conjugates: Synthesis and anti-tumor activity

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ABSTRACT

Novel neolymphostin-based antibody-drug conjugate (ADC) precursors were synthesized either through amide couplings between both cleavable and non-cleavable linkers and neolymphostin derivatives, or through Cu(I)-catalyzed acetylene-azide click cycloadditon between non-cleavable linkers and neolymphostin acetal derivatives. These precursors were site-specifically conjugated to cysteine mutant trastuzumab-A114C to provide neolymphostin-based ADCs. Preliminary *in vitro* data indicated that the corresponding ADCs were active against HER2-expressing tumor cell lines, thus providing a proof-of-concept for using neolymphostin as ADC-based anticancer agents.

Neolymphostin (**1**), a novel pyrrolo[4,3,2-*de*]quinolin-8-amine,[1] exhibits potent *in vitro* inhibitory activity against several phosphoinositide-3-kinase related kinases (PIKKs),[2] such as SMG1, PI3Ka, PI3Kγ, mTOR and ATR which regulate cell growth, cell proliferation, cell motility, DNA damage detection and repair, and protein synthesis and are thus implicated in a variety of cancer pathways. Neolymphostin also displays potent antiproliferative activity in cellular screens; however, achieving potent in vivo activity has been hampered by low bioavailability that is likely related to the poor physicochemical properties of members of this series. To overcome this problem, we have explored strategies which include the development of neolymphostin-based antibody-drug conjugates (ADCs), a strategy of using an antibody to target a cytotoxic agent to cancer cells.[3] As the first step toward achieving this objective, neolymphostin (**1**) was chosen to be attached to a maleimide-containing linker, which can be conjugated to free thiol groups that are either introduced on monoclonal antibodies (mAbs) through engineering (leading to site specific conjugates with maximum addition of 2 payloads per antibody) or generated by reduction of pre-existing disulfides (producing conventional or random conjugates with maximum addition of 8 payloads per antibody).

Fig. 1 illustrates our two initial approaches for attaching neolymphostin (**1**) to linkers suitable for conjugation. In approach (A), we sought to attach the payload to a protease sensitive dipeptide-*p*-aminobenzyl-carbamate (PABC)[4] linkage through the amino group at the C8 position. This particular amino group is involved in key hinge-region interaction with the targeted kinases, so employment of a self-immolative linkage that releases a free unsubstituted amine was considered to be essential. Approach (B) envisions that the payload is attached to the backbone of a linker through the acetal formation at the C11 position. In this case, the released payload would likely contain vestiges of the bound linker as an acetal-based prodrug of neolymphostin.

We began our investigation by attempting to incorporate a dipeptide-PABC linker to the neolymphostin nitrogen at the C8 position (Fig. 1, approach A). Activation of the maleimide-bearing valine-citruline (Val-Cit) *p*-aminobenzylalcohol linker **2**[5] was achieved by reaction with *p*-nitrophenyl carbonate (PNP) to generate **3** (Scheme 1). However, subsequent treatment of **3** with neolymphostin (**1**) in the presence of diisopropylethylamine at room temperature overnight only led to recovery of the starting material. Raising the reaction temperature to 50 °C, extending the reaction time to 24 h, and adding various additives (e.g. HOBt, 4-DMAP) did not drive the formation of the desired carbamate **4**.

We next attempted treatment of **2** with diphosgene/pyridine in

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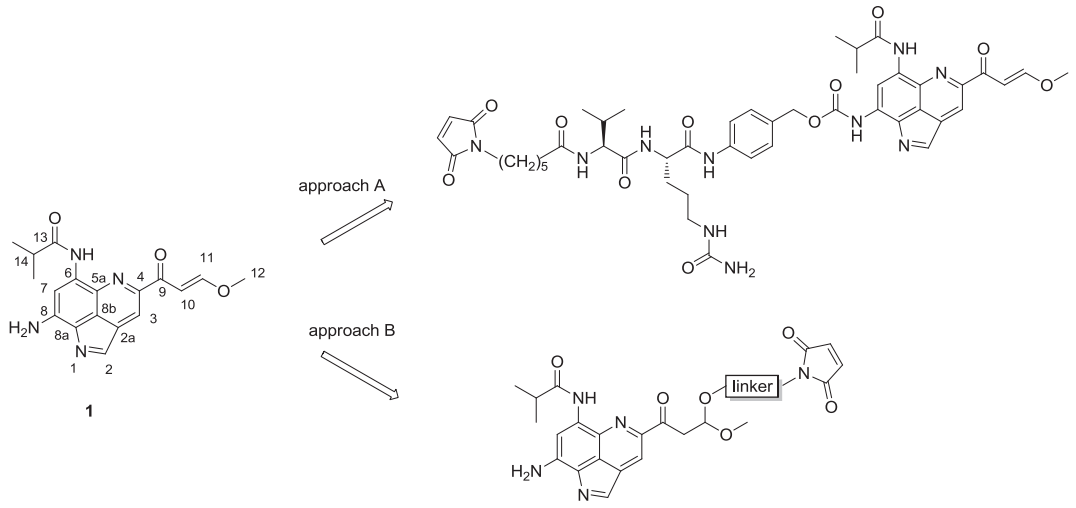
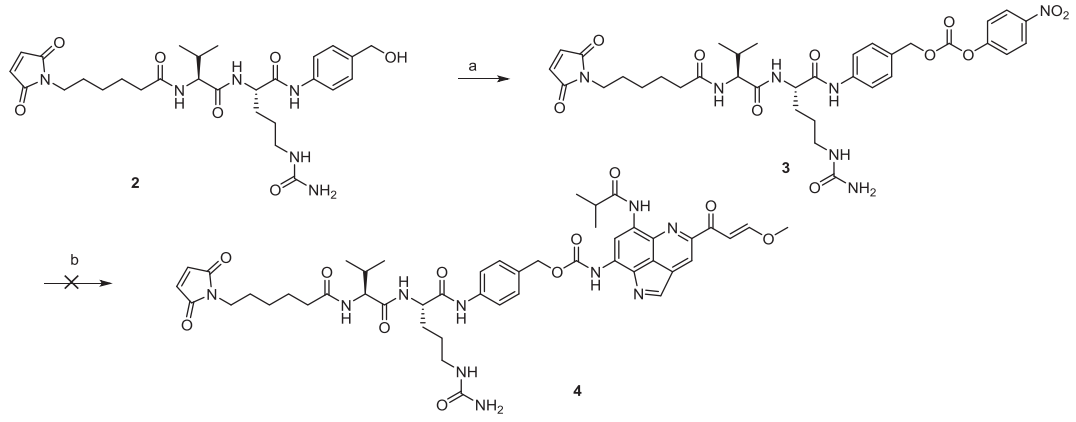


Fig. 1. Neolymphostin (**1**) and proposed approaches toward the conjugation of **1** with different linkers.



Scheme 1. [a]Reagents and conditions: a) *p*-nitrophenyl carbonate, DIPEA, DMF; (b) neolymphostin, DIPEA, DMF.

methylene chloride at – 42 °C[6] and reaction of the resulting crude dipeptide-PABC chloroformate with neolymphostin, but again the amino group at the C8 position proved to be too un-reactive and the desired conjugation precursor **4** was not formed in usable yields.

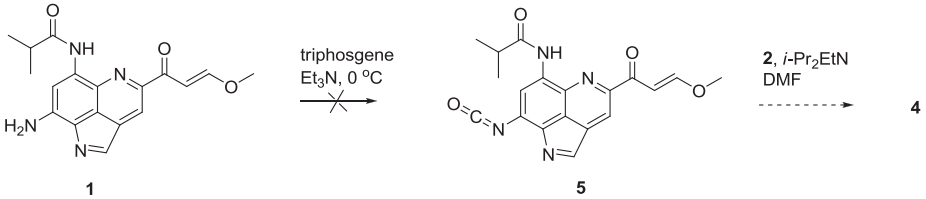
In light of the difficulties encountered in functionalizing the amino group at the C8 position, conversion of neolymphostin (**1**) to the corresponding isocyanate by treatment with triphosgene was attempted (Scheme 2). However, clean formation of **5** proved elusive; reactions with triphosgene led only to decomposition. Several conditions to generate isocyanate **5** and its synthetic equivalents (e.g. the acyl imidazole and triazole) were also attempted but were unsuccessful.

The markedly low nucleophilicity of the electron deficient aryl amine moiety at the C8 position as well as the fragility of the pyrrolo [4,3,2-*de*]quinoline skeleton provided the impetus to turn our attention on the β-methoxy enone moiety as an alternative site for attachment of a suitable linker.

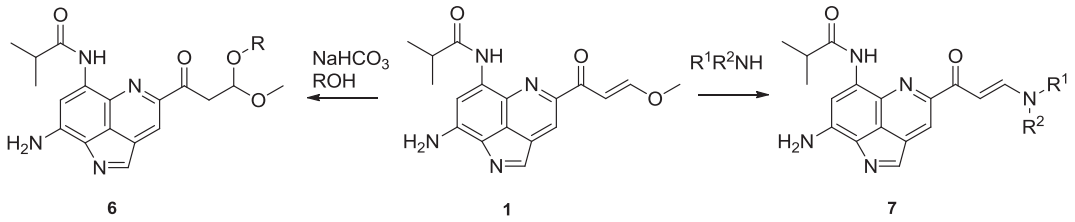
Previous work performed by our colleagues[7] indicated that the β-methoxy enone of neolymphostin (**1**) can be derivatized as the mixed acetals **6** (R = various, $NaHCO_3$, ROH as a solvent) or the vinylogous amides **7** (R^1R^2 = various, 1.2 eq of R^1R^2NH), and these reactions were known to be both general and reasonably efficient (Scheme 3). The resulting acetals **6** typically retained significant activity *in vitro* whereas the vinylogous amide products **7** were typically significantly less potent than **1** in an *in vitro* screen of kinase and cellular activity.[7] Despite the fact that these transformations led to somewhat less potent cytotoxic payloads when compared to **1**, we sought to capitalize on these facile functional group transformations to provide derivatives of neolymphostin linked to both cleavable and non-cleavable linkers. We hoped that we could quickly gain insights into the nature and activities of neolymphostin-based ADCs.

To this end, incorporation of a N^1,N^2-dimethyl ethylenediamine (DMEA) spacer[8] into the cleavable linker, mc-Val-Cit-PABC, was pursued. As depicted in Scheme 4, treatment of mc-Val-Cit-PABC-PNP **3** with Boc protected N^1,N^2-dimethylethane-1,2-diamine and subsequent Boc deprotection afforded mc-Val-Cit-PABC-DMEA **8**. Reaction of neolymphostin (**1**) with linker **8** in the presence of Hunig's base cleanly provided neolymphostin conjugate precursor **9** in a single step.

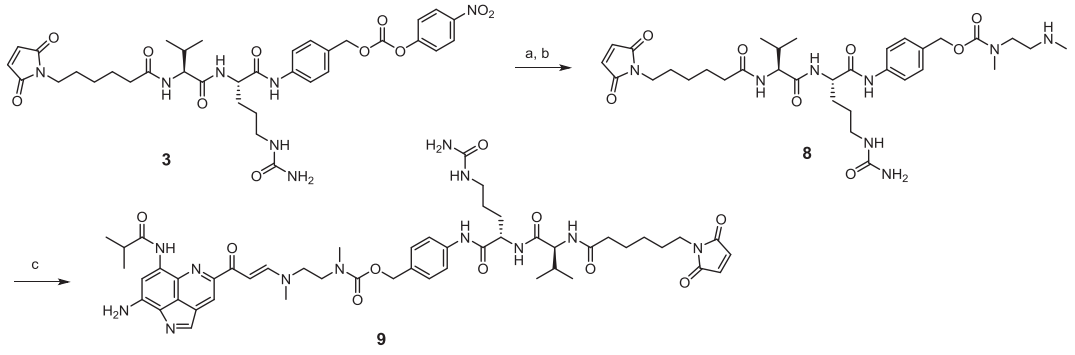
Neolymphostin (**1**) linked to a non-cleavable linker was also



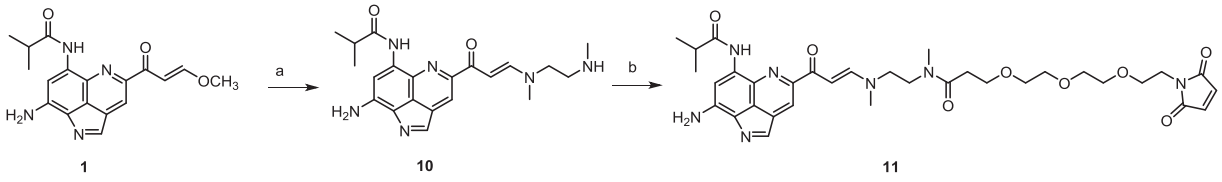
Scheme 2. [a]Reagents and conditions: (a) triphosgene, Et_3N, 0 °C; (b) DIPEA, DMF.



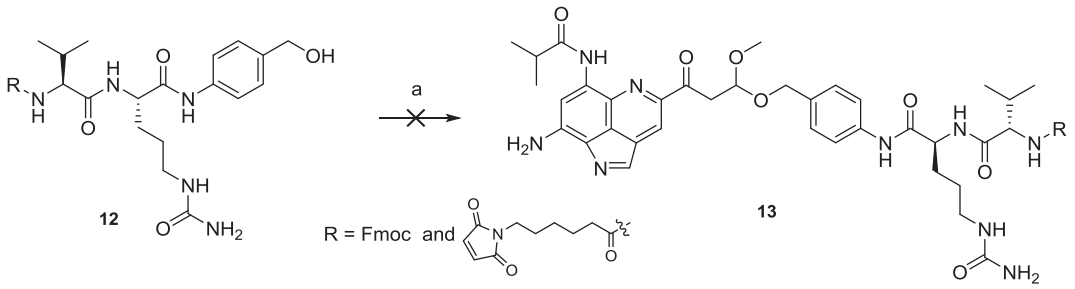
Scheme 3. [a]Reagents and conditions: (a) $NaHCO_3$, ROH; (b) R^1R^2NH.



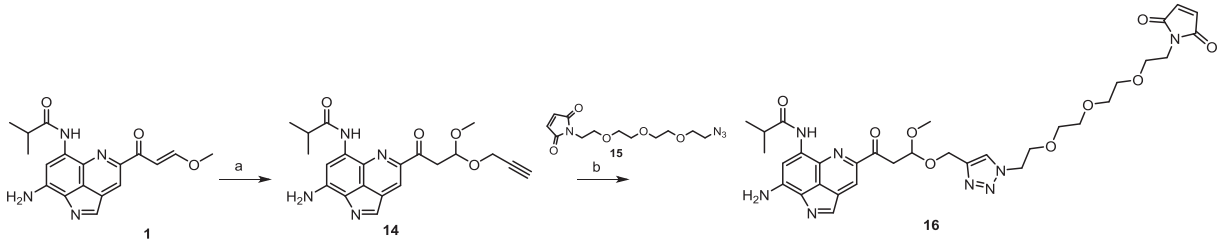
Scheme 4. [a]Reagents and conditions: (a) Boc-dimentylethylenediamine, DMF; (b) TFA; (c) neolymphostin, DMSO.



Scheme 5. [a]Reagents and conditions: (a) N^1,N^2-dimethyl ethylenediamine, DMSO; (b) mc-Peg3-propanoic acid, HATU, DIPEA, DMF.



Scheme 6. [a]Reagents and conditions: (a) Neolymphostin, $NaHCO_3$, DMF.

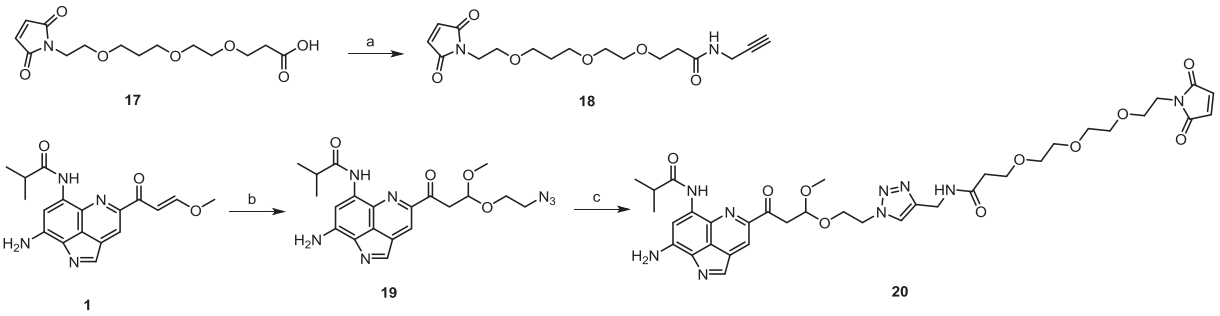


Scheme 7. [a]Reagents and conditions: (a) prop-2-yn-1-ol (neat), $NaHCO_3$; (b) **14**, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF.

prepared according to Scheme 5. Treatment of neolymphostin (**1**) with DMEA followed by amide coupling afforded a non-cleavable linker payload **11**.

In order to investigate the effects of neolymphostin acetal ADCs on *in vitro* potency against high and low HER2 expressing cell lines, we next turned our attention to the acetal scaffold **6**.

Our initial focus involved the construction of neolymphostin linker-payload **13** via direct acetal formation, in which a dipeptide-PABC linker **12** serves as an alcohol source (Scheme 6). However, all attempts to effect acetal formation using a number of reaction conditions proved unsuccessful, as this reaction requires large excesses of alcohol components that are typically used as a solvent. These results prompted us to revise our synthetic strategy and seek a stepwise approach to attach neolymphostin (**1**) to a linker.



Scheme 8. [a]Reagents and conditions: (a) propargylamine, HATU, $^{i}Pr_2EtN$; (b) 2-azidoethan-1-ol (neat), $NaHCO_3$; (c) **18,** $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF.

Table 1
Characterization of ADCs.

ADC	Linker-payload	DAR (drug/antibody)	Theoretical Δ mass	Observed Δ mass	Yield after purification
21	9	1.6	993	994	38%
22	11	1.6	678	678	54%
23	16	1.9	693	695	66%
24	20	17	764	765	52%

In searching for a practical method to conjugate neolymphostin (**1**) with a linker, we became interested in utilizing the copper (I)-catalyzed Huisgen cycloaddition of azides and terminal alkynes to form 1,2,3-triazoles[9] as the key transformation to construct a linker-payload. We envisioned that the generality and mild reaction conditions of this approach would be suitable for such a labile payload **1**.

To this end, we examined the feasibility of preparing neolymphostin alkyne **16** and using it in the Cu(I)-catalyzed reaction with PEGylated maleimide azide **15**. As shown in Scheme 7, the alkyne bearing neolymphostin substrate **14** was readily obtained using sodium bicarbonate in neat propynyl alcohol. However, the azide component **15**, prepared by reacting 11-azido-3,6,9-trioxaundecan-1-amine with *N*-(methyoxycarbonyl)maleimide, was unstable at room temperature. Azide-acetylene cycloaddition reaction was nonetheless carried out using copper sulfate and sodium ascorbate under ambient temperature within 15 min, but the desired linker payload **16** was only isolated in less than 10% yield (presumably due to the instability of **15**).

The poor reaction yield and unstable maleimido-azide partner led us to consider an alternative route wherein a maleimide-containing alkyne would be employed as a acetylenic counterpart in combination with suitable neolymphostin-azide for the Cu(I)-mediated "click" cycloaddition (Scheme 8).

The requisite PEGylated maleimido-alkyne **18** was prepared by reaction of propargyl amine with acid **17** in the presence of HATU. Although the reaction of neolymphostin (**1**) with neat 2-azidoethanol in the presence of sodium bicarbonate was sluggish, the resulting neolymphostin azide **19** was isolated in good yield and readily underwent Cu(I)-catalyzed [3 + 2] cycloaddition with acetylene **18** to afford neolymphostin-based linker-payload **20**.

The neolymphostin-based ADC precursors, linker-payloads **9**, **11**, **16** and **20,** were then conjugated to site-specific cysteine mutant trastuzumab-A114C by 1,4-addition across the maleimide. The crude ADCs were purified by size exclusion chromatography (*SEC*) using GE AKTA Explorer system with GE Superdex 200 (10/300 GL) column and DPBS (pH7.4) eluent. A mean drug-antibody ratio (DAR) of ADCs was determined by analytical LC-MS method. The results were listed in Table 1.

ADCs **21–24** were evaluated for cellular cytotoxicity. Cytotoxicity assays were conducted as reported previously.[10] Briefly, HER2 antigen expressing cells (BT474 breast cancer, N87 gastric cancer, MDA-MB-361-DYT2 breast cancer) or non-expressing cells (MDA-MB-468 breast cancer) were seeded in 96-well cell culture plates for 24 h before treatment. Cells were treated with 3-fold serially diluted antibody-drug conjugates or free compounds (i.e., no antibody conjugated to the drug) in duplicate at 10 concentrations. Cell viability was determined by CellTiter 96® AQueous One Solution Cell Proliferation MTS Assay (Promega, Madison, WI) 96 h after treatment. Relative cell viability was determined as percentage of untreated control. IC_{50} values were calculated using four parameter logistic model 203 with XLfit v4.2.

As shown in Table 2, the ADCs **21** and **22** displayed moderate cytotoxic potency against high HER2 (BT-474 and N87) expressing cell lines in the range of 77 to 423 nM. Moreover, both ADCs showed > 6-fold selectivity vs non-HER2 expressing cells (MDA-MB-468) compared with the neolymphostin (**1**) payload alone. ADC **23** was approximately as potent against BT-474 and N87 cell lines (IC_{50} = 286 nM and 274 nM, respectively) when compared to neolymphostin (**1**) alone (IC_{50} = 97 nM and 210 nM, respectively). ADC **24** demonstrated cytotoxic activity against BT474 and N87 cell lines with IC_{50} of 195 and 202, respectively. Selectivity of > 4-fold was observed with trastuzumab ADCs **23** and **24** vs non-HER2-expressing MDA-MB-468 cells compared with payload **1** alone. Furthermore, ADCs **23** and **24** demonstrated comparable *in vitro* cytotoxic activity against medium HER2 expressing MDA-MB-261-DYT2 cell line (IC_{50} of 162 and 216, respectively) compared with high HER2 (BT-474 and N87) expressing cell lines, providing additional evidence of the attractiveness of ADC-based

Table 2
In vitro cytotoxicity of neolymphostin ADCs.[a]

ADC	Linker-payload	DAR	IC_{50} (nM)			
		(drug/antibody)	HER2 + + + BT474	HER2 + + + N87	HER2 + + MDA-MB-361-DYT2	HER2-negative MDA-MB-468
–	1	–	97.5(8)	210(10)	92.9(3)	139(6)
21	9	1.6	77.3(4)	225(5)	ND	> 1000(6)
22	11	1.6	423(3)	251(3)	> 1000(1)	> 1000(1)
23	16	1.9	286(3)	274(3)	162(1)	> 1000(2)
24	20	1.7	195(4)	202(1)	216(2)	> 750(2)

[a] Relative antigen expression is classified as high (+ + +), medium (+ +), and low (−). Reported IC50 is the mean of the indicated number of independent determinations (parentheses).

targeted delivery of neolymphostin derivatives.

In summary, we have developed two synthetic methods for the preparation of neolymphostin-based ADC precursors for site-specific cysteine mutant trastuzumab-A114C conjugation. These methods capitalize on efficient chemistries employed for the preparation of vinylogous amide and acetal derivatives of the parent system **1** and have effectively overcome issues surrounding the tractability of the neolymphostin series as a potential ADC payload. Despite the fact that these derivatives are known to display reduced potency vs. the free payload **1**, we have demonstrated that ADCs that deliver these agents possess potent activity and selectivity in cancer cell lines. This work therefore provides a path forward to overcome the poor pharmacokinetics of the parent small molecule such that these PIKK inhibitors may prove useful as ADC-based anticancer agents.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.01.009.

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