Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Incorporation of Phosphonate into Benzonaphthyridine Toll-like Receptor 7 Agonists for Adsorption to Aluminum Hydroxide

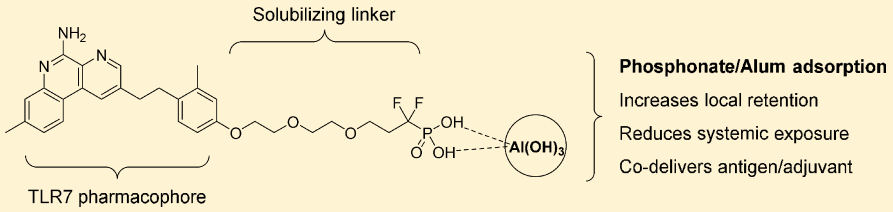
Alex Cortez,[†] Yongkai Li,[†] Andrew T. Miller,[†] Xiaoyue Zhang,[†] Kathy Yue,[†] Jillian Maginnis,[†] Janice Hampton,[†] De Shon Hall,[†] Michael Shapiro,[†] Bishnu Nayak,[†,∥] Ugo D'Oro,[§] Chun Li,[†] David Skibinski,[§] M. Lamine Mbow,[‡,⊥] Manmohan Singh,[‡] Derek T. O'Hagan,[‡] Michael P. Cooke,[†] Nicholas M. Valiante,[‡,#] and Tom Y.-H. Wu*[,†]

[†]Genomics Institute of Novartis Research Foundation, 10675 John Jay Hopkins Drive, San Diego, California 92121, United States

[‡]GSK Vaccines, 45 Sydney Street, Cambridge, Massachusetts 02139, United States

[§]GSK Vaccines, Via Florentina, 1, 53100, Siena, Italy

Supporting Information



ABSTRACT: Small molecule Toll-like receptor 7 (TLR7) agonists have been used as vaccine adjuvants by enhancing innate immune activation to afford better adaptive response. Localized TLR7 agonists without systemic exposure can afford good adjuvanticity, suggesting peripheral innate activation (non-antigen-specific) is not required for immune priming. To enhance colocalization of antigen and adjuvant, benzonaphthyridine (BZN) TLR7 agonists are chemically modified with phosphonates to allow adsorption onto aluminum hydroxide (alum), a formulation commonly used in vaccines for antigen stabilization and injection site deposition. The adsorption process is facilitated by enhancing aqueous solubility of BZN analogs to avoid physical mixture of two insoluble particulates. These BZN-phosphonates are highly adsorbed onto alum, which significantly reduced systemic exposure and increased local retention post injection. This report demonstrates a novel approach in vaccine adjuvant design using phosphonate modification to afford adsorption of small molecule immune potentiator (SMIP) onto alum, thereby enhancing co-delivery with antigen.

INTRODUCTION

Currently, several adjuvants have been approved for use in human vaccines. According to O'Hagan and De Gregorio, vaccine adjuvants can be classified into particulates/emulsions (generation 1) or synthetic component that activates the immune system (generation 2).[1] Alum is a mineral salt used in many commercial vaccines.[2] It is believed that alum particulates increase the effectiveness of vaccines by inducing local inflammation to help recruit antigen-presenting cells to the injection site, in addition to stabilizing the antigen and creating a depot at the injection site.[3] MF59 is a water-in-oil emulsion licensed for use in flu vaccine. Its mechanism of action appears to be the creation of a transient "immunocompetent" local environment at the injection site, resulting in the recruitment of key immune cells for antigen uptake.[4] AS04 is composed of alum and monophosphoryl lipid A (MPLA) and is used in vaccines against hepatitis B virus and human papilloma virus.[5] MPLA is a detoxified TLR4 agonist derived from cell wall lipopolysaccharide (LPS) of Gram-negative bacteria.[6]

Proteins can adsorb onto alum through hydrophobic interaction, electrostatic attraction, and ligand exchange.[3] Of these interactions, ligand exchange through phosphates has been determined to afford the strongest adsorption. We thought to leverage this physical interaction to create a small molecule adjuvant that can be adsorbed onto alum, as well as the antigen, to maximize antigen/adjuvant co-delivery. Recently, we have described the rational design of a series of BZN TLR7 agonists for use as vaccine adjuvants.[7] In this report, we disclose the BZN pharmacophore modified with phosphonate group for adsorption onto aluminum hydroxide $Al(OH)_3$ (Figure 1). Structure−activity relationship, alum adsorption study, and impact on pharmacokinetics (PK) are described.

CHEMISTRY

On the basis of our understanding of BZN TLR7 agonist structure−activity relationship, chemical modification was focused at the C8 position on the core (R_1) and at the C4′ position on the phenethyl ring (R_2). Preparation of

Received: April 1, 2016

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

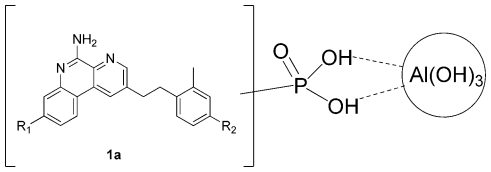


Figure 1. Benzonaphthyridine TLR7 pharmacophore modified with phosphonate group for adsorption onto aluminum hydroxide through ligand exchange.

phosphonates at R_1 was described in Schemes 1 and 2. The previously described benzyl alcohol **1b**[8] was oxidized to the aldehyde **2**, which then underwent an Abramov reaction with tris(trimethylsilyl) phosphite followed by another oxidation to yield keto-phosphonate **3** (Scheme 1). Aldehyde **2** can also react with lithiated difluoromethyl phosphonate ester to generate **4**, which is further oxidized and deprotected to give α-difluoro keto-phosphonate **6**. To prepare alkyl phosphonates (Scheme 2), Horner−Wadsworth−Emmons reactions were carried out using either methylene bis-phosphonate ester or fluoromethylene bis-phosphonate ester with aldehyde **2** to yield exclusively the (*E*)-alkenes (**7** and **8**). These intermediates can be deprotected to give the unsaturated phosphonic acids **9** and **10** or hydrogenated and deprotected to give saturated phosphonic acid **12**.

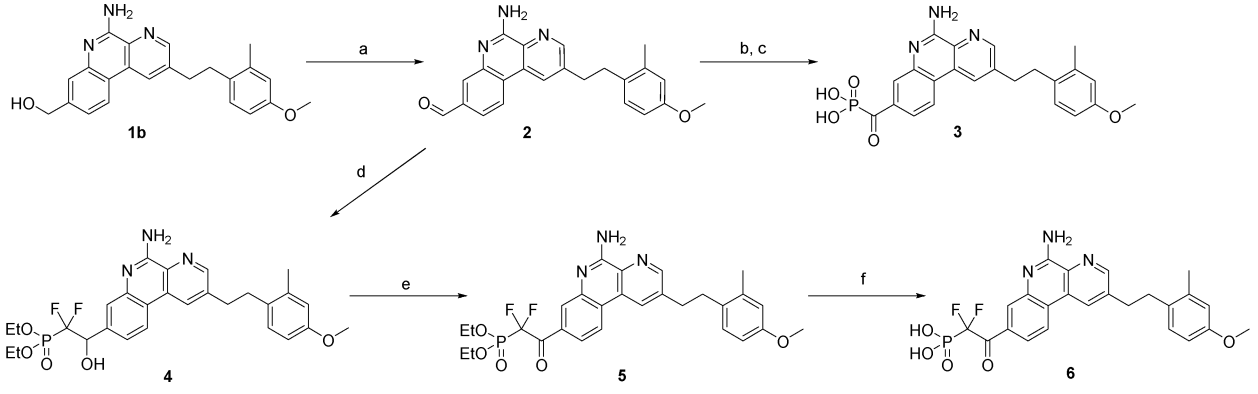
The BZN core was modified at R_2 by alkylation of the previously described phenol **13**[8] with various alkyl/benzyl halides containing phosphonate or phosphonate precursor (Scheme 3). The alkylation reaction generally tolerated internal ethylene glycol units and the presence of α-fluorine atoms next to the phosphonate. Phosphonate on **15** was introduced via a Pd-mediated cross-coupling of triethyl phosphite with aryl iodide **14**.[9] Deprotection using trimethylsilyl bromide in dichloromethane served as a general condition to afford the final phosphonic acids **15**, **16a−d**.

To improve the aqueous solubility of BZN-phosphonate, a propionic acid side chain was installed at R_1. This was achieved by first preparing the boronic ester **22** in three steps starting from Boc-protected 5-bromo-2-chloroaniline **17** (Scheme 4). Chemoselective hydrogenation of the alkene on **19** without reducing the aryl chloride was achieved using Wilkinson's catalyst. Boronic ester **22** was coupled with functionalized 3-chloropicolinonitrile **27** using Pd-mediated cross-coupling (Scheme 5). This was followed by hydrogenation of the triple bond and deprotection of the MOM group at R_2 to afford phenol **30**, which was subjected to further derivatization with various phosphonate-containing alkyl halides. Alkylation generally proceeded smoothly with either sodium hydride or cesium carbonate as the base. Phosphonate ester deprotection with trimethylsilyl bromide, followed by carboxylic ester hydrolysis with aqueous sodium hydroxide, afforded phosphonic acids **31a−d**. The high polarity of these final phosphonates generally required the use of reverse-phase HPLC for purification.

RESULTS AND DISCUSSION

Although proteins bearing phosphate groups (OPO_3H_2) were known to have ligand exchange interaction with aluminum hydroxide,[3] we hypothesized that phosphonate (CPO_3H_2) would retain high affinity toward alum but that C−P bond was more stable than O−P bond toward biological phosphatase hydrolysis. SAR studies were carried out around 2-(phenethyl)-8-methylbenzonaphthyridine (**1a**), a TLR7 agonist pharmacophore previously described by our lab.[7,8] Phosphonate groups were introduced on either R_1 or R_2 and tested in human embryonic kidney (HEK) TLR7 reporter, IL-6 mouse splenocyte, and IL-6 human peripheral blood mononuclear cell (hPBMC) assays. For R_1 substitution, carbonyl (**3**, **6**), alkenyl (**9**, **10**), and alkyl phosphonates (**12**) all showed appreciable activity across the assays (Table 1). Compound **12** suggested that a two-carbon alkyl chain is an optimal spacer between the pharmacophore and the R_1 phosphonate. Phosphonate installation at R_2 position generally involved O-alkylation of intermediate **13** or **30**. Different methylene units (CH_2) or ethylene glycol units [$(CH_2)_2O$] were explored as linkers between the benznonaphthyridine pharmacophore and R_2 phosphonate groups. Switching from R_1 = methyl to propionic acid generally boosted activity of corresponding R_2 analogs (**31a** vs **16a**, **31b** vs **16b**, **31c** vs **16c**, **31d** vs **16d**). It is unclear whether the length of R_2 linkers has a direct impact on the in vitro activity. Since phosphonate analogs were more soluble in water as the sodium salt than in DMSO, all in vitro assays were performed using 10 mM aqueous stock solutions of the monosodium salt (prepared in situ by adding 1 equiv of 1 N NaOH). In general, there is a 2- to 10-fold upward EC_{50} shift from HEK-TLR7 reporter cell line to primary cell assays, but most analogs still exhibit activities in the micromolar range (Table 1). The percentage of maximum IL-6 induction is much

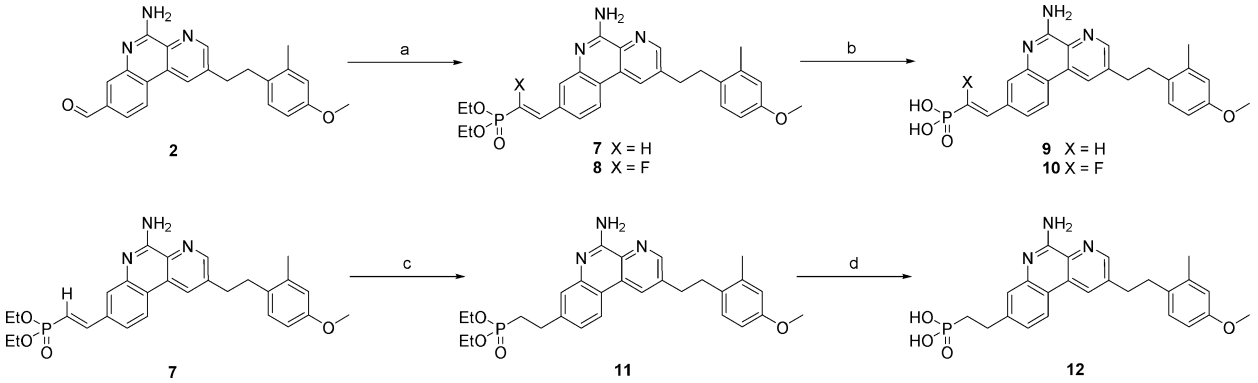
Scheme 1. Preparation of Keto-phosphonates at R_1[a]



[a]Reagents and conditions: (a) IBX, DMSO; (b) $(TMSO)_3P$, Tol, 80 °C; (c) IBX, DMSO; (d) $(EtO)_2P(O)CF_2H$, LDA, THF, − 78 °C; (e) IBX, DMSO/EtOAc, 80 °C; (f) TMSI, DCM, 0 °C.

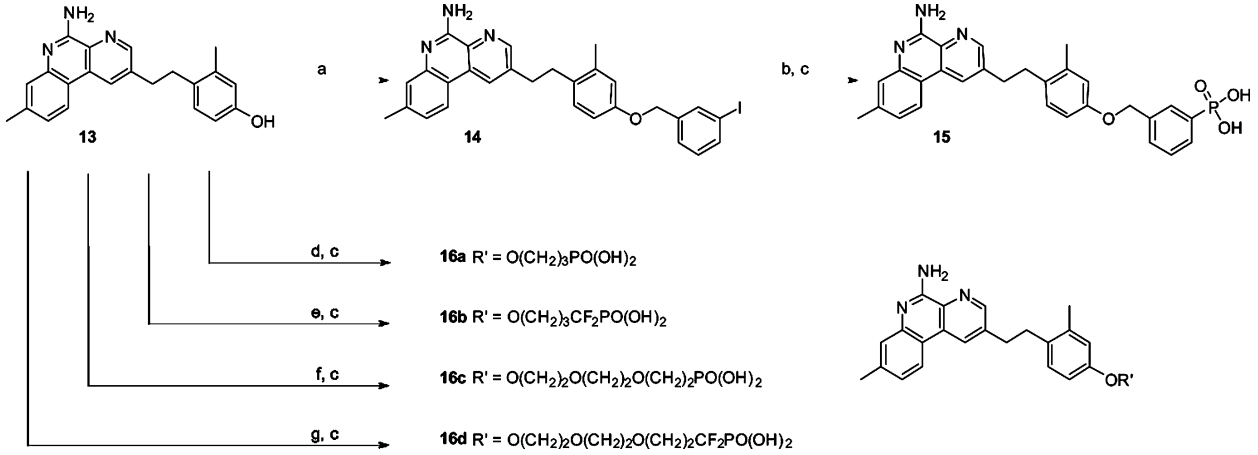
DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

Scheme 2. Preparation of Alkyl Phosphonates at R_1[a]



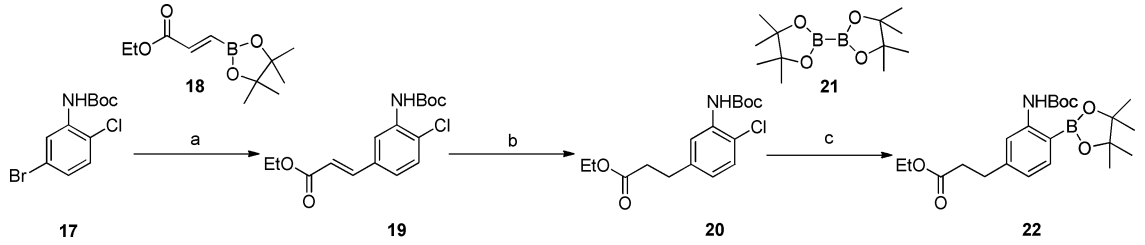
[a]Reagents and conditions: (a) NaH, $[(OEt)_2P(O)]_2CH_2$, THF 0 °C (for **7**) or LDA, $[(OEt)_2P(O)]_2CFH$, THF − 78 °C (for **8**); (b) TMSBr, DCM, 0 °C; (c) H_2, Pd/C, EtOH, DCM; (d) TMSBr, DCM, 0 °C.

Scheme 3. Preparation of Alkoxyphosphonates at R_2[a]



[a]Reagents and conditions: (a) Cs_2CO_3, 1-(bromomethyl)-3-iodobenzene, DMF; (b) $(EtO)_3P$, $Pd(OAc)_2$, 90 °C; (c) TMSBr, DCM, 0 °C; (d) NaH, $Br(CH_2)_3PO(OEt)_2$, DMF; (e) NaH, $Br(CH_2)_3CF_2PO(OEt)_2$, DMF; (f) NaH, $I(CH_2)_2O(CH_2)_2O(CH_2)_2PO(OEt)_2$, DMF; (g) NaH, $I(CH_2)_2O(CH_2)_2O(CH_2)_2CF_2PO(OEt)_2$, DMF.

Scheme 4. Preparation of Boronic Ester Suzuki Cross-Coupling Partner 22[a]



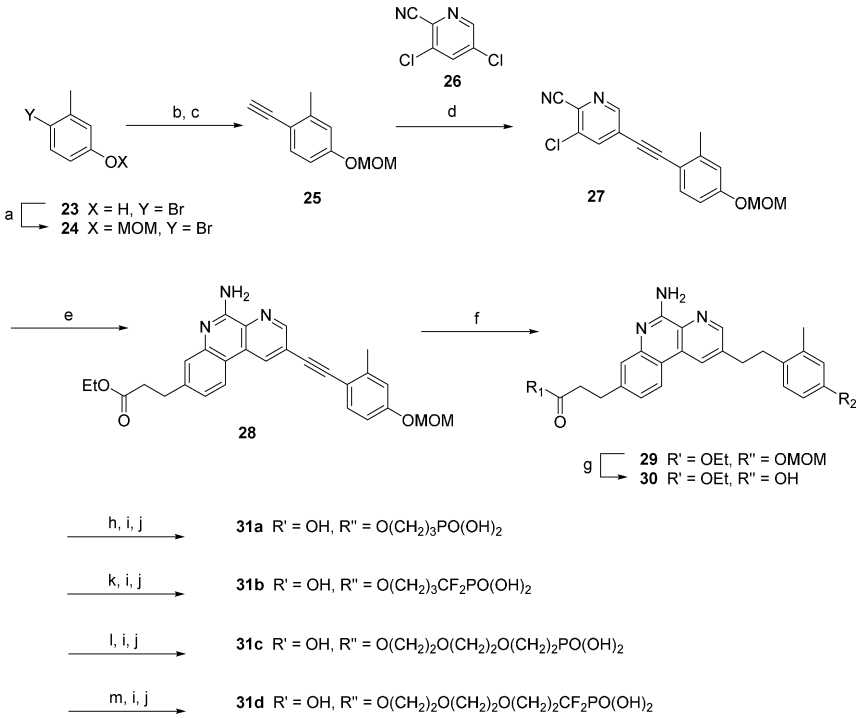
[a]Reagents and conditions: (a) **18**, $Pd(PPh_3)_4$, K_2CO_3, Tol/EtOH, 100 °C; (b) Wilkinson's catalyst, H_2, EtOAc/EtOH; (c) **21**, $Pd_2(dba)_3$, Xphos, KOAc, dioxane, 100 °C.

lower in hPBMC compared to mouse splenocytes likely because the reference compound resiquimod is a TLR7/8 agonist and can activate more cell types compared to TLR7-selective agonists within human PBMC mixed cell population. On the contrary, mouse lacks functional TLR8; therefore, mouse splenocytes respond to TLR7 agonists similarly to TLR7/8 agonist, as indicated by the similar percentage max activation.[10] All of the BZN-phosphonates were tested to be TLR7-selective agonists, with no appreciable TLR8 activity (<5% relative to resiquimod) tested up to 30 μM. (For comparison, resiquimod HEK-TLR8 EC_{50} = 4.0 μM, set to 100%.)

The process of alum adsorption involved first the preparation of a solution of BZN-phosphonate in an appropriate buffer (e.g., histidine, pH 6.5) and then adding to a suspension of aluminum hydroxide particulate. To avoid creating a physical comixture of two insoluble particulates, BZN-phosphonates needed to be completely soluble. Since antigens are expected to be adsorbed onto the same alum formulation, the pH range should not be detrimental to the fragile proteins. Hence, BZN-phosphonates must show high aqueous solubility within pH

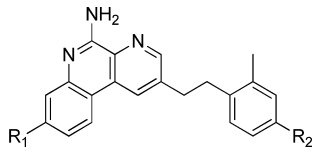
DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

Scheme 5. Preparation of Propionic Acid at R_1 and Alkoxyphosphonates at R_2[a]



[a]Reagents and conditions: (a) NaH, MOMCl, DMF; (b) CuI, $Pd(PPh_3)_2Cl_2$, TES-acetylene, Et_3N, DMF, 60 °C; (c) TBAF, THF; (d) CuI, $Pd(PPh_3)_2Cl_2$, Et_3N, DMF, 60 °C; (e) **22**, $Pd_2(dba)_3$, $NaHCO_3$, dicyclohexyl(2′,6′-dimethoxybiphenyl-2-yl)phosphine, *n*-BuOH/H_2O, 100 °C; (f) H_2, Pd/C, EtOH/THF; (g) HCl/EtOH; (h) Cs_2CO_3, $Br(CH_2)_3PO(OEt)_2$, DMF; (i) TMSBr, DCM, 0 °C; (j) NaOH, EtOH, 80 °C; (k) Cs_2CO_3, $Br(CH_2)_3CF_2PO(OEt)_2$, DMF; (l) Cs_2CO_3, $I(CH_2)_2O(CH_2)_2O(CH_2)_2PO(OEt)_2$, DMF; (m) Cs_2CO_3, $I(CH_2)_2O(CH_2)_2O(CH_2)_2CF_2PO(OEt)_2$, DMF.

Table 1. In Vitro Activities of Benzonaphthyridines Phosphonates



compd	R_1	R_2	HEK-TLR7[a]	IL-6 mouse spleno[a]	IL-6 human PBMC[a]
3	$C(O)PO_3H_2$	OMe	5.6 (112%)	14 (117%)	not active
6	$C(O)CF_2PO_3H_2$	OMe	0.39 (87%)	3.4 (100%)	7.8 (56%)
9	(*E*)-CH═$CHPO_3H_2$	OMe	0.15 (148%)	2.5 (81%)	1.7 (90%)
10	(*E*)-CH═$CFPO_3H_2$	OMe	0.20 (100%)	0.57 (220%)	3.4 (40%)
12	$CH_2CH_2PO_3H_2$	OMe	0.090 (146%)	0.13 (124%)	0.40 (107%)
16a	Me	$O(CH_2)_3PO_3H_2$	0.23 (94%)	1.1 (98%)	30 (26%)
16b	Me	$O(CH_2)_3CF_2PO_3H_2$	0.31 (97%)	3.2 (47%)	1.6 (31%)
16c	Me	$O(CH_2)_2O(CH_2)_2O(CH_2)_2PO_3H_2$	3.1 (94%)	3.6 (83%)	8.2 (55%)
16d	Me	$O(CH_2)_2O(CH_2)_2O(CH_2)_2CF_2PO_3H_2$	1.2 (95%)	0.81 (100%)	0.98 (36%)
31a	$CH_2CH_2CO_2H$	$O(CH_2)_3PO_3H_2$	0.065 (107%)	0.31 (100%)	0.29 (46%)
31b	$CH_2CH_2CO_2H$	$O(CH_2)_3CF_2PO_3H_2$	0.24 (95%)	2.4 (74%)	0.36 (35%)
31c	$CH_2CH_2CO_2H$	$O(CH_2)_2O(CH_2)_2O(CH_2)_2PO_3H_2$	0.35 (110%)	0.19 (140%)	0.49 (58%)
31d	$CH_2CH_2CO_2H$	$O(CH_2)_2O(CH_2)_2O(CH_2)_2CF_2PO_3H_2$	0.039 (100%)	1.2 (80%)	2.1 (35%)

[a]EC_{50} in μM. Shown in parentheses are % of max activation in reference to resiquimod (internal standard), which is set at 100%. Resiquimod typically produced 600−1200 pg/mL IL-6 in mSpleno assays and 20 000−12 500 pg/mL IL-6 in hPBMC assays. Data represent the median of at least two experiments performed in triplicate.

range 6.5−7.2 in order to be efficiently coadsorbed onto alum, together with the antigens. The initial phosphonate analog **16a** with hydrocarbon linker at R_2 showed poor solubility at neutral pH (Figure 2). It did, however, show increasing solubility with increasing pH, suggesting fraction of ionization (FI) is greatly enhanced when the phosphonate is fully deprotonated. We hypothesized that placement of fluorine atoms α to the phosphonate group can decrease the pK_a, resulting in higher fraction of ionization (FI) at more neutral pH (**16b**). Insertion of polyethylene glycol linker also provided added solubility (**16d**). Finally, installation of another ionizable group (propionic acid) at R_1 further increased solubility within the

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

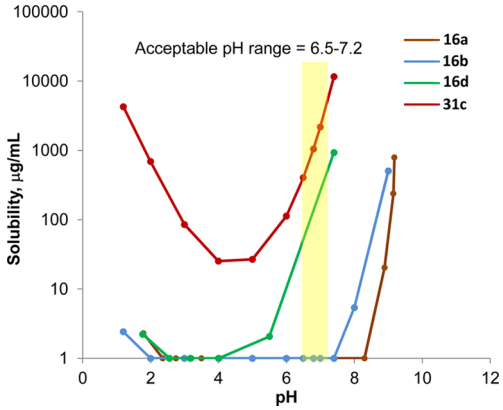


Figure 2. pH-dependent solubility of selected BZN-phosphonate analogs. The acceptable pH range for general protein antigen stability is 6.5−7.2. Each line represented one compound.

desired pH range (**31c**). The fraction of BZN-phosphonate bound to alum was determined indirectly from unbound fraction in the supernatant after centrifugation removal of insoluble alum particulate (Table 2). Alum adsorption of

Table 2. Benzonaphthyridine Phosphonate Adsorption Efficiency onto Alum

compd	% adsorbed onto alum
16a	nd (insoluble)[a]
16d	98
31b	95
31c	97
31d	97

[a]Adsorption efficiency cannot be determined using protocols described in Experimental Section due to poor compound solubility at pH range tested (6.5−7.2).

phosphonate **16a** could not be accurately determined due to insolubility. All soluble BZN-phosphonates tested show high degree of adsorption (>95%) in the conditions described. Addition of inorganic phosphate to the alum-bound formulations can desorb the TLR7 agonist, suggesting the adsorption is a reversible process (data not shown).

Previously, we have reported that benzonaphthyridine TLR7 agonists can exhibit different PK profiles with different physicochemical properties.[7] As expected, intramuscular injection of soluble BZN-phosphonate **16d** in aqueous buffer afforded high systemic exposure and low muscle concentration at 24 h (Figure 3). These are properties not desired for safe and effective adjuvants. Consistent with our design, when **16d** is adsorbed onto aluminum hydroxide, the formulation gave significantly reduced systemic C_{max} and increased injection site muscle retention. Alum created a local deposition for the adsorbed BZN-phosphonate, which is slowly released over time, likely from competition by small organic ions within the interstitial fluid (e.g., citrate).[3]

The BZN-phosphonates described here were extensively studied in mouse vaccine models and discussed in separate publications.[7,11] Alum adsorption offers one way to increase injection site local retention, reduce systemic exposure, and co-deliver antigen and adjuvant. Lu et al. have described a similar method of modifying antigen with phosphonate linker to enhance alum adsorption, which resulted in more efficient (antigen-sparing) vaccination.[12] Recently, there have been

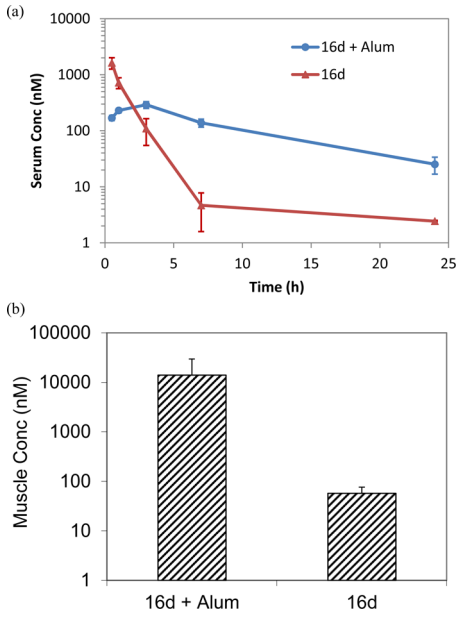


Figure 3. PK of BZN-phosphonate **16d**. Balb/C mice (3 per group) were injected intramuscularly with 100 μg of compounds with or without alum. (a) Compound concentrations in serum were measured at the indicated time points after injection. (b) Compound concentration in the injected muscles were measured 24 h after injection. Data represent the median ± SD.

other reports of similar concept utilizing different delivery methods. Local retention can be enhanced by manipulating physicochemical properties,[13] attaching phospholipid,[14] and forming dendrimers.[15] Tissue restriction can be achieved by conjugation of SMIP onto macromolecules such as antigen,[16] albumin,[17] or antibody.[18] Finally, small molecule adjuvants can be delivered by polymer encapsulation technologies.[19,20] All these modalities aim to facilitate antigen-specific immune response (exogenous or cancer) and reduce unnecessary systemic immune activation. It remains to be seen which will have significant translational impact in clinical settings.

CONCLUSION

Localized innate immune activation is a key attribute for safe and effective adjuvant. Here, low molecular weight TLR7 agonist is locally retained at the injection site not by its nonpolarity or insolubility but by physical adsorption onto alum, a particulate used to deliver protein antigens. Adsorption of antigens to alum is commonly found in many commercial human vaccines. Phosphonate modification of BZN TLR7 agonists allows coadsorption and colocalization of antigen and adjuvant to potentially elicit greater antigen-specific immune response.

EXPERIMENTAL SECTION

Chemistry. All reagents were obtained from commercial vendors unless specified. Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were obtained with a Bruker spectrometer operating at 400 MHz. Chemical shifts are given in parts per million (δ, ppm) and are referenced to the solvent in which they were run. Carbon, hydrogen, and nitrogen analyses were performed by Midwest Microlab, LLC, Indianapolis, IN, and were within 0.4% of the theoretical values unless otherwise indicated. Flash column chromatography was performed on an ISCO CombiFlash system using Redisep normal phase disposable columns (Teledyne ISCO, Lincoln, NE). For compound elution, a gradient of either methanol in dichloromethane or ethyl acetate in

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

hexanes was used as the mobile phase. The structures of all compounds were consistent with their spectral data. Unless noted otherwise, the reported yields were not optimized. All biologically evaluated compounds were determined to have purity of >95% using an Agilent LCMS system (C18 column; eluting gradient 10−90% acetonitrile in water).

5-Amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]-naphthyridine-8-carbonylphosphonic Acid (3). Scheme 1a−c. The synthesis of compound **1b** is described in ref 8, example 108. To a solution of (5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]-naphthyridin-8-yl)methanol (**1b**) (0.560 g, 1.50 mmol, 1.0 equiv) in DMSO (10 mL, 0.15 M) at room temperature was added IBX (0.630 g, 2.25 mmol, 1.5 equiv). The reaction was stirred for 2.5 h and then diluted with water. The aqueous layer was extracted with 2% MeOH/DCM (4×). The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated in vacuo. The resulting residue was purified by a CombiFlash system (ISCO) using a gradient of 0−5% MeOH/DCM to provide 5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*]-[1,7]naphthyridine-8-carbaldehyde (**2**) (325 mg, 58%) as a solid. To a stirred suspension of **2** (100 mg, 0.27 mmol, 1.0 equiv) in toluene (1 mL, 0.27 M) was added tris(trimethylsilyl) phosphite (81 mg, 0.27 mmol, 1.0 equiv). The reaction was stirred at 80 °C for 60 min, then solvents were removed, and the resulting residue was taken up in DMSO (1 mL, 0.27 M), and IBX (113 mg, 0.41 mmol, 1.5 equiv) was added. The reaction was stirred at rt for 2.5 h, filtered, and directly purified on RP-HPLC using a C18 column, eluting with 10−40% 95:5 (MeCN/5 mM NH_4OAc) in 10 mM NH_4OAc (pH 9) gradient. The fractions containing the product were combined and concentrated in vacuo to give **3** (12 mg, 9%) as a solid. ^{1}H NMR (dimethylsulfoxide-d_6): δ 9.84 (s, 1H), 9.35 (s, 1H), 9.09 (s, 1 H), 8.89 (s, 1 H), 8.76 (d, 1H, J = 8.4 Hz), 8.60 (s, 1H), 8.19 (d, 1H, J = 8.8 Hz), 7.04 (d, J = 8.8 Hz, 1H), 6.70 (d, 1H, J = 2.8 Hz), 6.62 (dd, 1H, J = 2.8, 8.4 Hz), 3.64 (s, 3H), 3.15−3.09 (m, 2H), 2.97−2.91 (m, 2H), 2.23 (s, 3H). LRMS [M + H] = 452.1.

2-(5-Amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]-naphthyridin-8-yl)-1,1-difluoro-2-oxoethylphosphonic Acid (6). Scheme 1d−f. To a solution of diethyl difluoromethylphosphonate (244 mg, 1.30 mmol, 3.0 equiv) in THF (4 mL, 0.3 M) at −78 °C under nitrogen atmosphere was added dropwise 2 M LDA (0.65 mL, 1.3 mmol, 3.0 equiv, commercial grade). The reaction was stirred at −78 °C for 25 min, and a solution of 5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]naphthyridine-8-carbaldehyde (**2**) (0.160 g, 0.431 mmol, 1.0 equiv) in THF (4 mL, 0.1 M) was added slowly. The reaction was stirred at −78 °C for 1 h, 0 °C for 1 h, and then warmed to room temperature over 30 min. The reaction was quenched with saturated aqueous ammonium chloride solution and extracted twice with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The resulting residue was purified by a CombiFlash system (ISCO) using a gradient of 0−5% MeOH/DCM to provide diethyl 2-(5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]-naphthyridin-8-yl)-1,1-difluoro-2-hydroxyethylphosphonate (**4**) (79 mg, 33%) as a solid. ^{1}H NMR (acetone-d_6): δ 8.79 (s, 1H), 8.74 (s, 1H), 8.37 (d, 1H, J = 8.4 Hz), 7.94 (s, 1H), 7.45 (d, 1H, J = 8.4 Hz), 7.08 (d, 1H, J = 8.4 Hz), 6.8 (br, 2H), 6.73 (s, 1H), 6.68 (d, 1H, J = 8.4 Hz), 5.36 (dt, 1H, J = 21.4, 6.1 Hz), 4.41−4.31 (m, 4H), 3.73 (s, 3H), 3.19−3.02 (m, 4H), 2.28 (s, 3H), 1.37−1.26 (m, 6H). LRMS [M + H] = 560.2

To a solution of **4** (79.0 mg, 0.141 mmol, 1.0 equiv) in 1:1 DMSO/ethyl acetate (2 mL, 0.07 M) was added IBX (60.0 mg, 0.214 mmol, 1.5 equiv). The reaction was heated to 80 °C for 1 h and then cooled to room temperature. The mixture was diluted with ethyl acetate and filtered through Celite. The filtrate was washed with water (2×), brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The resulting residue was purified by a CombiFlash system (ISCO) using a gradient of 0−5% MeOH/DCM to provide diethyl 2-(5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]naphthyridin-8-yl)-1,1-difluoro-2-oxoethylphosphonate (**5**) (29 mg, 37%) as a solid. ^{1}H NMR (acetone-d_6): δ 8.92 (s, 1H), 8.85 (s, 1H), 8.59 (d, 1H, J = 8.5 Hz), 8.44 (s, 1H), 7.96 (d, 1H, J = 8.5 Hz), 7.09 (d, 1H, J = 8.5 Hz), 6.75 (s, 1H), 6.68 (d, 1H, J = 8.3 Hz), 4.39−4.28 (m, 4H), 3.73 (s, 3H), 3.21 (t, 2H, J = 8.5 Hz), 3.06 (t, 2H, J = 8.5 Hz), 2.30 (s, 3H), 1.37−1.34 (m, 6H). LRMS [M + H] = 558.2

To a solution of **5** (29.0 mg, 0.0521 mmol, 1.0 equiv) in DCM (1 mL, 0.05 M) at 0 °C was added TMSI (37 μL, 0.26 mmol, 5.0 equiv). The reaction was warmed to room temperature over 2 h, and more TMSI was added (19 μL, 0.13 mmol, 2.5 equiv). The reaction was stirred for another 30 min and then quenched with small amounts of water. The DCM was removed by evaporation, and then DMSO/water was added. The mixture was adjusted to pH 9 and directly purified on RP-HPLC using a C18 column, eluting with 10−40% 95:5 (MeCN/5 mM NH_4OAc) in 10 mM NH_4OAc (pH 9) gradient. The fractions containing the product were combined and concentrated in vacuo to give **6** (28 mg, 88%) as a solid. ^{1}H NMR (dimethylsulfoxide-d_6): δ 8.82 (s, 1H), 8.5 (br, 1H), 8.44 (s, 1H), 8.2 (br, 1H), 7.98 (d, 1H, J = 8.2 Hz), 7.2 (br, 2H), 7.05 (d, 1H, J = 8.3 Hz), 6.73 (s, 1H), 6.67 (d, 1H, J = 8.3 Hz), 3.70 (s, 3H), 2.99−2.87 (m, 4H), 2.25 (s, 3H). LRMS [M + H] = 502.2

(*E*)-2-(5-Amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*]-[1,7]naphthyridin-8-yl)vinylphosphonic Acid (9). Scheme 2a,b. To a stirred suspension of NaH (8 mg, 60% in mineral oil, 0.19 mmol, 1.2 equiv) in THF (2 mL, 0.1 M) cooled at 0 °C was added a solution of tetraethyl methylenediphosphonate (61 mg, 0.21 mmol, 1.3 equiv) in THF (1 mL, 0.21 M). To the resulting reaction mixture was added a solution of 5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]-naphthyridine-8-carbaldehyde (**2**) (60 mg, 0.16 mmol, 1.0 equiv) in THF (2 mL, 0.08 M). The reaction was stirred at rt for 30 min, then solvents were removed in vacuo, and the resulting residue was purified by a CombiFlash system (ISCO) using a gradient of 0−5% MeOH/DCM to provide (*E*)-diethyl 2-(5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]naphthyridin-8-yl)vinylphosphonate (**7**) as colorless solid (32 mg, 40%). To a solution of **7** (48 mg, 0.095 mmol, 1.0 equiv) in DCM (1 mL, 0.095 M) at 0 °C was added TMSBr (145 mg, 0.95 mmol, 10 equiv). The reaction was warmed to room temperature over 2 h and then quenched with small amounts of MeOH. The DCM was removed by evaporation, and then DMSO/water was added. The mixture was adjusted to pH 9 and directly purified on RP-HPLC using a C18 column, eluting with 10−40% 95:5 (MeCN/5 mM NH4OAc) in 10 mM NH_4OAc (pH 9) gradient. The fractions containing the product were combined and concentrated in vacuo to give **9** (25 mg, 59%) as a solid. ^{1}H NMR (dimethylsulfoxide-d_6): δ 9.76 (s, 1H), 9.33 (s, 1H), 9.03 (s, 1H), 8.82 (s, 1H), 8.60 (d, 1H, J = 8.4 Hz), 7.87 (d, 1H, J = 8.4 Hz), 7.78 (s, 1H), 7.31 (dd, 1H, J = 17.6, 21.6 Hz), 7.03 (d, 1H, J = 8.4 Hz), 6.69 (m, 2H), 6.61 (dd, 1H, J = 2.8, 8.4 Hz), 3.64 (s, 3H), 3.14−3.06 (m, 2H), 2.97−2.91 (m, 2H), 2.23 (s, 3H). LRMS [M + H] = 450.2

(*E*)-2-(5-Amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*]-[1,7]naphthyridin-8-yl)-1-fluorovinylphosphonic Acid (10). Scheme 2a,b. To a stirred solution of tetraethyl fluoromethylenediphosphonate (412 mg, 1.35 mmol, 2.5 equiv) in THF (5 mL, 0.27 M) cooled at −78 °C was added LDA solution (1.8 M in ethylbenzene/pentane/hexane, 0.60 mL, 1.08 mmol, 2.0 equiv). The resulting reaction mixture was warmed up to rt and stirred for 30 min before it was cooled back down to −78 °C. A solution of 5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]naphthyridine-8-carbaldehyde (**2**) (200 mg, 0.54 mmol, 1.0 equiv) in THF (3 mL, 0.18 M) was added, and the reaction mixture was allowed to warm up to rt slowly. The reaction was quenched with saturated aqueous NH_4Cl solution. Aqueous phase was extracted with DCM (3×). The combined organic phases were combined and concentrated in vacuo. The residue was purified by a CombiFlash system (ISCO) using a gradient of 0−5% MeOH/DCM to provide (*E*)-diethyl 2-(5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]naphthyridin-8-yl)-1-fluorovinylphosphonate (**8**) as a colorless solid (137 mg, 48%). To a solution of **8** (137 mg, 0.26 mmol, 1.0 equiv) in DCM (5 mL, 0.05 M) at 0 °C was added TMSBr (401 mg, 2.6 mmol, 10 equiv). The reaction was warmed to room temperature over 2 h and then quenched with small amounts of MeOH. The DCM was removed by evaporation, and then DMSO/water was added. The mixture was adjusted to pH 9 and directly purified on RP-HPLC using a C18 column, eluting with 10−

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

40% 95:5 (MeCN/5 mM NH4OAc) in 10 mM NH_4OAc (pH 9) gradient. The fractions containing the product were combined and concentrated in vacuo to give **10** (60 mg, 49%) as a solid. ^{1}H NMR (dimethylsulfoxide-d_6): δ 9.80 (s, 1H), 9.41 (s, 1H), 9.05 (s, 1H), 8.87 (s, 1H), 8.65 (d, 1H, J = 8.8 Hz), 8.08 (s, 1H), 7.76 (d, 1H, J = 8.4 Hz), 7.08 (d, 1H, J = 8.4 Hz), 7.02 (d, 1H, J = 8.4 Hz), 6.83−6.65 (m, 2H), 3.69 (s, 3H), 3.18−3.12 (m, 2H), 3.02−2.96 (m, 2H), 2.28 (s, 3H). LRMS [M + H] = 468.1

2-(5-Amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]-naphthyridin-8-yl)ethylphosphonic Acid (12). Scheme 2c,d. To a solution of (*E*)-diethyl 2-(5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]naphthyridin-8-yl)vinylphosphonate (**7**) (120 mg, 0.24 mmol, 1.0 equiv) in DCM (5 mL, 0.05 M) and EtOH (3 mL, 0.08 M) was added 10% palladium on carbon (24 mg, 0.022 mmol, 0.09 equiv). The reaction vessel was charged with a hydrogen balloon and stirred at rt overnight. After the reaction was complete as monitored by LCMS, solvents were removed, and the resulting residue was purified by a CombiFlash system (ISCO) using a gradient of 0−5% MeOH/DCM to provide diethyl 2-(5-amino-2-(4-methoxy-2-methylphenethyl)benzo[*f*][1,7]naphthyridin-8-yl)-ethylphosphonate (**11**) (60 mg, 50%). To a solution of **11** (60 mg, 0.118 mmol, 1.0 equiv) in DCM (5 mL, 0.02 M) at 0 °C was added TMSBr (180 mg, 1.18 mmol, 10 equiv). The reaction was warmed to room temperature over 2 h and then quenched with small amounts of MeOH. The DCM was removed by evaporation, and then DMSO/water was added. The mixture was adjusted to pH 9 and directly purified on RP-HPLC using a C18 column, eluting with 10−40% 95:5 (MeCN/5 mM NH4OAc) in 10 mM NH_4OAc (pH 9) gradient. The fractions containing the product were combined and concentrated in vacuo to give **12** (33 mg, 62%) as a solid. ^{1}H NMR (dimethylsulfoxide-d_6): δ 9.66 (s, 1H), 9.30 (s, 1H), 8.95 (s, 1H), 8.78 (s, 1H), 8.50 (d, 1H, J = 8.4 Hz), 7.54 (s, 1H), 7.45 (d, 1H, J = 8.4 Hz), 7.02 (d, 1H, J = 8.4 Hz), 6.69 (d, 1H, J = 2.8 Hz), 6.61 (dd, 1H, J = 2.8, 8.4 Hz), 3.64 (s, 3H), 3.14−3.06 (m, 2H), 3.00−2.90 (m, 4H), 2.22 (s, 3H), 2.02−1.92 (m, 2H). LRMS [M + H] = 452.2

3-((4-(2-(5-Amino-8-methylbenzo[*f*][1,7]naphthyridin-2-yl)-ethyl)-3-methylphenoxy)methyl)phenylphosphonic Acid (15). Scheme 3a−c. The synthesis of compound **13** is described in ref 8, example 50. To a solution of 4-(2-(5-amino-8-methylbenzo[*f*][1,7]-naphthyridin-2-yl)ethyl)-3-methylphenol (**13**) (100 mg, 0.29 mmol, 1.0 equiv) in dimethylformamide (3 mL, 0.10 M) at 22 °C was added cesium carbonate (142 mg, 0.44 mmol, 1.5 equiv), and the resulting mixture was allowed to stir for 30 min. At this point, 1-(bromomethyl)-3-iodobenzene (131 mg, 0.44 mmol, 1.5 equiv) was added to this mixture. The reaction mixture was allowed to stir at 55 °C for 18 h, after which it was diluted with ethyl acetate and water. The biphasic layers were separated, and the organic layer was washed twice with water. The organic layer was dried over anhydrous Na_2SO_4, and the volatiles were removed in vacuo. The resulting residue was purified by a CombiFlash system (ISCO) using 0−50% ethyl acetate in hexanes gradient to provide 2-(4-(3-iodobenzyloxy)-2-methylphenethyl)-8-methylbenzo[*f*][1,7]naphthyridin-5-amine (**14**) (151 mg, 93%) as a solid. To a stirred solution of **14** (151 mg, 0.27 mmol, 1.0 equiv) in triethyl phosphate (47 mg, 0.28 mmol, 1.05 equiv) was added palladium acetate (4.8 mg, 0.022 mmol, 0.08 equiv). The resulting reaction mixture was heated at 90 °C overnight. After the reaction was cooled down to rt, the residue was taken up in DCM (1 mL, 0.27 M) at 0 °C and was treated with TMSBr (0.4 mL, 3.0 mmol, 11 equiv). The reaction was warmed to room temperature over 2 h and then quenched with small amounts of MeOH. The DCM was removed by evaporation, and then DMSO/water was added. The mixture was adjusted to pH 9 and directly purified on RP-HPLC using a C18 column, eluting with 10−40% 95:5 (MeCN/5 mM NH4OAc) in 10 mM NH_4OAc (pH 9) gradient. The fractions containing the product were combined and concentrated in vacuo to give **15** (15 mg, 10%) as a solid. ^{1}H NMR (dimethylsulfoxide-d_6): δ 8.84 (s, 1H), 8.72 (s, 1H), 8.35 (d, 1H, J = 8.4 Hz), 7.67 (d, 1H, J = 12 Hz), 7.60−7.54 (m, 1H), 7.30−7.20 (m, 2H), 7.15 (d, 1H, J = 8.4 Hz), 7.11 (d, 1H, J = 8.4 Hz), 7.04 (s, 1H), 6.84 (s, 1H), 6.77 (m, 1H), 4.99 (s, 2H), 3.12−2.92 (m, 4H), 2.44 (s, 3 H), 2.27 (s, 3H). LRMS [M + H] = 514.2

A representative example for the synthesis of compounds **16a−d** is illustrated with the synthesis of **16d**.

3-(2-(2-(4-(2-(5-Amino-8-methylbenzo[*f*][1,7]naphthyridin-2-yl)ethyl)-3-methylphenoxy)ethoxy)ethoxy)-1,1-difluoropropylphosphonic Acid (16d). Scheme 3g,c. Representative Preparation of Fluorophosphonate Electrophiles: Diethyl 1,1-difluoro-3-(2-(2-iodoethoxy)ethoxy)-propylphosphonate. To a solution of diethyl difluoromethylphosphonate (2.00 mL, 12.7 mmol, 1.0 equiv) in THF (16 mL, 0.8 M) at −78 °C was slowly added a solution of LDA (7.00 mL, 14.0 mmol, 2.0 M, 1.1 equiv) in heptane/THF/ethylbenzene, and the mixture was vigorously stirred for 30 min. In a separate reaction flask, a solution of 1,2-bis(2-iodoethoxy)ethane (4.70 g, 12.7 mmol, 1.0 equiv) in THF (16 mL, 0.8 M) was cooled to −78 °C. To this solution was transferred, by cannula, the freshly prepared alkyllithium solution, and the reaction mixture was allowed to stir for 1 h at −78 °C. At this point, the cooling bath was removed and the reaction mixture was allowed to warm to room temperature. The reaction mixture was then quenched with a 1 M aqueous solution of HCl. The resulting mixture was transferred to a separatory funnel and washed with CH_2Cl_2 three times. The combined organic layers were dried over anhydrous Na_2SO_4, and the volatiles were removed in vacuo. The resulting residue was purified by a CombiFlash system (ISCO) using CH_2Cl_2 to provide diethyl 1,1-difluoro-3-(2-(2-iodoethoxy)ethoxy)-propylphosphonate (1.09 g, 20%) as yellow oil. ^{1}H NMR ($CDCl_3$): δ 4.33−4.21 (m, 4H), 3.80−3.73 (m, 4H), 3.68−3.59 (m, 4H), 3.27 (t, 2H, J = 6.7 Hz), 2.51−2.31 (m, 2H), 1.39 (t, 6H, J = 7.1 Hz). LRMS [M + H] = 431.1

The synthesis of compound **13** is described in ref 8, example 50. To a solution of **13** (0.400 g, 1.17 mmol, 1.0 equiv) in dimethylformamide (11.7 mL, 0.10 M) at 22 °C was added 60% dispersion of sodium hydride in mineral oil (0.700 g, 1.75 mmol, 1.5 equiv), and the resulting mixture was allowed to stir for 30 min. At this point, diethyl 1,1-difluoro-3-(2-(2-iodoethoxy)ethoxy)propylphosphonate (0.600 mg, 1.40 mmol, 1.2 equiv) was added to this mixture. The reaction mixture was then allowed to stir for 18 h, after which it was diluted with ethyl acetate and water. The biphasic layers were separated, and the organic layer was washed twice with water. The organic layer was dried over anhydrous Na_2SO_4, and the volatiles were removed in vacuo. The resulting residue was purified by a CombiFlash system (ISCO) using 0−50% ethyl acetate in hexanes gradient to provide diethyl 3-(2-(2-(4-(2-(5-amino-8-methylbenzo[*f*][1,7]naphthyridin-2-yl)ethyl)-3-methylphenoxy)ethoxy)ethoxy)-1,1-difluoropropyl-phosphonate (302 mg, 40%) as a solid. ^{1}H NMR ($CDCl_3$): δ 8.56 (s, 1H), 8.38 (s, 1H), 8.10 (d, 1H, J = 8.2 Hz), 7.51 (s, 1H), 7.19 (d, 1H, J = 9.7 Hz), 6.98 (d, 1H, J = 8.4 Hz), 6.75 (s, 1H), 6.67 (d, 1H, J = 8.3 Hz), 6.07 (br, 2H), 4.30−4.23 (m, 4H), 4.10 (t, 2H, J = 5.0 Hz), 3.85 (t, 2H, J = 4.7 Hz), 3.79−3.71 (m, 4H), 3.66−3.64 (m, 2H), 3.09 (t, 2H, J = 8.5 Hz), 2.96 (t, 2H, J = 8.5 Hz), 2.51 (s, 3H), 2.48−2.35 (m, 2H), 2.26 (s, 3H), 1.37 (t, 6H, J = 7.1 Hz). LRMS [M + H] = 646.7

To a solution of diethyl 3-(2-(2-(4-(2-(5-amino-8-methylbenzo[*f*]-[1,7]naphthyridin-2-yl)ethyl)-3-methylphenoxy)ethoxy)ethoxy)-1,1-difluoropropylphosphonate (0.300 g, 0.465 mmol, 1.0 equiv) in CH_2Cl_2 (4.65 mL, 0.10 M) at 0 °C was slowly added trimethylsilyl bromide (0.950 mL, 6.98 mmol, 15 equiv). After 1 h the ice bath was removed and the reaction mixture was allowed to stir at 22 °C for 18 h. At this point, the volatiles were removed in vacuo and the resulting residue was purified by reverse phase HPLC using a 20−90% 0.5 mM NH_4OAc (in MeCN) to 10 mM NH_4OAc (in water) gradient to deliver **16d** (160 mg, 60%) as a solid. ^{1}H NMR (dimethylsulfoxide-d_6): δ 8.83 (s, 1H), 8.68 (s, 1H), 8.32 (d, 1H, J = 8.3 Hz), 7.34 (s, 1H), 7.14 (d, 1H, J = 8.4 Hz), 7.09 (br, 2H), 7.08 (d, 1H, J = 8.4 Hz), 6.74 (s, 1H), 6.68 (d, 1H, J = 8.3 Hz), 4.01 (t, 2H, J = 4.5 Hz), 3.70 (t, 2H, J = 3.3 Hz), 3.61 (t, 2H, J = 7.6 Hz), 3.59−3.54 (m, 2H), 3.50−3.48 (m, 2H), 3.07 (t, 2H, J = 6.8 Hz), 2.94 (t, 2H, J = 6.8 Hz), 2.43 (s, 3H), 2.25 (s, 3H), 2.21−2.06 (m, 2H). LRMS [M + H] = 590.2.

3-(4-(2-(5-Amino-8-methylbenzo[*f*][1,7]naphthyridin-2-yl)-ethyl)-3-methylphenoxy)propylphosphonic Acid (16a). Scheme 3d,c. The title compound was prepared according to the procedure described for **16d** but first using commercially available

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

diethyl 3-bromopropylphosphonate as the electrophile followed by TMSBr hydrolysis of the diethyl phosphonate.

Diethyl (3-(4-(2-(5-Amino-8-methylbenzo[f][1,7]naphthyridin-2-yl)-ethyl)-3-methylphenoxy)propyl)phosphonate, the Product after Alkylation of ***13*** *with Diethyl 3-bromopropylphosphonate.* ^{1}H NMR (dimethylsulfoxide-d_6): δ 9.91 (s, 1H), 9.14 (s, 1H), 8.72 (d, 1H, J = 8.2 Hz), 7.86 (s, 1H), 7.59 (d, 1H, J = 9.8 Hz), 7.51 (d, 1H, J = 8.3 Hz), 7.20 (s, 1H), 7.12 (d, 1H, J = 8.3 Hz), 7.00 (br, 2H), 4.56−4.45 (m, 6H), 3.61 (t, 2H, J = 8.5 Hz), 3.49 (t, 2H, J = 8.5 Hz), 2.90 (s, 3H), 2.74 (s, 3H), 2.47−2.39 (m, 2H), 2.36−2.28 (m, 2H), 1.72 (t, 6H, J = 7.0 Hz). LRMS [M + H] = 522.6.

Title Compound ***16a****.* TFA was added to the ^{1}H NMR sample to solubilize the compound for analysis. ^{1}H NMR (dimethylsulfoxide-d_6): δ 9.72 (br, 1H), 9.01 (s, 1H), 8.96 (br, 1H), 8.85 (s, 1H), 8.54 (d, 1H, J = 8.4 Hz), 7.54 (s, 1H), 7.42 (d, 1H, J = 8.2 Hz), 7.08 (d, 1H, J = 8.4 Hz), 6.74 (s, 1H), 6.66 (d, 1H, J = 8.3 Hz), 3.95 (t, 2H, J = 6.4 Hz), 3.14 (t, 2H, J = 8.6 Hz), 2.97 (t, 2H, J = 8.6 Hz), 2.50 (s, 3H), 2.27 (s, 3H), 1.91−1.81 (m, 2H), 1.67−1.56 (m, 2H). LRMS [M + H] = 466.2.

4-(4-(2-(5-Amino-8-methylbenzo[*f*][1,7]naphthyridin-2-yl)-ethyl)-3-methylphenoxy)-1,1-difluorobutylphosphonic Acid (16b). Scheme 3e,c. The title compound was prepared according to the procedure described in **16d** but using diethyl 4-bromo-1,1-difluorobutylphosphonate as the electrophile which was prepared from 1,3-dibromopropane.

Diethyl 4-Bromo-1,1-difluorobutylphosphonate. ^{1}H NMR ($CDCl_3$): δ 4.33−4.24 (m, 4H), 3.46 (t, 2H, J = 6.1 Hz), 2.31−2.13 (m, 4H), 1.39 (t, 6H, J = 7.1 Hz). LRMS [M + H] = 309.1

Diethyl (4-(4-(2-(5-Amino-8-methylbenzo[f][1,7]naphthyridin-2-yl)-ethyl)-3-methylphenoxy)-1,1-difluorobutyl)phosphonate, the Product after Alkylation of ***13*** *with Diethyl 4-Bromo-1,1-Difluorobutylphosphonate.* ^{1}H NMR ($CDCl_3$): δ 8.61 (s, 1H), 8.38 (s, 1H), 8.08 (d, 1H, J = 8.2 Hz), 7.53 (s, 1H), 7.20 (d, 1H, J = 6.8 Hz), 7.00 (d, 1H, J = 8.2 Hz), 6.73 (s, 1H), 6.66 (d, 1H, J = 8.3 Hz), 6.02 (br, 2H), 4.33−4.26 (m, 4H), 3.98 (t, 2H, J = 6.1 Hz), 3.11 (t, 2H, J = 8.4 Hz), 2.98 (t, 2H, J = 8.4 Hz), 2.52 (s, 3H), 2.35−2.28 (m, 2H), 2.27 (s, 3H), 2.13−2.08 (m, 2H), 1.40 (t, 6H, J = 7.1 Hz). LRMS [M + H] = 572.6

Title Compound ***16b****.* TFA was added to the ^{1}H NMR sample to solubilize the compound for analysis. ^{1}H NMR (dimethylsulfoxide-d_6): δ 9.71 (br, 1H), 9.33 (br, 1H), 9.00 (s, 1H), 8.85 (s, 1H), 8.54 (d, 1H, J = 8.4 Hz), 7.53 (s, 1H), 7.42 (d, 1H, J = 8.3 Hz), 7.08 (d, 1H, J = 8.4 Hz), 6.76 (s, 1H), 6.70 (d, 1H, J = 8.3 Hz), 3.97 (t, 2H, J = 6.2 Hz), 3.15 (t, 2H, J = 8.5 Hz), 2.98 (t, 2H, J = 8.5 Hz), 2.50 (s, 3H), 2.28 (s, 3H), 2.21−2.06 (m, 2H), 1.97−1.87 (m, 2H). LRMS [M + H] = 516.2.

2-(2-(2-(4-(2-(5-Amino-8-methylbenzo[*f*][1,7]naphthyridin-2-yl)ethyl)-3-methylphenoxy)ethoxy)ethoxy)ethylphosphonic Acid (16c). Scheme 3f,c. Representative Preparation of Phosphonate Electrophile: Diethyl 2-(2-(2-Iodoethoxy)-ethoxy)ethylphosphonate. A microwave tube was charged with a stirring bar, commercially available 1,2-bis(2-iodoethoxy)ethane (2.74 mL, 15 mmol, 1.0 equiv), and triethylphosphite (2.61 mL, 15 mmol, 1.0 equiv). The microwave tube was capped and then irradiated at 160 °C for 40 min with stirring. The reaction mixture was cooled down to rt and was purified by CombiFlash using 0−75% EtOAc in hexanes, or alternatively by RP-HPLC (0.035% TFA in ACN/0.05% TFA in H_2O, C18 column), to give diethyl 2-(2-(2-iodoethoxy)ethoxy)-ethylphosphonate (2.85 g, 50%) as pale yellow oil. ^{1}H NMR ($CDCl_3$): δ 4.14−4.02 (m, 4H), 3.76−3.70 (m, 4H), 3.65−3.60 (m, 4H), 3.25 (t, 2H, J = 7.0 Hz), 2.17−2.08 (m, 2H), 1.31 (t, 6H, J = 7.1 Hz). LRMS [M + H] = 381.1.

The title compound **16c** was then prepared according to the procedure described for **16d** but utilizing diethyl 2-(2-(2-iodoethoxy)-ethoxy)ethylphosphonate as the electrophile.

Diethyl (2-(2-(2-(4-(2-(5-Amino-8-methylbenzo[f][1,7]naphthyridin-2-yl)ethyl)-3-methylphenoxy)ethoxy)ethoxy)ethyl)phosphonate, the Product after Alkylation of ***13*** *with Diethyl 2-(2-(2-Iodoethoxy)ethoxy)-ethylphosphonate.* ^{1}H NMR ($CDCl_3$): δ 8.60 (s, 1H), 8.35 (s, 1H), 8.09 (d, 1H, J = 8.3 Hz), 7.57 (s, 1H), 7.23 (d, 1H, J = 8.3 Hz), 6.96 (d, 1H, J = 8.3 Hz), 6.80 (br, 2H), 6.75 (s, 1H), 6.66 (d, 1H, J = 8.3 Hz), 4.14−4.05 (m, 6H), 3.85−3.82 (m, 2H), 3.77−3.70 (m, 4H), 3.66−3.63 (m, 2H), 3.11 (t, 2H, J = 8.3 Hz), 2.97 (t, 2H, J = 8.3 Hz), 2.52 (s, 3H), 2.25 (s, 3H), 2.18−2.09 (m, 2H), 1.31 (t, 6H, J = 7.1 Hz). LRMS [M + H] = 596.3.

Title Compound ***16c****.* ^{1}H NMR (MeOD-d4): δ 8.73 (s, 1H), 8.66 (s, 1H), 8.38 (d, 1H, J = 8.4 Hz), 7.52 (s, 1H), 7.47 (d, 1H, J = 8.3 Hz), 7.36 (s, 1H), 6.93 (d, 1H, J = 8.4 Hz), 6.75 (s, 2H), 6.64 (d, 1H, J = 10.8 Hz), 4.09−4.06 (m, 2H), 3.80−3.76 (m, 2H), 3.69−3.64 (m, 2H), 3.64−3.59 (m, 2H), 3.53−3.49 (m, 2H), 3.25 (t, 2H, J = 7.5 Hz), 3.09 (t, 2H, J = 7.5 Hz), 2.58 (s, 3H), 2.28 (s, 3H), 2.13−2.01 (m, 2H). LRMS [M + H] = 540.2.

Ethyl 3-(3-(*tert*-Butoxycarbonylamino)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propanoate (22). Scheme 4a−c. To a solution of *tert*-butyl 5-bromo-2-chlorophenylcarbamate (**17**) (1.22 g, 4.0 mmol, 1.0 equiv) in acetonitrile (12 mL, 0.3 M) and EtOH (8 mL, 0.5 M) was added K_2CO_3 (1.1 g, 8.0 mmol, 2.0 equiv). The reaction was degassed and flushed with N_2, then to it were added (*E*)-ethyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)acrylate (**18**) (1.1 g, 4.8 mmol, 1.2 equiv) and $Pd(PPh_3)_4$ (0.46 g, 0.4 mmol, 0.1 equiv). The reaction was flushed again with N_2 and stirred at 100 °C overnight. After cooling to room temperature, hexane was added, and the mixture was filtered through a pad of silica, eluting with EA/Hex (1:1) until the product was completely eluted. The filtrate was concentrated and purified on CombiFlash, eluting with 0−15% EA in Hex to give (*E*)-ethyl 3-(3-(*tert*-butoxycarbonylamino)-4-chlorophenyl)acrylate (**19**) as a white solid (0.8 g, 62%). ^{1}H NMR ($CDCl_3$): δ 8.40 (s, 1H), 7.60 (d, 1H, J = 16.0 Hz), 7.30 (s, 1H, J = 8.0 Hz), 7.08 (d, 1H, J = 8.0 Hz), 7.02 (s, 1H), 6.43 (d, 1H, J = 16.0 Hz), 4.23 (q, 2H, J = 8.0 Hz), 1.52 (s, 9H), 1.31 (t, 3H, J = 8.0 Hz). LRMS [M + H] = 326.1.

To a solution of **19** (5.00 g, 15.4 mmol, 1.0 equiv) in ethyl acetate/ethanol (1:1, 0.3 M) was added Wilkinson's catalyst (1.40 g, 1.54 mmol, 0.10 equiv). Hydrogen gas was introduced via a balloon, and the reaction was stirred at room temperature for 24 h. The mixture was filtered through a pad of Celite, washing with dichloromethane. The filtrate was concentrated in vacuo and purified by CombiFlash using 0−10% ethyl acetate in hexane to give ethyl 3-(3-(*tert*-butoxycarbonylamino)-4-chlorophenyl)propanoate (**20**) as a solid (4.80 g, 96%). ^{1}H NMR ($CDCl_3$): δ 8.05 (s, 1H), 7.23 (d, 1H, J = 8.0 Hz), 6.98 (s, 1H), 6.81 (d, 1H, J = 8.0 Hz), 4.13 (q, 2H, J = 8.0 Hz), 2.92 (t, 2H, J = 8.0 Hz), 2.61 (t, 2H, J = 8.0 Hz), 1.53 (s, 9H), 1.24 (t, 3H, J = 8.0 Hz). LRMS [M + H] = 328.1.

A solution of **20** (6.56 g, 20 mmol, 1.0 equiv), 4,4,4′,4′,5,5,5′,5′-octamethyl-2,2′-bi(1,3,2-dioxaborolane) (**21**) (10.16 g, 40.0 mmol, 2.0 equiv), tris(dibenzylideneacetone)dipalladium(0) (916 mg, 1.0 mmol, 0.05 equiv), 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (1.9 g, 4.0 mmol, 0.20 equiv), and potassium acetate (3.92 g, 40.0 mmol, 2.0 equiv) in 1,4-dioxane (0.2 M) was degassed and stirred at 100 °C overnight. After cooling to ambient temperature, the reaction content was concentrated in vacuo. The crude material was purified by CombiFlash using 0−50% ethyl acetate in hexane to afford **22** (8.4 g, >99% overestimate) as brown oil. The product was stored at −20 °C and used within a month of synthesis. ^{1}H NMR ($CDCl_3$): δ 8.67 (s, 1H), 8.06 (s, 1H), 7.61 (d, 1H, J = 8.0 Hz), 6.81 (d, 1H, J = 8.0 Hz), 4.10 (t, 2H, J = 8.0 Hz), 2.91 (t, 2H, J = 8.0 Hz), 2.59 (t, 2H, J = 8.0 Hz), 1.50 (s, 9H), 1.23 (s, 12H), 1.22 (t, 3H, J = 8.0 Hz). LRMS [M + H] = 420.2.

Ethyl 3-(5-Amino-2-(4-hydroxy-2-methylphenethyl)benzo-[*f*][1,7]naphthyridin-8-yl)propanoate (30). Scheme 5a−g. To a solution of 4-bromo-3-methylphenol (**23**) (25.0 g, 134 mmol, 1.0 equiv) in DMF (285 mL, 0.5 M) at 0 °C was added portionwise 60 wt % NaH (8.06 g, 202 mmol, 1.5 equiv). The addition was controlled such that internal reaction temperature never went above 10 °C. The reaction was stirred at room temperature for 45 min, then a solution of chloro(methoxy)methane (13.1 mL, 161 mmol, 1.2 equiv) in DMF (50 mL, 3 M) was added dropwise via additional funnel. The reaction was stirred at room temperature for 3.5 h and then quenched by pouring into ice. The resulting mixture was stirred at room temperature for 1 h. Ether was added, and the two layers were separated. The aqueous layer was extracted (1×) with ether. The

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

combined organic layers were washed with water (2×), brine, dried over $MgSO_4$, and concentrated to give colorless oil, 1-bromo-4-(methoxymethoxy)-2-methylbenzene (**24**) (>100% yield due to mineral oil in the NaH). The crude material was used in the next step without further purification. A solution of **24** (23.4 g, 102 mmol overestimate, 1.0 equiv), triethylamine (70 mL, 510 mmol, 5.0 equiv) in DMF (200 mL, 0.5 M) was degassed and flushed with nitrogen. To the reaction was added TES-acetylene (19.2 mL, 107 mmol, 1.05 equiv), CuI (1.9 g, 10 mmol, 0.098 equiv), and $Pd(PPh_3)_2Cl_2$ (7.1 g, 10 mmol, 0.098 equiv). The reaction was heated to 60 °C and stirred overnight. After cooling to room temperature, water and ether were added. The layers were separated, and the organic layer was washed with water (2×). The organic layer was separated and passed through a pad of silica (packed with hexane). The silica was eluted with 10% EA in Hex. The fractions were combined and concentrated to give triethyl((4-(methoxymethoxy)-2-methylphenyl)ethynyl)silane as a black oil (>100% yield). The crude material was used in the next step without further purification. To a solution of triethyl((4-(methoxymethoxy)-2-methylphenyl)ethynyl)silane (39.31 g, 135.6 mmol, 1.0 equiv) at 0 °C was slowly added tetrabutylammonium fluoride (1 M solution in THF, 27 mL, 27.11 mmol, 0.20 equiv). At this point, the ice bath was removed and the reaction mixture was allowed to stir at room temperature for 45 min. The reaction mixture was then passed through a pad of silica (packed with hexane) and eluted with 20% EtOAc in hexanes to remove insoluble salts. The crude product was then purified by CombiFlash using 0−10% EtOAc in hexanes to give 1-ethynyl-4-(methoxymethoxy)-2-methylbenzene (**25**) as slightly brown liquid (98% yield). A solution of **25** (11.0 g, 62.5 mmol, 1.0 equiv), 3,5-dichloropicolinonitrile (**26**) (9.73 g, 56.3 mmol, 0.90 equiv), CuI (1.19 g, 6.25 mmol, 0.10 equiv), $Pd(PPh_3)_2Cl_2$ (4.40 g, 6.25 mmol, 0.10 equiv), and triethylamine (43 mL, 313 mmol, 5.0 equiv) in DMF (250 mL, 0.25 M) was degassed and flushed with nitrogen. The reaction mixture was then heated to 60 °C and stirred overnight. After cooling to room temperature, water was added. The mixture was extracted with EtOAc (2×). The combined organic layers were washed with 10% aq NH_4OH (2×), brine and concentrated. The crude material was filtered through a pad of silica (wetted with hexane). The silica was eluted with 10% EtOAc in hexane. The fractions were combined and concentrated. The resulting solids were washed in hot ether and filtered to give a yellow solid, which was used in the next step without further purification. The filtrate was concentrated and purified by CombiFlash using 0−10% EtOAc in hexanes to give 3-chloro-5-((4-(methoxymethoxy)-2-methylphenyl)-ethynyl)picolinonitrile (**27**) as a yellow solid. Combined yield was 45%. ^{1}H NMR ($CDCl_3$): δ 8.64 (s, 1H), 7.90 (s, 1H), 7.45 (d, 1H, J = 8.0 Hz), 6.94 (s, 1H), 6.89 (d, 1H, J = 8.0 Hz), 5.20 (s, 2H), 3.49(s, 3H), 2.49 (s, 3H). LRMS [M + H] = 313.1.

A solution of **27** (1.87 g, 6.00 mmol, 1.0 equiv), **22** (3.14 g, 7.50 mmol, 1.25 equiv), tris(dibenzylideneacetone)dipalladium(0) (549 mg, 0.60 mmol, 0.10 equiv), dicyclohexyl(2′,6′-dimethoxybiphenyl-2-yl)phosphine (493 mg, 1.20 mol, 0.20 equiv), and sodium bicarbonate (1.5 g, 18 mmol, 3.0 equiv) in n-butanol/H_2O (5:1, 0.2 M) was degassed and stirred at 100 °C overnight. After cooling to ambient temperature, the reaction content was diluted with ethyl acetate and water. The two phases were separated, and the aqueous layer was extracted twice with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude material was purified by flash chromatography on a CombiFlash system (ISCO) using 0−40% ethyl acetate in DCM first to remove the impurity, then 0−4% MeOH in DCM to give ethyl 3-(5-amino-2-((4-(methoxymethoxy)-2-methylphenyl)ethynyl)benzo-[f][1,7]naphthyridin-8-yl)propanoate (**28**). The product can be further purified by precipitating and washing in hot ether. Depending on the purity of the product, the isolated yield ranges 40−60%. A solution of **28** (3.0 g, 6.4 mmol, 1.0 equiv) in EtOH/THF (3:1, 40 mL, 0.16 M) was flushed with nitrogen. Then, 10 wt % Pd/C (0.6 g, 0.20 equiv by weight) was added. The reaction was flushed with hydrogen (2×) and stirred under a hydrogen balloon. After 24 h, the reaction was filtered through a pad of Celite, washing with 5% MeOH in DCM. The filtrate was checked for presence of starting material using LCMS. The hydrogenation reaction was repeated until no more of the alkyne starting material or alkene intermediate can be detected. The crude product was purified by CombiFlash using 0−4%MeOH in DCM to give ethyl 3-(5-amino-2-(4-(methoxymethoxy)-2-methylphenethyl)benzo[f][1,7]naphthyridin-8-yl)propanoate (**29**) as a white solid (>90%). ^{1}H NMR ($CDCl_3$): δ 8.63 (s, 1H), 8.37 (s, 1H), 8.11 (d, 1H, J = 8.0 Hz), 7.54 (s, 1H), 7.22 (d, 1H, J = 8.0 Hz), 7.01 (d, 1H, J = 8.0 Hz), 6.86 (s, 1H), 6.81 (d, 1H, J = 8.0 Hz), 6.22 (s, 2H), 5.15 (s, 2H), 4.15 (q, 2H, J = 8.0 Hz), 3.48(s, 3H), 3.13−3.08 (m, 4H), 2.97 (t, 2H, J = 8.0 Hz), 2.72 (t, 2H, J = 8.0 Hz), 2.27(s, 3H), 1.24 (t, 3H, J = 8.0 Hz). LRMS [M + H] = 474.2.

Compound **29** (2.04 g, 4.3 mmol, 1.0 equiv) was dissolved in EtOH (21 mL, 0.2 M), and then to the mixture was added a solution of 4 M HCl in dioxane (21 mL, 0.2 M). The product precipitated out as a yellow salt. After stirring for 3 h, the reaction was poured into a stirring solution of ether. The mixture was stirred for 10 min, then filtered and washed with ether. The yellow solids were dried in vacuum overnight to give ethyl 3-(5-amino-2-(4-hydroxy-2-methylphenethyl)benzo[f]-[1,7]naphthyridin-8-yl)propanoate (**30**) (2.08 g of bis-HCl salt, 97%). Alternatively, the crude product can be purified by CombiFlash using 0−5% MeOH in DCM to give the free base. ^{1}H NMR ($CDCl_3$): δ 8.53 (s, 1H), 8.33 (s, 1H), 8.09 (d, 1H, J = 8.0 Hz), 7.56 (s, 1H), 7.24 (d, 1H, J = 8.0 Hz), 6.84 (d, 1H, J = 8.0 Hz), 6.69 (s, 1H), 6.60 (d, 1H, J = 8.0 Hz), 4.14 (q, 2H, J = 8.0 Hz), 3.12−3.08 (m, 4H), 2.97 (t, 2H, J = 8.0 Hz), 2.71 (t, 2H, J = 8.0 Hz), 2.21 (s, 3H), 1.25 (t, 3H, J = 8.0 Hz). LRMS [M + H] = 430.2

A representative example for the synthesis of compounds **31a−d** is illustrated with the synthesis of **31c**.

3-(5-Amino-2-(2-methyl-4-(2-(2-(2-phosphonoethoxy)-ethoxy)ethoxy)phenethyl)benzo[*f*][1,7]naphthyridin-8-yl)-propanoic Acid (31c). Scheme 5l,i,j. The title compound was prepared utilizing the same phosphonate electrophile that was used to produce **16c**.

To a solution of **30** (108.0 mg, 0.252 mmol, 1.0 equiv) dissolved in DMF (4.2 mL, 0.06 M) was added a solution of diethyl 2-(2-(2-iodoethoxy)ethoxy)ethylphosphonate (190.0 mg, 0.50 mmol, 2.0 equiv) in DMF (0.36 mL, 0.7 M) and cesium carbonate (328.4 mg, 1.01 mmol, 4 equiv). The reaction was stirred at 60 °C. After 1.5 h (or until reaction is complete by LCMS), DCM (2 vol equiv) was added to the reaction. The solids (inorganic) were filtered, and the filtrate was concentrated. The crude product was purified by CombiFlash using 0−5%MeOH in DCM to give ethyl 3-(5-amino-2-(4-(2-(2-(2-(diethoxyphosphoryl)ethoxy)ethoxy)ethoxy)-2-methylphenethyl)-benzo[f][1,7]naphthyridin-8-yl)propanoate as an oil which upon standing became a white solid (154.6 mg, 90%). To a solution of ethyl 3-(5-amino-2-(4-(2-(2-(2-(diethoxyphosphoryl)ethoxy)ethoxy)-ethoxy)-2-methylphenethyl)benzo[f][1,7]naphthyridin-8-yl)-propanoate (154.6 mg, 0.227 mmol, 1.0 equiv) in DCM (1.42 mL, 0.16 M) at 0 °C was added slowly TMSBr (347 mg, 2.27 mmol, 10 equiv). The reaction was stirred at room temperature overnight. Additional TMSBr (173 mg, 1.13 mmol, 5.0 equiv) was added at 0 °C, and the reaction was again stirred at room temperature overnight. The solvent was removed by evaporation, and the crude orange solids were dried in vacuum briefly. The solids were suspended in EtOH (0.454 mL, 0.5 M), and to the mixture was added 2.5 N NaOH (0.908 mL, 2.27 mmol, 10.0 equiv). The reaction was stirred at 80 °C for 3 h. After cooling to room temperature, the mixture was adjusted to pH 9 and directly purified on RP-HPLC using a C18 column, eluting with 10−40% 95:5 (MeCN/5 mM NH_4OAc) in 10 mM NH4OAc (pH 9) gradient. The fractions containing the product were combined and concentrated in vacuo to give **31c** as a solid (105.4 mg, 70% yield from **30**). ^{1}H NMR (dimethylsulfoxide-d_6): δ 9.02 (s, 1H), 8.82 (s, 1H), 8.55 (d, 1H, J = 8.0 Hz), 7.58 (s, 1 H), 7.49 (d, 1H, J = 8.4 Hz), 7.06 (d, 1H, J = 8.0 Hz), 6.76 (s, 1 H), 6.68 (d, 1H, J = 8.0 Hz), 4.03−4.00 (m, 2H), 3.71−3.69 (m, 2H), 3.60−3.54 (m, 4H), 3.51−3.49 (m, 2H), 3.16−3.12 (m, 2H), 3.03−2.96 (m, 4H), 2.67−2.66 (m, 2H), 2.33−2.32 (m, 2H), 2.26 (s, 3H). LRMS [M + H] = 598.2.

3-(5-Amino-2-(2-methyl-4-(3-phosphonopropoxy)-phenethyl)benzo[*f*][1,7]naphthyridin-8-yl)propanoic Acid (31a). Scheme 5h,i,j. The title compound was prepared according

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

to the procedure described in example **31c** but using commercially available diethyl 3-bromopropylphosphonate as the electrophile. ^{1}H NMR (MeOD-d_4): δ 8.60 (s, 1H), 8.27 (s, 1H), 8.07 (d, 1H, J = 8.4 Hz), 7.52 (s, 1H), 7.30 (d, 1H, J = 8.4 Hz), 6.87 (d, 1H, J = 8.4 Hz), 6.67 (s, 1H), 6.60 (d, 1H, J = 8.4 Hz), 3.93 (t, J = 6.4 Hz, 2H), 3.49−3.47 (m, 2H), 3.14−3.09 (m, 2H), 2.99−2.95 (m, 2H), 2.69−2.64 (m, 2H), 2.17 (s, 3H), 2.02−2.00 (m, 2H), 1.74−0.66 (m, 2H). LRMS [M + H] = 524.2.

3-(5-Amino-2-(4-(4,4-difluoro-4-phosphonobutoxy)-2-methylphenethyl)benzo[*f*][1,7]naphthyridin-8-yl)propanoic Acid (31b). Scheme 5k,i,j. The title compound was prepared according to the procedure described in example **31c** and utilizing the same fluorophosphonate electrophile that was used to produce **16b**. ^{1}H NMR (MeOD-d4): δ 8.69 (s, 1H), 8.45 (s, 1H), 8.22 (d, 1H, J = 8.4 Hz), 7.53 (s, 1H), 7.45 (d, 1H, J = 8.4 Hz), 6.89 (d, J = 8.4 Hz, 1H), 6.69 (s, 1H), 6.60 (d, 1H, J = 8.4 Hz), 3.95 (t, 2H, J = 6.4 Hz), 3.92−3.90 (m, 2H), 3.49−3.47 (m, 2H), 3.20−3.16 (m, 2H), 3.14−3.10 (m, 2H), 3.03−2.99 (m, 2H), 2.74−2.70 (m, 2H), 2.22 (s, 3H). LRMS [M + H] = 574.2.

3-(5-Amino-2-(4-(2-(2-(3,3-difluoro-3-phosphonopropoxy)-ethoxy)ethoxy)-2-methylphenethyl)benzo[*f*][1,7]-naphthyridin-8-yl)propanoic Acid (31d). Scheme 5m,i,j. The title compound was prepared according to the procedure described in example **31c** and utilizing the same fluorophosphonate electrophile that was used to produce **16d**. ^{1}H NMR (DMSO-d_6): δ 9.02 (s, 1H), 8.82 (s, 1H), 8.55 (d, 1H, J = 8.4 Hz), 7.58 (s, 1 H), 7.49 (d, 1H, J = 8.4 Hz), 7.07 (d, 1H, J = 8.4 Hz), 6.75 (s, 1H), 6.68 (d, 1H, J = 8.0 Hz), 4.03−4.00 (m, 2H), 3.72−3.70 (m, 2H), 3.66−3.62 (m, 2H), 3.58−3.56 (m, 2H), 3.53−3.52 (m, 2H), 3.16−3.12 (m, 2H), 3.03−2.96 (m, 4H), 2.68−2.64 (m, 2H), 2.33−2.31 (m, 2H), 2.27 (s, 3H). LRMS [M + H] = 648.2.

TLR7 Reporter Gene Assay. HEK293 cells were stably transfected with a reporter vector expressing the firefly luciferase gene under the control of an NF-κB dependent promoter and the resistance gene to puromycin. Selected HEK293-NF-κB-luciferase cells were then stably transfected with pUno plasmid expressing human TLR7 and the resistance gene to blasticidin (Invivogen). Transfected cells were cultured in the presence of selection antibiotics puromycin (2 μg/mL) and blasticidin (5 μg/mL), and individual resistant clones were picked, expanded, and tested for expression of luciferase upon stimulation with the TLR7/8 agonist resiquimod. The best responding clone of HEK293-hTLR7-NF-κB-luciferase cells was then selected for experiments. The cell line was grown in DMEM (high glucose) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 μg/mL puromycin, and 5 μg/mL blasticidin (Hyclone). For the assay, the cells were seeded in DMEM media containing 3% FCS, 2 mM L-glutamine, 100 U penicillin, 100 μg/mL streptomycin. 25 000 cells were plated onto 384-well plates in 50 μL volume of assay media. The test compounds were dissolved in endotoxin-free water, with addition of 1 mol equiv of 1 N NaOH to generate the sodium salt in situ. The resulting solutions (10 mM) were serially diluted 3-fold for 10 points starting at 30 μM to obtain EC_{50} values. The compounds were then added into the wells. After 6 h of incubation at 37 °C and 5% CO_2, 30 μL of Bright-Glo luciferase (Promega) detection reagent was added, and luminescence intensity was measured by CLIPR (Molecular Devices). Activity is reported as percentage of relative luminescence unit (RLU) over that achieved by resiquimod (set to 100%), which is used as an internal standard for every experiment.

Murine Splenocyte Assay. Spleens were removed aseptically from female BALB/c mice (6−10 weeks old) and processed to single cell suspension in PBS supplemented with 5% heat inactivated FCS (Hyclone). Following RBC lysis, cells were resuspended ((5×10^6)/mL) in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 1 mM MEM, 10 mM HEPES, and 50 μM 2-ME. 5×10^5 splenocytes in 100 μL volume ware plated onto 96-well flat-bottom plates. The test compounds were dissolved in endotoxin-free water, with addition of 1 mol equiv of 1 N NaOH to generate the sodium salt in situ. The resulting solutions (10 mM) were serially diluted 3-fold for 10 points starting at 30 μM to obtain EC_{50} values. The compounds were then added into the wells. After 18 h of incubation at 37 °C and 5% CO_2, supernatants were analyzed for different cytokines by Meso Scale Discovery (MSD) multicytokine detection kit as described by the manufacturer. Activity is reported as percentage of IL-6 over that achieved by resiquimod (set to 100%), which is used as an internal standard for every experiment.

Human PBMC Assay. Heparin anticoagulated whole blood was obtained from healthy volunteers registered with The Scripps Research Institute Normal Blood Donor Service. PBMCs were isolated by density gradient centrifugation using Ficol-paque PLUS (GE Healthcare) as recommended by the manufacturer. The isolated PBMCs were washed twice with PBS and resuspended in RPMI1640 supplemented with heat inactivated FBS (10% final), 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 1 mM MEM, 10 mM HEPES (Hyclone). The test compounds were dissolved in endotoxin-free water, with addition of 1 mol equiv of 1 N NaOH to generate the sodium salt in situ. The resulting solutions (10 mM) were serially diluted 3-fold for 10 points starting at 30 μM to obtain EC_{50} values. The compounds were then added into the wells containing 100 μL of culture media with hPBMCs ((1×10^6 cells)/mL). After 18 h of incubation at 37 °C and 5% CO_2, supernatants were analyzed for different cytokines by Meso Scale Discovery (MSD) multicytokine detection kit as described by the manufacturer. Activity is reported as percentage of IL-6 over that achieved by resiquimod (set to 100%), which is used as an internal standard for every experiment.

Representative SMIP-Alum Formulation Protocol. To a suspension of SMIP (1 mg) in endotoxin-free water (0.25 mL) was added 1 N NaOH solution (1 mol equiv) to obtain a clear solution. To this solution was added 0.21 mL of alum suspension (9.0−11.0 mg/mL, obtained as Alhydrogel from Invivogen), 0.1 mL of histidine buffer (100 mM, pH 6.5), and 0.44 mL endotoxin-free water. The final suspension (1 mg/mL SMIP and 3 mg/mL alum in 10 mM histidine) was gently agitated for 1 h at room temperature. To determine fraction of unbound SMIP, an aliquot (100 μL) was placed into an Eppendorf tube and centrifuged (13 000 rpm for 10 min). The supernatant was analyzed on LCMS using 0−90% MeCN in water gradient and compared to the nonadsorbed SMIP reference.

Protocol for Acquisition of pH-Dependent Solubilities. The solubility−pH profiles of **16d** and **31c** were determined by potentiometric titration using Sirius T3 titrator (Sirius Analytical, East Sussex, U.K.), whereas the solubility−pH profiles of **16a** and **16b** were obtained by saturation shake-flask solubility assay as the solubilities of these two compounds were poor and no aqueous pK_a values were able to be obtained. For the potentiometric titration approach, the pK_a values of each compound were determined using the same Sirius T3 titrator prior to solubility determination using MeOH as the cosolvent and data were processed with T3 accompanying software. In the potentiometric solubility assay, about 2 mg of the solid sample was weighed into a T3 vial and titration was performed between pH 10 and pH 1.5. Precipitation occurred during the titration range, causing pK_a values to shift. The intrinsic solubility was calculated according to the pK_a shift based on Noyes−Withney theory using T3 accompanying software, and solubility−pH profile was extrapolated based on Henderson−Hasselbalch equation. In the saturated shake-flask approach, saturated solubility samples were prepared at different pHs and incubated for 24 h. The samples were then filtered through 0.22 mm PVDF membranes, and the filtrates were analyzed and quantified against a standard calibration curve of each compound. The solubility−pH profiles were constructed by connecting solubility data at different pH values.

Animal Studies. For PK experiments female BALB/c mice (6−8 weeks old) were injected intramuscularly with a total of 100 μL of 1 mg/mL compound in alum (50 μL in each hind leg) and then bled retro-orbitally at different time points postinjection. Blood was processed into serum by centrifugation followed by protein precipitation, reverse-phase gradient elution, and MRM detection via ESI+ mass spectrometry to determine SMIP serum concentration (PK). In some studies, animals were sacrificed after the final blood

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX

collection (24 h) and muscle was removed and flash frozen in liquid nitrogen for tissue PK analyses.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00489.

Molecular formula strings and in vitro activities (CSV)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: tyhwu@yahoo.com. Phone: (858) 284-8839.

Present Addresses

[‖]B.N.: HealthTell, San Ramon, CA 94583, U.S.

[⊥]M.L.M.: Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT 06877, U.S.

[#]N.M.V.: Moderna Therapeutics, Cambridge, MA 02139, U.S.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Liping Zhou for providing the solubility determination. T.Y.-H.W. thanks Xu Wu for help in initiating the project. T.Y.-H.W. also thanks Peter G. Schultz for giving him the opportunity and support to lead this project. This work was supported in part by the Transformational Medical Technologies Program Contract HDTRA1-07-9-0001 from the Department of Defense Chemical and Biological Defense program through the Defense Threat Reduction Agency.

■ ABBREVIATIONS USED

TLR7, Toll-like receptor 7; BZN, benzonaphthyridine; SMIP, small molecule immune potentiator; MPLA, monophosphoryl lipid A; PK, pharmacokinetics; hPBMC, human peripheral blood mononuclear cell; FI, fraction of ionization

■ REFERENCES

(1) O'Hagan, D. T.; De Gregorio, E. The path to a successful vaccine adjuvant—"the long and winding road". *Drug Discovery Today* **2009**, *14*, 541−551.

(2) Clements, C. J.; Griffiths, E. The global impact of vaccines containing aluminium adjuvants. *Vaccine* **2002**, *20*, S24−S33.

(3) Hem, S. L.; Hogenesch, H. Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation. *Expert Rev. Vaccines* **2007**, *6*, 685−698.

(4) O'Hagan, D. T.; Otten, G. S.; De Gregorio, E.; Seubert, A. The mechanism of action of MF59—an innately attractive adjuvant formulation. *Vaccine* **2012**, *30*, 4341−4348.

(5) Garçon, N.; Segal, L.; Tavares, F.; Van Mechelen, M. The safety evaluation of adjuvants during vaccine development: the AS04 experience. *Vaccine* **2011**, *29*, 4453−4459.

(6) Garçon, N.; Chomez, P.; Van Mechelen, M. GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. *Expert Rev. Vaccines* **2007**, *6*, 723−739.

(7) Wu, T. Y.; Singh, M.; Miller, A. T.; De Gregorio, E.; Doro, F.; D'Oro, U.; Skibinski, D. A.; Mbow, M. L.; Bufali, S.; Herman, A. E.; Cortez, A.; Li, Y.; Nayak, B. P.; Tritto, E.; Filippi, C. M.; Otten, G. R.; Brito, L. A.; Monaci, E.; Li, C.; Aprea, S.; Valentini, S.; Calabró, S.; Laera, D.; Brunelli, B.; Caproni, E.; Malyala, P.; Panchal, R. G.; Warren, T. K.; Bavari, S.; O'Hagan, D. T.; Cooke, M. P.; Valiante, N. M. Rational design of small molecules as vaccine adjuvants. *Sci. Transl. Med.* **2014**, *6*, 263ra160.

(8) Wu, T. Y.-H.; Li, Y.; Cortez, A.; Zou, Y.; Mishra, P.; Zhang, X.; Skibinski, D.; Signh, M.; Valiante, N. WO2009111337 2009.

(9) Nordqvist, A.; Nilsson, M. T.; Röttger, S.; Odell, L. R.; Krajewski, W. W.; Evalena Andersson, C.; Larhed, M.; Mowbray, S. L.; Karlén, A. Evaluation of the amino acid binding site of Mycobacterium tuberculosis glutamine synthetase for drug discovery. *Bioorg. Med. Chem.* **2008**, *16*, 5501−5513.

(10) Jurk, M.; Heil, F.; Vollmer, J.; Schetter, C.; Krieg, A. M.; Wagner, H.; Lipford, G.; Bauer, S. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat. Immunol.* **2002**, *3*, 499.

(11) Bagnoli, F.; Fontana, M. R.; Soldaini, E.; Mishra, R. P.; Fiaschi, L.; Cartocci, E.; Nardi-Dei, V.; Ruggiero, P.; Nosari, S.; De Falco, M. G.; Lofano, G.; Marchi, S.; Galletti, B.; Mariotti, P.; Bacconi, M.; Torre, A.; Maccari, S.; Scarselli, M.; Rinaudo, C. D.; Inoshima, N.; Savino, S.; Mori, E.; Rossi-Paccani, S.; Baudner, B.; Pallaoro, M.; Swennen, E.; Petracca, R.; Brettoni, C.; Liberatori, S.; Norais, N.; Monaci, E.; Bubeck Wardenburg, J.; Schneewind, O.; O'Hagan, D. T.; Valiante, N. M.; Bensi, G.; Bertholet, S.; De Gregorio, E.; Rappuoli, R.; Grandi, G. Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against Staphylococcus aureus. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 3680−3685.

(12) Lu, F.; Boutselis, I.; Borch, R. F.; Hogenesch, H. Control of antigen-binding to aluminum adjuvants and the immune response with a novel phosphonate linker. *Vaccine* **2013**, *31*, 4362−4367.

(13) Smirnov, D.; Schmidt, J. J.; Capecchi, J. T.; Wightman, P. D. Vaccine adjuvant activity of 3M-052: an imidazoquinoline designed for local activity without systemic cytokine induction. *Vaccine* **2011**, *29*, 5434−5442.

(14) Hayashi, T.; Chan, M.; Norton, J. T.; Wu, C. C.; Yao, S.; Cottam, H. B.; Tawatao, R. I.; Corr, M.; Carson, D. A.; Daniels, G. A. Additive melanoma suppression with intralesional phospholipid-conjugated TLR7 agonists and systemic IL-2. *Melanoma Res.* **2011**, *21*, 66−75.

(15) Shukla, N. M.; Salunke, D. B.; Balakrishna, R.; Mutz, C. A.; Malladi, S. S.; David, S. A. Potent adjuvanticity of a pure TLR7-agonistic imidazoquinoline dendrimer. *PLoS One* **2012**, *7*, e43612.

(16) Wille-Reece, U.; Wu, C. Y.; Flynn, B. J.; Kedl, R. M.; Seder, R. A. Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8+ T cell responses. *J. Immunol.* **2005**, *174*, 7676−7683.

(17) Wu, C. C.; Hayashi, T.; Takabayashi, K.; Sabet, M.; Smee, D. F.; Guiney, D. D.; Cottam, H. B.; Carson, D. A. Immunotherapeutic activity of a conjugate of a Toll-like receptor 7 ligand. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 3990−3995.

(18) Gadd, A. J.; Greco, F.; Cobb, A. J.; Edwards, A. D. Targeted Activation of Toll-Like Receptors: Conjugation of a Toll-Like Receptor 7 Agonist to a Monoclonal Antibody Maintains Antigen Binding and Specificity. *Bioconjugate Chem.* **2015**, *26*, 1743−1752.

(19) Liu, H.; Moynihan, K. D.; Zheng, Y.; Szeto, G. L.; Li, A. V.; Huang, B.; Van Egeren, D. S.; Park, C.; Irvine, D. J. Structure-based programming of lymph-node targeting in molecular vaccines. *Nature* **2014**, *507*, 519−522.

(20) Ali, O. A.; Verbeke, C.; Johnson, C.; Sands, R. W.; Lewin, S. A.; White, D.; Doherty, E.; Dranoff, G.; Mooney, D. J. Identification of immune factors regulating antitumor immunity using polymeric vaccines with multiple adjuvants. *Cancer Res.* **2014**, *74*, 1670−1681.

DOI: 10.1021/acs.jmedchem.6b00489
J. Med. Chem. XXXX, XXX, XXX−XXX