Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

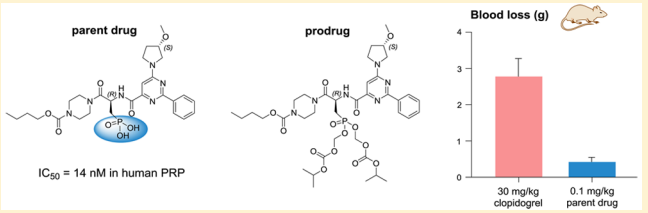
4-((*R*)-2-{[6-((*S*)-3-Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-3-phosphonopropionyl)piperazine-1-carboxylic Acid Butyl Ester (ACT-246475) and Its Prodrug (ACT-281959), a Novel $P2Y_{12}$ Receptor Antagonist with a Wider Therapeutic Window in the Rat Than Clopidogrel

Eva Caroff,* Francis Hubler, Emmanuel Meyer, Dorte Renneberg, Carmela Gnerre, Alexander Treiber, Markus Rey, Patrick Hess, Beat Steiner, Kurt Hilpert, and Markus A. Riederer

Drug Discovery and Preclinical Research and Development, Actelion Pharmaceuticals Ltd., Gewerbestrasse 16, CH-4123 Allschwil, Switzerland

Supporting Information

ABSTRACT: Recent post hoc analyses of several clinical trials with $P2Y_{12}$ antagonists showed the need for new molecules being fully efficacious as antiplatelet agents and having a reduced propensity to cause major bleeding. We have previously reported the discovery of the 2-phenylpyrimidine-4-carboxamide analogs as $P2Y_{12}$ antagonists with nanomolar potency in the disease-relevant platelet aggregation assay in human plasma. Herein we present the optimization steps that led to the discovery of clinical candidate ACT-246475 (**30d**). The key step was the replacement of the carboxylic acid functionality by a phosphonic acid group which delivered the most potent molecules of the program. In addition, low in vivo clearance in rat and dog was achieved for the first time. Since the bioavailability of **30d** was low in rat and dog, we developed the bis((isopropoxycarbonyl)oxy)methyl ester prodrug (ACT-281959, **45**). Compound **30d** showed efficacy in the rat ferric chloride thrombosis model when administered intravenously as parent or orally as its prodrug **45**. Moreover, **30d** displays a wider therapeutic window as compared to clopidogrel in the rat surgical blood loss model.



INTRODUCTION

Following a lesion of the vascular wall, multiple signals will trigger activation of circulating platelets that subsequently adhere and aggregate at the site of injury, forming a seal to prevent blood loss. Similarly, rupture of an atherosclerotic plaque may lead to uncontrolled platelet thrombus formation. Vessel occlusion might occur followed by ischemia of the downstream located tissue with consecutive myocardial infarction (MI).[1] Inhibiting the activation and aggregation of platelets is the recommended therapeutic option to prevent atherothrombotic events in patients with atherosclerotic disease in the coronary, peripheral, or cerebrovascular circulation.[2,3]

The ADP receptor $P2Y_{12}$ is a key player in the process of activating platelets and aggregation.[4,5] Platelet activation is initially triggered by the interaction of glycoproteins on the surface of platelets with components of the subendothelial matrix or ruptured atherosclerotic plaque and is amplified by the release of soluble mediators, which also recruit new platelets. One of the most important soluble mediators is ADP, which is an agonist at the two G protein-coupled receptors (GPCRs), $P2Y_1$, and $P2Y_{12}$. Both $P2Y_1$ and $P2Y_{12}$ participate in the activation of integrin glycoprotein IIb/IIIa, which binds fibrinogen, contributing to the creation of the cross-linked aggregates that form a thrombus. Whereas $P2Y_1$ is important for the initial activation at low levels of ADP, it appears that $P2Y_{12}$ is required to amplify and sustain the responses leading to stable thrombus formation. The $P2Y_{12}$ receptor is a validated target in antiplatelet therapy for patients with acute coronary syndrome (ACS) or undergoing percutaneous coronary intervention (PCI).[6]

In patients with ACS, the current guidelines recommend a dual antiplatelet therapy with acetylsalicylic acid and a $P2Y_{12}$ receptor antagonist, such as clopidogrel, prasugrel, or ticagrelor.[7−9] Clopidogrel is an irreversible $P2Y_{12}$ receptor antagonist with proven antithrombotic efficacy.[10,11] Nevertheless, the antiplatelet effect is delayed by the need for conversion to the active metabolite, and additionally about 14−30% of the patients are resistant to clopidogrel treatment due to genetic polymorphisms of the CYP-450 enzymes responsible for its metabolism.[12,13] Prasugrel is also an irreversible $P2Y_{12}$ receptor antagonist from the thienopyridine class, with a different mechanism for the formation of the active metabolite.[14] It achieves stronger inhibition of platelet aggregation, but the superior efficacy versus clopidogrel in moderate- to high-risk patients with ACS undergoing PCI[15,16]

Received: June 17, 2015

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

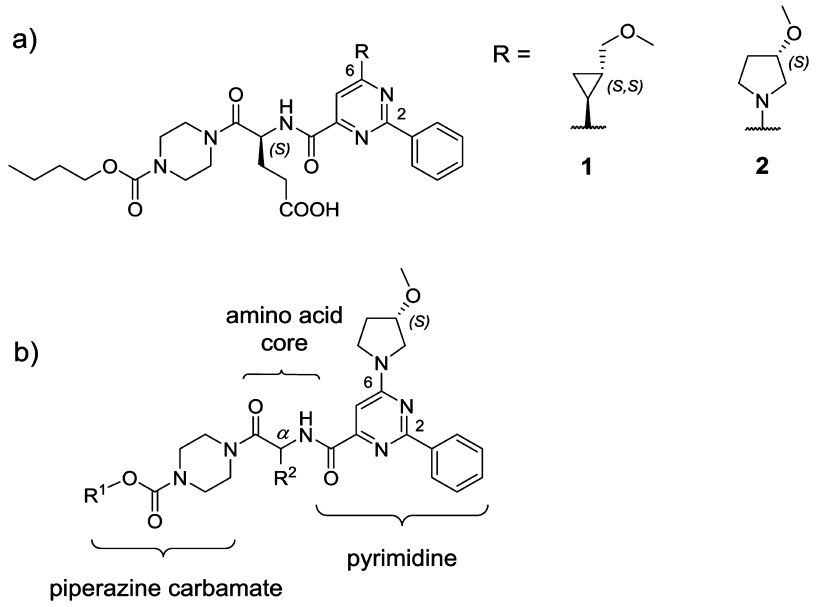
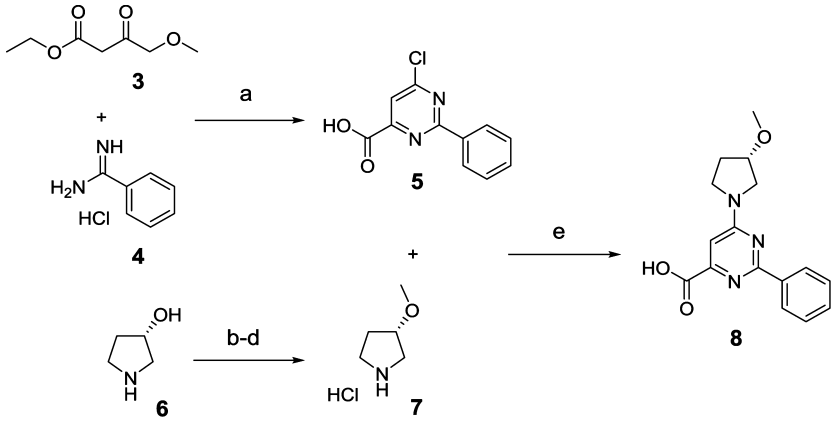


Figure 1. (a) Previous work: 2-phenylpyrimidine-4-carboxamide analogs **1** and **2** where the central core was set as L-glutamic acid. (b) This disclosure: general structure of (*S*)-6-(3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carboxamide analogs. R^1 is ethyl or *n*-butyl. R^2 and stereochemistry at the α carbon of the amino acid core are detailed in the tables.

Scheme 1. Preparation of Building Block 8[a]



[a]Reagents and conditions: (a) see ref 28, 4 steps, 51%; (b) BOC_2O, Et_3N, DCM, 100% (crude); (c) NaH, MeI, THF, 93% (crude); (d) HCl in EA, 95%; (e) DIPEA, THF, 60 °C, 4 days, 93%.

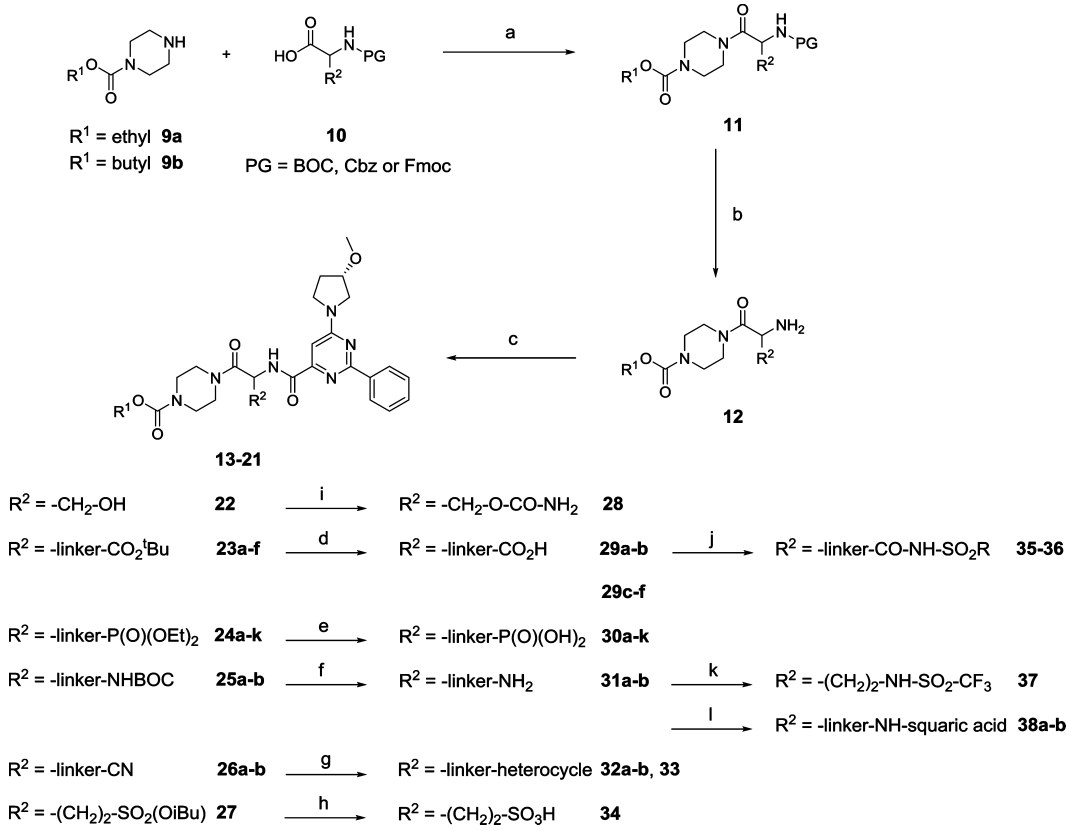
is associated with an increased bleeding risk.[17] Ticagrelor, the first reversible $P2Y_{12}$ receptor antagonist tested in clinical trials, demonstrated superiority over clopidogrel in the prevention of ischemic events with no statistically significant increase of bleeding.[18] Nevertheless, it is still discussed which $P2Y_{12}$ antagonist is the best choice and which subgroups of patients should be treated with clopidogrel, prasugrel, or ticagrelor.[19] Consequently, new $P2Y_{12}$ inhibitors combining high inhibition of platelet aggregation with low risk of bleeding are still actively searched.[20] Recent publication of X-ray structural information[21,22] as well as molecular modeling studies docking known $P2Y_{12}$ antagonists (including a close analog of the molecules disclosed herein)[23] will likely support this dynamic research field.

At Actelion, we aimed at discovering a reversible and selective $P2Y_{12}$ antagonist, which may provide an improved therapeutic window[24] compared to the benchmark drug clopidogrel. Indeed, reversible antagonism may cause less bleeding than irreversible blockade, since the receptor remains responsive to high local concentrations of ADP.[25,26] In addition, some of the excess bleeding associated with thienopyridines may be due to off-target effects, as shown by comparing clopidogrel and prasugrel treatment with genetic ablation of the $P2Y_{12}$ receptor in mice.[27]

We have previously reported the discovery of the 2-phenylpyrimidine-4-carboxamide series and the SAR of the substituent on position 6 of the pyrimidine ring.[28] We identified two sets of highly potent $P2Y_{12}$ antagonists, of which compounds **1** and **2** are the most active representatives (Figure 1a). The best molecules showed IC_{50} values in the low nanomolar range in the binding assay and in the light transmission aggregometry (LTA) assay. No inhibition of the major P450 enzymes or of the hERG channel was observed. Moderate to high clearance in the rat represented the main caveat of the two chemical classes. We set the substituent in position 6 of the pyrimidine ring to (*S*)-3-methoxypyrrolidin-1-yl as in analog **2** and the SAR of the R^2 group was explored (Figure 1b). In the present paper we report the properties of

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

Scheme 2. Preparation of $P2Y_{12}$ Antagonist Analogs 13−22, 28, 29a−f, 30a−k, 31a,b, 32a,b, 33−37, and 38a,b (for R^1 and R^2, See Tables 1, 2, and 4)[a]



[a]Reagents and conditions: (a) HOBt/EDC-Cl or HATU or PyBOP, DIPEA, DCM or DCM/THF, rt. (b) PG = BOC: TFA/DCM, rt, or HCl in EA or EA/dioxane. PG = Cbz: Pd/C (wet, 5%), H_2, EtOH or EtOH/AcOH, rt. PG = Fmoc: Et_2NH, DCM, rt. (c) **8**, similar conditions as described in (a); (d) TFA, DCM, rt, 22−100%; (e) TMSBr, MeCN, 0 °C to rt overnight, then DCM, water, rt, 1 h, 17−98%; (f) 4 M HCl in dioxane, rt, 30 min, 99−100%; (g) $ZnBr_2$, NaN_3, H_2O, 100 °C, 20 h, 22% (**32a,b**), or NH_2OH−HCl, $NaHCO_3$, MeOH, 70 °C, 24 h, then CDI, DBU, dioxane, 105 °C, 2 h, 2% (**33**); (h) Me_4NCl, DMF, 150 °C, overnight, 23%; (i) trichloroacetyl isocyanate, EtOH, 0 °C, 20 min, then $NaBH_4$, EtOH, 0 °C to rt overnight, 5%; (j) $SOCl_2$, rt, 1 h, then Et_3N, $CF_3SO_2NH_2$, DCM, rt, overnight, 6% (**35**), or $MeSO_2NH_2$, DCC, DMAP, DCM, 0 °C, 1 h, then rt, 20 h, 36% (**36**); (k) CF_3SO_2Cl, Et_3N, THF, 0 °C to rt, overnight, 11%; (l) 3,4-diethoxy-3-cyclobutene-1,2-dione, Et_3N, EtOH, rt, overnight, 52−65%, then 4 M HCl in dioxane, THF, 50 °C, 2 days, 52−56%.

(*S*)-6-(3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carboxamide analogs and the path that led to the discovery of a new and potent $P2Y_{12}$ antagonist with a unique efficacy/safety ratio.

RESULTS AND DISCUSSION

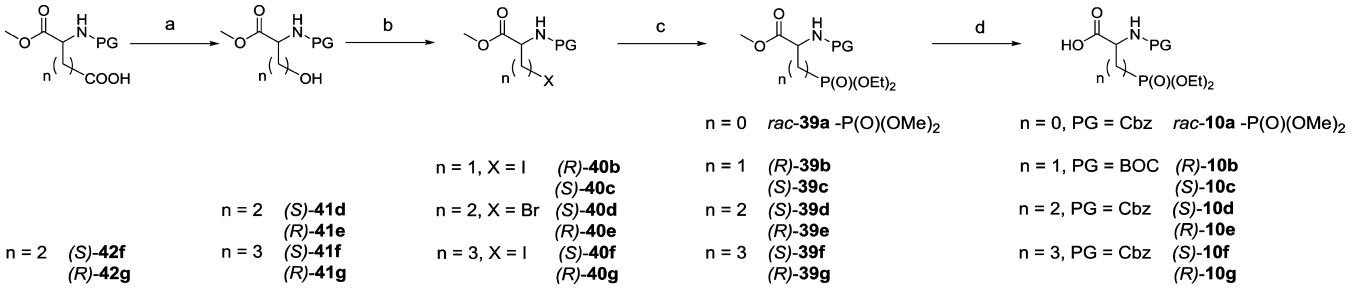
Chemistry. Retrosynthetically, the target molecules were divided into three parts, the piperazine carbamate, the central amino acid core, and the pyrimidine moiety. Scheme 1 shows the synthesis of the pyrimidinecarboxylic acid building block **8**. Intermediate **5** was obtained in four consecutive steps starting from commercially available ethyl 4-methoxy-3-oxobutanoate **3** and benzamidine **4**. Chloropyrimidine **5** was reacted with (*S*)-3-methoxypyrrolidine **7** at 60 °C in THF in the presence of DIPEA to give the acid **8**.

The synthesis of the $P2Y_{12}$ antagonists was designed and carried out as shown in Scheme 2. The chirality at the amino acid Cα was introduced with intermediates **10** and was conserved throughout the synthesis. The piperazine carbamate **9** was coupled to the amino acid **10** using as activating agent EDC-Cl/HOBt or HATU or PyBOP. The deprotection step performed on the resulting intermediate **11** was a HCl- or TFA-mediated BOC cleavage or a palladium catalyzed hydrogenolysis of Cbz or a Fmoc removal with Et_2NH. The second amide coupling step assembled the amine **12** and the acid building block **8** to give the final scaffold in good overall yield. Analogs **13−22** were directly obtained without any additional transformation. Compound **28** was obtained by reacting the hydroxyl group of analog **22** with trichloroacetyl isocyanate, followed by $NaBH_4$ mediated trichloroacetamide cleavage.[29]

When R^2 contained a *tert*-butyl protected acidic function such as in intermediates **23a−f**, TFA treatment in DCM afforded analogs **29a−f** in high yields. Hydrolysis of the diethyl phosphonate group of intermediates **24a−k** was achieved using TMSBr in anhydrous MeCN and provided analogs **30a−k**.[30] Analogs **31a,b** were quantitatively obtained upon treatment of BOC-protected amino derivatives **25a,b** with HCl in dioxane. The cyano group of intermediates **26a,b** was transformed into a tetrazole ring upon treatment with NaN_3 in the presence of $ZnBr_2$ to provide analogs **32a,b**.[31] Furthermore, the cyano group was reacted with NH_2OH and the resulting hydroxyamidine was subsequently cyclized to the oxadiazolone analog **33** upon treatment with CDI.[32] Analog **34** was obtained by cleavage of the isobutyl sulfonate using Me_4NCl.[33] Carboxylic acid analogs **29a,b** were further transformed into sulfonylamide analogs **35** and **36** using the corresponding primary

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

Scheme 3. Preparation of Intermediates 10a–g for the Synthesis of Phosphonic Acid-Containing Analogs 30a–k[a]



[a]Reagents and conditions: (a) isobutyl chloroformate, NMM, THF, −15 °C, 14 h, then $NaBH_4$, MeOH, −10 °C, 100% (crude) (**41f–g**); (b) CBr_4, PS–PPh_3, DCM, 0 °C, 2–3 h, 25–52% (**40d–e**), or imidazole, PPh_3, I_2, THF, 0 °C to rt, 11 h, 65–100% (**40f–g**); (c) $P(OEt)_3$, 120–130 °C, overnight, 57–100% (crude); (d) LiOH, MeOH, EtOH, H_2O, 0 °C, 1 h, 100% (**10a**), or rt, 3 h to overnight, 48–100% (**10e–g**), or LiOH, THF, H_2O, rt, 3 h to overnight, 72–100% (**10b–d**).

sulfonamide reagent in combination with DCC/DMAP or $SOCl_2$. Compound **37** was prepared starting from analog **31b** and reacted with CF_3SO_2Cl. Analogs **38a,b** were obtained by reacting 3,4-diethoxy-3-cyclobutene-1,2-dione with analogs **31a,b**.[34,35]

The key intermediates **10** leading to intermediates **23a–f**, **25a,b**, **26a,b**, and **27** and to final analogs **13–22** were commercially available. For the preparation of diethyl phosphonate derivatives **24a–k** leading to analogs **30a–k**, intermediates **10a–g** were synthesized as shown in Scheme 3. When the phosphonic acid group was directly attached to the amino acid backbone, racemic intermediate **10a** was easily prepared from commercially available racemic dimethyl phosphonate **39a** by saponification with aqueous LiOH. For the enantiomers **10b,c**, application of the Arbuzov protocol on the enantiomerically pure iodoserine derivatives **40b,c** gave the corresponding phosphonate analogs **39b,c**.[36] Whenever the appropriate protected amino acid derivative bearing a halogenated side chain was not commercially available, it was prepared starting from the hydroxyl analog. Enantiomers **41d,e** were reacted with CBr_4 in the presence of PPh_3, leading to enantiomers **40d** and **40e**, respectively. Likewise, hydroxyl precursors **41f–g** were reacted with iodine in the presence of PPh_3 and imidazole leading to enantiomers **40f** and **40g**.[37] In case the phosphonic acid group was attached to the amino acid backbone through a *n*-propyl linker (**10f–g**), the side chain carboxylic groups of protected (L)- and (D)-glutamic acid derivatives **42f–g** were reduced to the corresponding alcohols by activation with isobutyl chloroformate followed by $NaBH_4$ treatment affording enantiomers **41f–g**.[38]

Analogs **43** and **44** were prepared following a different route (not shown) and the synthetic protocols and schemes can be found in the Experimental Section and Supporting Information.

Structure–Activity Relationships (SARs). The affinity of the synthesized analogs to the $P2Y_{12}$ receptor was measured in the binding assay. The most potent compounds (generally IC_{50} below 100 nM) were further profiled ex vivo; i.e., inhibition of ADP-induced platelet aggregation was measured in human platelet-rich plasma (PRP) using light transmission aggregometry (LTA). An extensive screening of the amino acid side chain R^2 was undertaken, and a panel of representative examples are shown in Table 1. Generally, most R^2 residues were well tolerated, leading to moderate to good IC_{50} values in the binding assay. R^2 being hydrogen (**13**) and alkyl groups of moderate size (**14–16** and **18**) provided IC_{50} values in the hundred nanomolar range. Lipophilic groups with more steric bulk at the amino acid C*β* such as *tert*-butyl (**17**) led to micromolar activities. Polar functional groups such as carboxyl (**2**, **29a–f**), amine (**31a,b**), hydroxyl (**22**, **43**), ether (**19**), sulfone (**20**), primary amide, and carbamate (**21**, **28**) were introduced, varying the length of the linker to the amino acid C*α*. Among those, the most potent analogs with binding IC_{50} values below 100 nM were hydroxymethyl (**22**), aminomethyl (**31a**), and primary carbamate (**28**) derivatives and carboxylic acids (**2**, **29a,b**, **29e,f**). Interestingly, analog **29c**, where the stereochemistry at the amino acid core is reversed to (*R*), was 11-fold less active than the corresponding (*S*) analog **29a**. The trend favoring the (*S*) orientation was even more striking when the linker was longer, as shown with analogs (*S*)-**29b** and (*R*)-**29d**. In the LTA assay in human plasma, only carboxyl-containing analogs showed IC_{50} values below 100 nM. Finally, elongating R^1 from ethyl to *n*-butyl led to analogs with better affinity, increased lipophilicity, and slightly higher potency in human plasma (see **16** vs **15**, **2** vs **29b**, **29e** vs **29a**).

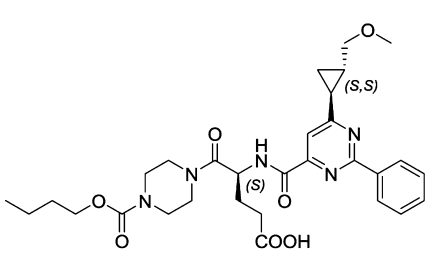
These results supported the prevalence of the carboxyl group to reach good potency in human plasma. Furthermore, the carboxyl group was most likely responsible for the medium clearance observed in the rat via excretion as unchanged drug in the bile (data not shown). Therefore, we synthesized a number of carboxylic acid bioisosteres[39,40] with the goal to achieve superior PK behavior while keeping the high potency. Biological data are reported in Table 2 (PK data are discussed in Table 4).

Indeed, apart from the sulfonic acid analog **34**, all carboxylic acid bioisosteres (**30f**, **32a–38b**, **44**) were potent in the binding assay ranging from 3.7 to 43 nM. In the LTA assay, analog **35** came out as an outlier with an IC_{50} value of 2100 nM. High lipophilicity might account for this low affinity, since the two analogs **35** and **37** having the highest clogP (>3.2) also show the highest plasma shift and modest potencies in the LTA assay. All other molecules showed medium to good potencies in the LTA assay similar to carboxyl-containing derivatives. Notably, analogs **30f** and **44** showed two of the lowest plasma shifts ever encountered in the course of our medicinal chemistry program, 1.4 and 1.7, respectively. In the next step, we focused on analog **30f** and expanded the phosphonic acid-containing compound class by varying the linker length and the stereochemistry at the amino acid core. The biological data are listed in Table 3.

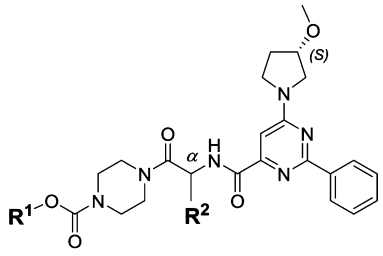
Analog **30a** (epimeric mixture) with the phosphonic acid directly attached to the amino acid backbone displayed moderate activity in the binding assay. The influence of the

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX–XXX

Table 1. Screening of the Amino Acid Side Chain R^2: Binding Affinity and LTA IC_{50} Values



1



2, 13-22, 28, 29a-f, 31a-b, 43

Compound	R^1	R^2	Stereochemistry [a]	Binding IC_{50} [b] (nM)	LTA IC_{50} [c] (nM)
1[d]	Bu		(*S*)	4.9 ± 1.0	31
2[d]	Bu		(*S*)	6.3 ± 2.0	21
13	Bu	-H	-	180 ± 40	
14	Bu		(*S*)	160 ± 40	
15	Et		(*S*)	220 ± 50	
16	Bu		(*S*)	110 ± 10	
17	Bu		(*S*)	2800 ± 900	
18	Bu		(*S*)	360 ± 100	
19	Bu		(*S*)	240 ± 30	
20	Et		(*S*)	960 ± 170	
21	Bu		(*S*)	150 ± 30	
22	Bu		(*S*)	34 ± 6	600
28	Bu		(*S*)	87 ± 16	1300
29a	Et		(*S*)	39 ± 9	140
29b	Et		(*S*)	35 ± 8	32
29c	Et		(*R*)	440 ± 110	
29d	Et		(*R*)	>10000	
29e	Bu		(*S*)	8.2 ± 2.3	91
29f	Et		(*S*)	87 ± 14	230
31a	Bu		(*S*)	83 ± 20	480
31b	Bu		(*S*)	600 ± 230	
43	Et		(*S*)	2500 ± 700	

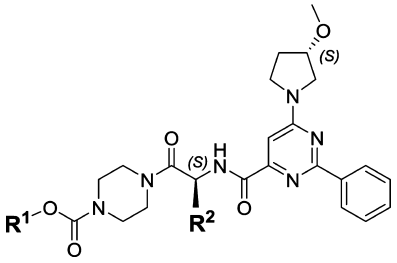
[a]Absolute stereochemistry at the amino acid Cα. [b]CHO cells expressing recombinant $P2Y_{12}$ receptors incubated with [^{33}P]-2MeSADP and compound. Data are presented as the mean ± SEM of n = 2–4. [c]Incubation for 2 min in human PRP with 3 μM ADP. n = 1. [d]See ref 28.

stereocenter was analogous to what was observed with carboxyl-containing analogs **29a–d** favoring the forward orientation of R^2, irrespective of the linker length. Indeed, analogs extending R^2 in the backward direction (as depicted in Table 3), **30e**, **30g**, **30i**, and **30k**, were at least 10 times less active in the binding assay than the corresponding analogs **30d**, **30f**, **30h**, and **30j**. Unexpectedly, **30c** was only twice less active as **30b**. In general, elongation of R^1 from ethyl to butyl led to increased binding affinity. However, notable exceptions are **30e** and **30i** wherein R^1 is butyl which are equally or less active than **30c** and **30g** wherein R^1 is ethyl. Overall, refinements of the SAR led to five phosphonic acid-containing analogs **30b–d**, **30h**, and **30j** that were extremely potent in human plasma.

Pharmacokinetic Analysis. On the basis of in vitro and ex vivo potencies, a selection of analogs from Tables 1, 2, and 3 were progressed for evaluation of PK parameters in rat (see Table 4; data for **1** and **2** are indicated for comparison). Systemic clearance was close to liver blood flow for hydroxymethyl **22**, benzoic acid-containing analog **29f**, compounds **32a,b**, **36**, **38b**, and **44** containing a carboxylic acid bioisostere other than a phosphonic acid and analog **30j** extending a phosphonic acid group on a *n*-propyl linker. However, compounds **30c**, **30d**, and **30h** extending a phosphonic acid group on shorter linkers showed low clearances of 3.7, 2.1, and 5.4 mL/(min·kg), respectively. With the exception of hydroxyl-containing analog **22**, volumes of distribution at steady state were in the range of total body water. Consequently half-lives were short, with a maximum of 1.8 h for **30d**. Oral bioavailability was 16% and 28% for **22** and **29f**, respectively, but below 3% for all molecules containing a

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX–XXX

Table 2. Replacement of the Carboxylic Acid by Acid Bioisosteres: Binding Affinity and LTA IC_{50} Values, Plasma Shift, and clogP



Compound	R^1	R^2 [a]	Binding IC_{50} [b] (nM)	LTA IC_{50} [c] (nM)	Plasma shift[d]	clogP
30f	Et		15 ± 4	21	1.4	-1.3
32a	Et		24 ± 3	130	5.3	1.1
32b	Et		38 ± 8	380	10	1.5
33	Et		36 ± 9	230	6.4	2.2
34	Et		120 ± 30			0.26
35	Et		43 ± 5	2100	49	3.2
36	Et		22 ± 6	62	2.8	1.6
37	Bu		16 ± 6	340	21	4.5
38a	Bu		11 ± 3	95	8.7	1.1
38b	Bu		3.7 ± 0.3	30	8.1	1.5
44	Et		14 ± 3	24	1.7	2.0

[a]Absolute stereochemistry at the amino acid Cα is (*S*). [b]CHO cells expressing recombinant $P2Y_{12}$ receptors incubated with [^{33}P]-2MeSADP and compound. Data are presented as the mean ± SEM of n = 2−4. [c]Incubation for 2 min in human PRP with 3 μM ADP. n = 1. [d]Plasma shift = (LTA IC_{50})/(binding IC_{50}).

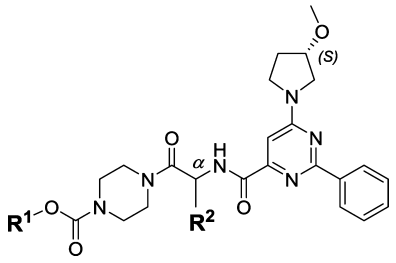
carboxylic acid bioisostere including a phosphonic acid. Whether the poor oral exposure of analogs **32b**, **36**, **38b**, and **44** is the result of high clearance combined with limited oral absorption cannot be concluded from these basic pharmacokinetic experiments. However, considering the predicted pK_a of the carboxylic acid bioisosteric moiety of the four analogs (**32b**, 4.86; **36**, 4.45; **38b**, 1.23; **44**, 8.06),[41] one can hypothesize that the oral exposure of **38b** below limit of quantification is likely due to the ionized state of the molecule at the pH of the upper intestine (6.8) leading to poor permeability, and to a clearance close to liver blood flow. Likewise, since the phosphonic acid group bears at least one negative charge at intestinal pH,[42] analogs **30c−d** and **30h** may not readily undergo passive diffusion across cellular membranes and intestinal mucosa, resulting in low oral bioavailability, despite a low in vivo clearance. As a conclusion, the new $P2Y_{12}$ antagonists described in Table 4 show poorer bioavailabilities and shorter half-lives compared to compound **1**. Interestingly, the phosphonic acid-containing analogs **30c**, **30d**, and **30h** exhibit markedly lower clearances than compound **1**. Among these three phosphonates, analog **30d** was deemed the best combination of potency, clearance, and exposure in the rat. Therefore, it was further progressed together with compound **1**.

In Vivo Efficacy/Safety Assessment. Compounds **1** and **30d** were tested for in vivo efficacy and safety and were compared with clopidogrel. The ferric chloride model was performed in Wistar rats to determine the antithrombotic efficacy of the two $P2Y_{12}$ antagonists. The deposition of a 20% aqueous ferric chloride solution onto the right carotid artery of the rat induced a rapid decrease in carotid blood-flow velocity. Within 11 ± 1 min, the flow was not measurable anymore due to histologically confirmed thrombotic occlusion of the artery (data not shown). In vehicle treated rats, the vessel injury resulted in 100% thrombotic occlusion (Figure 2, vehicle). Oral treatment with clopidogrel as well as intravenous bolus injection of compound **1** dose-dependently prevented the ferric chloride-induced thrombus formation (Figure 2A,B). At the highest doses of 30 mg/kg of clopidogrel and 1 mg/kg of compound **1**, thrombus formation was fully blocked. The observed increase of 26% in blood flow at 30 mg/kg of clopidogrel was attributed to off-target effects at the vessel wall.[27] Intravenous bolus injection of compound **30d** dose-dependently prevented the ferric chloride-induced thrombotic occlusions (Figure 2C). At the highest dose tested (0.1 mg/kg), full antithrombotic efficacy was achieved and no increase in carotid blood flow versus baseline flow was measured. Thus, the $P2Y_{12}$ antagonists **1** and **30d** were shown to be potent antithrombotic agents. Compound **30d** showed maximal efficacy in this rat model at about 10-fold lower dose than compound **1**.

To determine the efficacy/safety ratio, the two compounds and clopidogrel were profiled in a rat surgical blood loss model at doses inducing the maximal antithrombotic effect (Figure 3). The surgical blood loss was defined as the amount of blood

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

Table 3. Replacement of the Carboxylic Acid by a Phosphonic Acid: Binding Affinity and LTA IC_{50} Values



Compound	R^1	R^2	Stereochemistry [a]	Binding IC_{50} [b] (nM)	LTA IC_{50} [c] (nM)
30a	Bu		2 epimers	120 ± 20	
30b	Et		(*R*)	4.9 ± 0.4	10
30c	Et		(*S*)	12 ± 2	11
30d	Bu		(*R*)	1.0 ± 0.2	8.0
30e	Bu		(*S*)	12 ± 2	210
30f	Et		(*S*)	15 ± 4	21
30g	Et		(*R*)	340 ± 40	
30h	Bu		(*S*)	2.2 ± 0.3	1.0
30i	Bu		(*R*)	1100 ± 200	
30j	Bu		(*S*)	1.7 ± 0.5	4.0
30k	Bu		(*R*)	190 ± 60	

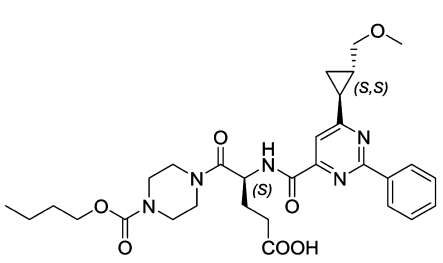
[a]Absolute stereochemistry at the amino acid Cα. [b]CHO cells expressing recombinant $P2Y_{12}$ receptors incubated with [^{33}P]-2MeSADP and compound. Data are presented as the mean ± SEM, with n = 2–4. [c]Incubation for 2 min in human PRP with 3 μM ADP. n = 1.

oozing out of the rat spleen after placing a standardized surgical wound. In the absence of any antithrombotic treatment (vehicle group), the surgical blood loss was quantified at 0.16 and 0.18 g of blood, after oral gavage and intravenous bolus injection, respectively. At maximal antithrombotic dose of clopidogrel (30 mg/kg), the surgical blood loss was increased from 0.16 to 2.8 g of blood. At maximal antithrombotic dose of compound **1** (1 mg/kg), the surgical blood loss was increased from 0.18 to 1.5 g of blood. In striking contrast, at maximal antithrombotic dose of compound **30d** (0.1 mg/kg), the observed surgical blood loss was only 0.4 g. On the basis of these in vivo data, compound **30d**, at maximal antithrombotic efficacy, displayed a wider therapeutic window in the rat than clopidogrel and compound **1**. The clear difference in blood loss between compound **30d** and clopidogrel at maximal efficacious doses warranted further characterization of **30d** toward clinical development.

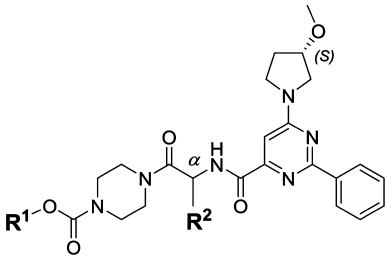
Preclinical Characterization of Compound 30d. In radioligand displacement assays, [^{33}P]-2MeSADP was able to displace prebound compound **30d** and to reach maximal binding capacity, hence confirming **30d** as a reversible $P2Y_{12}$ antagonist. During SAR optimization, inhibition of platelet aggregation was assessed in human PRP, with incubation time of 2 min and 3 μM ADP for platelet activation. In this setting compound **30d** displayed an IC_{50} value of 8.0 nM. As a preparation for clinical profiling, assay conditions were adapted to the protocols used in hospital setting. Using a PAP-8 aggregometer with a 20 min incubation time and 20 μM ADP, the IC_{50} value was confirmed to be 14 nM (n = 8).[43] No antagonistic activity on genetically related P2Y receptors was measured. Up to 10 μM concentration of **30d** did not interfere with agonist binding to $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{13}$. No antagonistic activity was detected on $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_7$ receptors. Furthermore, compound **30d** was tested for potential off-target activity against a broad panel of receptors, transporters, and enzymes. Radioligand displacement assays were performed against 68 targets, including GPCRs, ion channels, and transporters, and activity assays were performed with 30 enzymes. To expand the profiling and include potential agonism, PathHunter technology was used on a panel of 161 recombinant GPCRs. All assays were performed in duplicate using a final concentration of 10 μM. Compound **30d** did not interact with the target in any of these assays. At 50 μM concentration of **30d**, less than 10% reduction of the hERG K^+ current was observed.[44] Compound **30d** did not inhibit cytochrome P450 enzymes in assays assessing competitive inhibition using human liver microsomes up to 50 μM and did not exert time-dependent inhibition of CYP2C9, CYP2D6, or CYP3A4.[45] Plasma protein binding in animal species used for preclinical safety testing, i.e., rat, dog, as well as in man, was above 99%. In liver microsomes of human, rat, dog, rabbit, and cynomolgus monkey, the turnover was very low and the cleavage of the −CO−N− bond of the carbamate moiety was not observed. Similar to rat (Table 4), compound **30d** showed a low plasma clearance and volume of distribution in the dog, resulting in a half-life of 4.8 h. Oral bioavailability in the dog was around 1%. Compound **30d** successfully passed preclinical toxicology and safety studies.

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

Table 4. Rat Pharmacokinetic Profiles of Selected $P2Y_{12}$ Antagonists[c]



1



2, 22, 29f, 30c-d, 30h, 30j, 32a-b, 36, 38b, 44

Compound	R^1	R^2	CL[a] (mL/min·kg)	Vss (L/kg)	$T_{1/2}$ (h)	AUC_{oral} (ng·h/mL)	$Cmax_{oral}$ (ng/mL)	F (%)
1[b]	Bu		17	0.41	2.0	3490	4430	33
2[b]	Bu		24	0.50	1.2	701	1010	9.5
22	Bu		58	3.1	1.1	463	300	16
29f	Et		56	0.49	0.23	833	682	28
30c	Et		3.7	0.17	1.1	132	37.5	<1
30d	Bu		2.1	0.18	1.8	244	61.2	<1
30h	Bu		5.4	0.20	0.56	51.0	31.0	<1
30j	Bu		40	0.33	0.43			
32a	Et		45	0.43	0.39			
32b	Et		59	0.78	0.55	36.3	41.4	1.3
36	Et		37	0.30	0.28	57.8	72.8	1.3
38b	Bu		65	0.99	0.41	BLQ	BLQ	
44	Et		32	0.50	0.84	145	289	2.8

[a]Low clearance: CL < 30% of liver blood flow (LBF). Moderate clearance: 30% < CL < 70% LBF. High clearance: >70% LBF. LBF in rat is 55−90 mL/(min·kg). For correct classification, plasma clearance needs to be converted to blood clearance considering the blood/plasma partitioning ratio, which in the absence of data is assumed to be 1. [b]See ref 28. [c]Male Wistar rats. Dose: iv infusion at 1 mg/kg (n = 1 or 2); po at 10 mg/kg (n = 2 or 3). BLQ: below limit of quantification (~1 ng/mL). SDs and ranges are provided as Supporting Information.

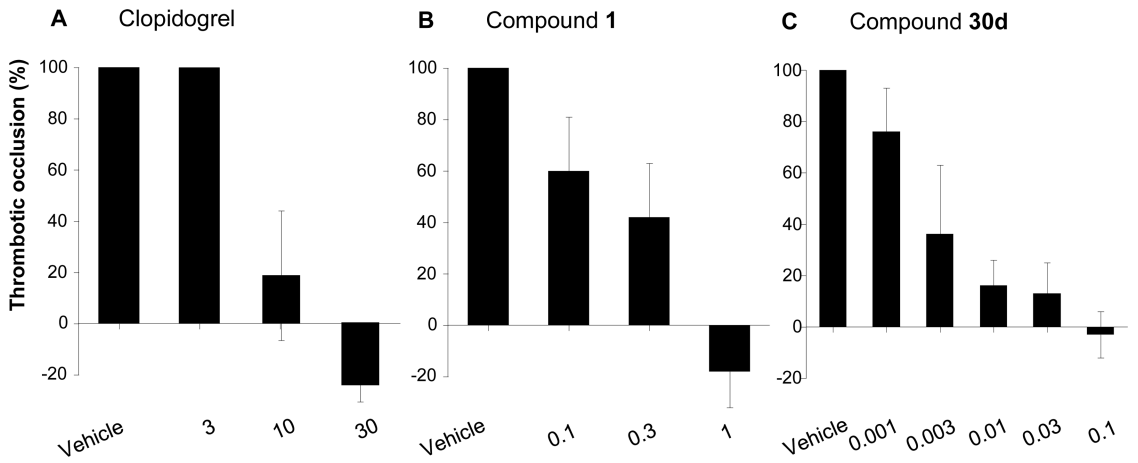


Figure 2. Effects of (A) clopidogrel, (B) compound **1**, and (C) compound **30d** on thrombotic occlusion after $FeCl_3$-induced arterial injury in anesthetized Wistar rats (n = 3−4 per group (A), n = 5−11 per group (B), and n = 3−11 per group (C)). Doses are in mg/kg. Thrombotic occlusion is defined as % reduction in blood flow velocity from baseline, 30 min after arterial injury. Data are presented as the mean ± SEM. (A) Vehicle and clopidogrel were administered orally as single dose 2 h before arterial injury. Vehicle was saline 9 g/L, pH adjusted to 2.5 with 3 M HCl. (B, C) Vehicle and compounds **1** and **30d** were administered as intravenous bolus injection 5 min before arterial injury. Vehicle was PEG 400 (7.5%), PG (7.5%), Cremophor (5%), phosphate buffer, pH = 7.4.

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

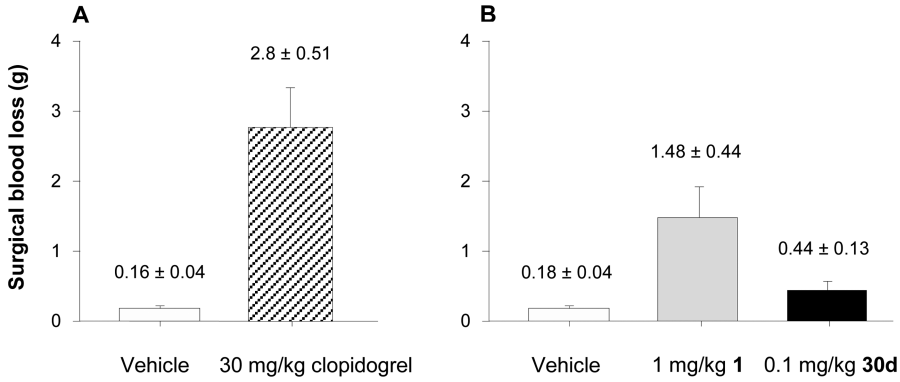


Figure 3. Effects of (A) clopidogrel and (B) compounds **1** and **30d** on surgical blood loss after spleen puncture in anesthetized Wistar rats (n = 8–14 per group (A) or n = 5–20 per group (B)). Surgical blood loss was measured 30 min after spleen puncture. Data are presented as the mean ± SEM. (A) Vehicle and clopidogrel were administered orally as single dose 2 h before spleen puncture. Vehicle was saline 9 g/L, pH adjusted to 2.5 with 3 M HCl. Open bar = vehicle. Hatched bar = clopidogrel at 30 mg/kg. (B) Vehicle and compounds **1** and **30d** were administered as intravenous bolus injection 5 min before spleen puncture. Vehicle was PEG 400 (7.5%), PG (7.5%), Cremophor (5%), phosphate buffer, pH = 7.4. Open bar = vehicle. Gray bar = compound **1** at 1 mg/kg. Black bar = compound **30d** at 0.1 mg/kg.

In Vivo Efficacy of Compound 45 as Prodrug of Compound 30d. On the basis of in vitro metabolic studies and PK data in rat and dog, we expected a low oral absorption of compound **30d** in human. Therefore, we decided to mask the phosphonic acid group as a double ester prodrug.[46,47] Briefly, we synthesized a variety of phosphonate ester prodrugs and evaluated the chemical stability, metabolic stability in plasma and liver microsomes, and PK in rat and dog. Finally, the bis((isopropoxycarbonyl)oxy)methyl ester prodrug **45** (Figure 4) was selected for preclinical development. An overview of the synthetic procedures and optimization program toward this prodrug will be published separately.

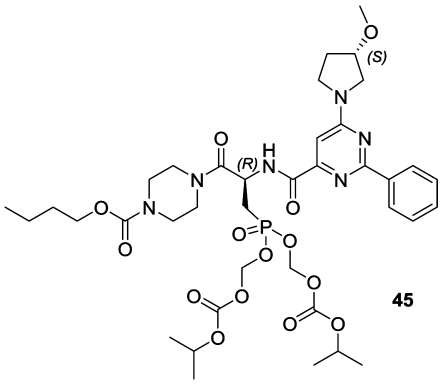


Figure 4. Compound **45**, bis((isopropoxycarbonyl)oxy)methyl ester prodrug of **30d**.

To confirm the oral antithrombotic efficacy of compound **45**, the ferric chloride model in Wistar rats was repeated comparing oral administration of the prodrug **45** with intravenous infusion[48] of the parent drug **30d**. The prodrug **45** was below analytical detection limits in rat blood after oral gavage. Plasma concentration of the active **30d** was measured in the two experiments at the time of vessel injury and 30 min later. In vehicle treated rats, the vessel injury resulted in complete thrombotic occlusion of the artery, i.e., 100% decrease of blood flow (Figure 5, vehicle). Intravenous infusion of 0.01 mg/kg/50 min of analog **30d** led to a blood flow velocity decrease 30 min after injury of 35 ± 16% compared to baseline flow in the absence of vessel injury; i.e., thrombotic occlusion was inhibited by 65 ± 16% (Figure 5A). A similar reduction in blood flow velocity 30 min after injury of 47 ± 18% (i.e., inhibition of thrombotic occlusion by 53 ± 18%) was measured after oral administration of a 1 mg/kg dose of analog **45** (Figure 5B). During the 30 min time period of the experiments, the plasma concentration of **30d** was between 72 and 141 ng/mL for the 0.01 mg/kg intravenous dose of **30d** (Figure 6A) and between 101 and 190 ng/mL for the 1 mg/kg oral dose of **45** (Figure 6B). Plasma concentrations of the parent drug **30d** were comparable in both experiments, which supports the observed similar antithrombotic efficacy. Therefore, one can reasonably conclude that (1) the prodrug **45** liberates the parent drug **30d** in circulation, (2) the observed antithrombotic effect after oral administration of **45** is entirely due to the parent drug **30d**, and (3) a 1 mg/kg oral dose of **45** leads to a comparable antithrombotic efficacy as a 0.01 mg/kg iv dose of **30d** in the ferric chloride rat thrombosis model. Compound **45** showed no toxicity or safety signals in preclinical studies.

CONCLUSION

The first optimization sequence of the 2-phenylpyrimidine-4-carboxamide scaffold led to analogs **1** and **2** showing high potencies in both the binding and LTA assays albeit with moderate PK profiles in rat and dog (data not shown). In a second step, keeping the newly discovered (*S*)-3-methoxypyrrolidinyl substituent in position 6 of the pyrimidine ring, we focused on the amino acid core. Introduction of various functional groups on the amino acid side chain confirmed our initial observation that an acidic group was needed to reach high potency in human plasma. We also suspected the acidic group to be responsible for the medium clearance observed in vivo in rat and dog. Therefore, we embarked on a third optimization sequence with the goal to identify a bioisosteric replacement for the carboxylic acid functionality. The phosphonic acid group was found beneficial, since it delivered the most potent molecules in the LTA assay as well as low in vivo clearance in the rat. Consequently, the phosphonic acid-containing analog **30d** was selected for preclinical development. In vitro, it was shown to be a reversible and highly selective $P2Y_{12}$ antagonist, potent in human plasma at a low nanomolar concentration. **30d** dose-dependently blocked thrombus formation in the rat ferric chloride model and displayed a wide therapeutic window. Overall, **30d** exhibited a very safe

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

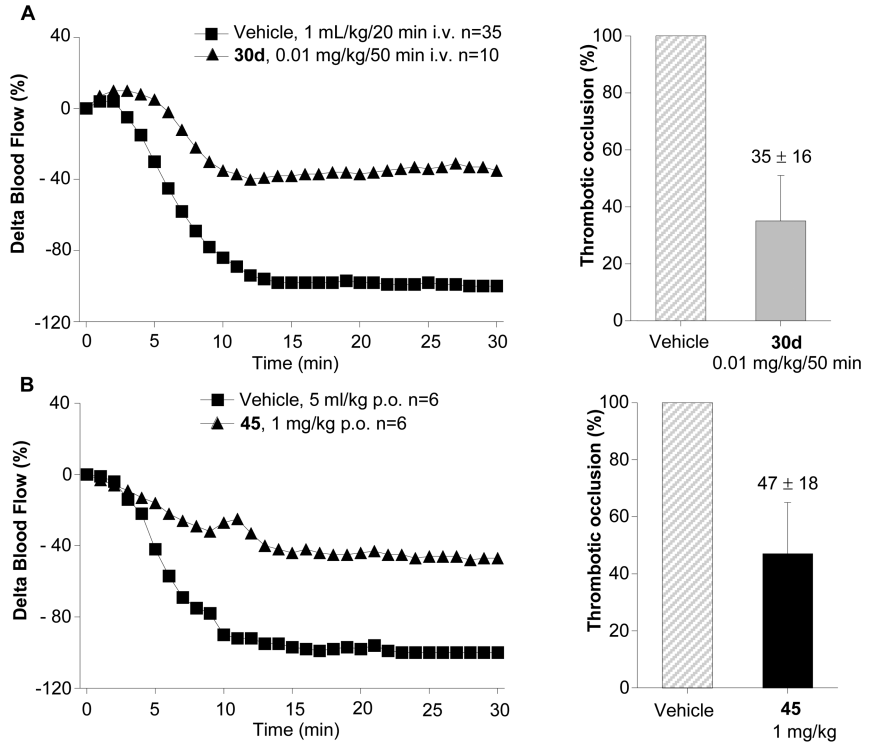


Figure 5. Effects of (A) compound **30d** and (B) compound **45** on thrombotic occlusion after $FeCl_3$-induced arterial injury in anesthetized Wistar rats (n = 10−35 per group (A) or n = 6 per group (B)). Thrombotic occlusion is defined as % reduction in blood flow velocity from baseline, 30 min after arterial injury. Data are presented as the mean ± SEM. (A) Vehicle and compound **30d** were administered as continuous intravenous infusion over 50 min at the rate of 1 (mL/kg)/20 min, starting 20 min before arterial injury. Vehicle was PEG 400 (7.5%), PG (7.5%), Cremophor (5%), phosphate buffer, pH = 7.4. (B) Vehicle and compound **45** were administered orally as single dose 1 h before arterial injury. Vehicle was PEG 400 (7.5%), PG (7.5%), Cremophor (5%), water, 5 mL/kg.

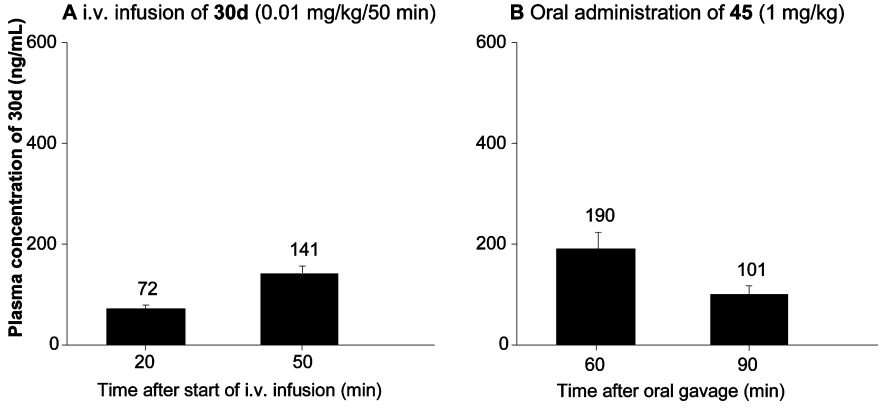


Figure 6. Plasma concentrations of the active drug **30d** (A) at 20 and 50 min after starting iv infusion of **30d** in the experiment described in Figure 5A and (B) at 60 and 90 min after oral gavage of **45** in the experiment described in Figure 5B. Plasma was obtained from arterial blood sampling (EDTA). Experimental details are given in Figure 5.

profile in in vitro and in vivo preclinical experiments. However, on the basis of in vitro metabolic studies and PK data in rat and dog, we expected a low oral absorption of compound **30d** in human. Therefore, we developed the bis((isopropoxycarbonyl)-oxy)methyl ester prodrug **45** which showed antithrombotic efficacy after oral administration in the rat ferric chloride model. Both compounds **45** and the parent drug **30d** entered clinical studies in healthy volunteers.[43]

EXPERIMENTAL SECTION

$P2Y_{12}$ Radioligand Displacement Assay (Binding Assay). Recombinant Chinese hamster ovary (CHO) cells with recombinant expression the human $P2Y_{12}$ gene were grown in Ham's F12 medium with phenol red and L-glutamine (Gibco no. 21765-029) containing 250 μg/mL Geneticin (Gibco), 100 μg/mL gygromycin B (Invitrogen), and 10% FCS Sera Plus (PAN Biotech; Aidenbach, DE). Cells were cultivated in 96-well plates at a density of 75 000 cells/well in medium supplemented with apyrase 0.1 U/mL and grown overnight at 37 °C and 5% CO_2. The next day, the cells were washed three times with binding buffer (Ham's F12 medium (Gibco) with phenol red and L-glutamine (Gibco no. 21765-029), supplemented with 0.01% protease-free BSA). The assay was performed as follows: First, 100 μL of binding buffer was added to each well. Second, 50 μL of binding buffer containing 5% DMSO or compound was added. Third, 100 μL of 0.25 nM radioligand [^{33}P]-2MeSADP (PerkinElmer CUS55563) was added to each well and incubated for 90 min at room

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

temperature, with gentle agitation. The assay was stopped by three washes with binding buffer, followed by addition of 200 μL of scintillation liquid (Microscint 40, Packard) and quantification in a scintillation counter (Packard, Top Count NXT).

In Vitro Light Transmission Aggregometry (LTA) Assay.[49] *Preparation of Platelet-Rich Plasma (PRP).* Blood was collected from healthy, aspirin-free volunteers, after obtaining informed consent, by venipuncture of the large arm vein. The blood was immediately anticoagulated with 100 μM direct thrombin inhibitor napsagatran[50] (RO 46-6240, kindly provided by Hoffmann La Roche Ltd.). With this anticoagulant, the physiological calcium concentration is maintained. 10 mL aliquots of blood were centrifuged at 200*g* (1000 rpm) for 10 min. The top layer, representing the platelet-rich plasma (PRP), was carefully transferred into a fresh tube. The remaining blood was again centrifuged at 1800*g* (3000 rpm) for 10 min. The top layer constituting the platelet-poor plasma (PPP) was carefully transferred into a new tube. Finally, platelet numbers in PRP were determined using a CASY cell counter system (Roche Diagnostics (Schweiz) AG) and, if necessary, platelet count was adjusted to 314 × 106 platelets/mL using PPP from the same donor.

In Vitro Platelet Aggregation Assay Using LTA. All experiments were performed with a four-channel aggregometer (Chrono-Log Corporation, Lumi-Aggregometer 490-4D) with the AggroLink software package. First, 400 μL of PRP at 314 × 10^6 Plts/mL were placed into a siliconized glass cuvette (ref 70180 from Probe and Go). With napsagatran-anticoagulated PRP, 85 μL of Tyrode's solution (NaCl 133 mM, KCl 2.7 mM, $NaHCO_3$ 11.9 mM, NaH_2PO_4−H_2O 0.36 mM, Hepes 10 mM, glucose 5 mM, BSA 0.1%, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, pH 7.4) was added, mixed, and equilibrated at 37 °C for at least 15 min without stirring. Second, 5 μL of DMSO or test compound in DMSO was added and incubated for 2 min at 37 °C with brief stirring for 2 min only. The samples were stirred with siliconized magnetic stir bars (ref 70188 from Probe and Go) at 1100 rpm at 37 °C. In parallel, the aggregometer system was calibrated with PRP (0% aggregation control = baseline) and PPP (100% aggregation control). Third, aggregation was started by addition of 10 μL of ADP (during SAR optimization at 3 μM final concentration) to the cuvette. The addition of these various volumes resulted in final concentration of 250 × 10^6 Plts/mL. Aggregation was monitored for up to 8 min. The results are expressed as the maximal change in light transmission at peak response (=maximal aggregation response) or as the change in light transmission at 6 min after ADP addition (=final aggregation response).[51] IC_{50} values were calculated using XLfit software. All compounds were tested in parallel with one reference compound. The geometric mean IC_{50} value for this reference compound was 167 nM with a 95% confidence interval of 143−196 nM over 19 independent IC_{50} determinations.

Pharmacology. Normotensive male Wistar rats delivered from Harlan Netherlands were group-housed in climate-controlled conditions with a 12 h light/dark cycle in accordance with the guidelines of the Basel Cantonal Veterinary Office, with appropriate environmental enrichment (shelter, polycarbonate rat tunnel, chew blocks, aspen bricks). All animals were maintained under identical conditions and had free access to drinking water and normal pelleted food (no. 3336, Provimi Kliba SA, Switzerland). Plavix, 75 mg, from Sanofi/BMS (clopidogrel) was purchased in a pharmacy and stored at room temperature (max, 25 °C) protected from light. Clopidogrel was dissolved in saline (9 g/L, containing 3 μL/mL of 3 M HCl) at a concentration of 6 mg/mL pH = 2.5. Compounds **1** and **30d** were dissolved in PEG 400 (7.5% v/v), PG (7.5% v/v), Cremophor (5% v/v) (Cremophor EL, Fluka no. 27963), in phosphate buffer pH = 7.4. Compound **45** was dissolved in PEG 400 (7.5%), PG (7.5%), Cremophor (5%), water.

Rat $FeCl_3$ Thrombosis Model. The $FeCl_3$ model was performed based on previous reports.[52] Specifically, after an acclimatization period of at least 7 days, the rats were anesthetized by an intraperitoneal injection of thiobutabarbital sodium salt hydrate at a dose of 150 mg/kg (Inactin, Sigma-Aldrich GmbH). The animals were then placed on a thermostatically controlled heating table to maintain body temperature at 36−38 °C. After tracheotomy, a catheter was inserted into the right femoral vein for bolus injection or continuous infusion at a rate of 1 (mL/kg)/20 min of compounds **1** and **30d** or vehicle. A second catheter was inserted in the right femoral artery to measure mean arterial pressure (MAP) and heart rate (HR). The right carotid artery was gently dissected free of connective tissue, and a silastic tubing flow transducer (D-20-0.8mm, Triton Technologies Inc.) was placed on the artery for blood flow velocity measurement. Ferric chloride ($FeCl_3$, Fluka no. 44943) was dissolved in distilled water at a concentration of 200 mg/mL (20%). At 5 min after bolus injection or 20 min after infusion start of vehicle or compound, two filter papers (Tempo handkerchief, 4 mm × 4 mm) were soaked in this solution and placed on the carotid artery anterior to the flow transducer for 5 min to induce thrombus formation. Mean arterial pressure (MAP), heart rate (HR), and carotid blood flow velocity were continuously recorded on a PowerLab data acquisition system (IOX data acquisition, Emka Technologies, France) during 30 min after removal of the ferric chloride filter papers by using a IOX software (Emka Technologies, France). Data were exported to a Dell Dimension 733R computer for analysis (Datanalyst, version 2.10.17, Emka Technologies, France). At the end of the experiment, rats were euthanized by an intravenous infusion of pentobarbital (100 mg/kg, Esconarkon, Streuli Pharma AG, Switzerland). Clopidogrel and its vehicle were administered by oral gavage at 5 mL/kg 2 h before $FeCl_3$-soaked filter papers were applied to the carotid artery. The time for clopidogrel gavage was determined in a pilot experiment where the $FeCl_3$ arterial induced injury was performed 2 and 4 h after oral administration of 10 mg/kg clopidogrel. The same antithrombotic efficacy was observed in both cases; therefore, the time for clopidogrel administration was set to 2 h before arterial injury.[53] Compound **45** and its vehicle were administered by oral gavage at 5 mL/kg 1 h before $FeCl_3$-soaked filter papers were applied to the carotid artery. An arterial blood sample (EDTA) was withdrawn immediately before $FeCl_3$-soaked filter papers were applied to the carotid artery and 30 min after. Blood was centrifuged, and supernatant plasma was removed and stored at −20 °C until plasma concentration measurements.

Rat Blood Loss Model. The blood loss model was developed in house. After an acclimatization period of at least 7 days, the rats were anesthetized by an intraperitoneal injection of thiobutabarbital sodium salt hydrate at a dose of 150 mg/kg (Inactin, Sigma-Aldrich GmbH). The animals were then placed on a thermostatically controlled heating table to maintain body temperature at 36−38 °C. After tracheotomy, a catheter was inserted into the right jugular vein for injection of compound. A laparotomy was performed and vehicle or compounds **1** and **30d** were intravenously injected as a bolus of 1 mL/kg. Five minutes later, the spleen was punctured with a 4 mm biopsy punch (Stiefel) to induce a surgical wound, from which blood was collected for 30 min. The amount of blood loss was measured and reported in gram. At the end of the experiment, rats were euthanized by an intravenous infusion of pentobarbital (100 mg/kg, Esconarkon, Streuli Pharma AG, Switzerland). Clopidogrel and its vehicle were administered by oral gavage at 5 mL/kg 2 h before the spleen was punctured.

Chemistry. All reagents and solvents were used as purchased from commercial sources. Moisture sensitive reactions were carried out under an argon atmosphere. Progress of the reactions was followed either by thin-layer chromatography (TLC) analysis (Merck, 0.2 mm silica gel 60 F_{254} on glass plates) or by liquid chromatography−mass spectrometry (LC−MS). LC−MS: Thermo Finnigan MSQ Plus, with Agilent G4220A binary pump and DAD Agilent G4212A; column, Zorbax SB-AQ, 5 μm, 120 Å, 4.6 mm × 50 mm (Agilent); gradient, 5−95% acetonitrile in water containing 0.04% of TFA, within 1 min, then 95% acetonitrile in water containing 0.04% of TFA for 0.5 min; flow, 4.5 mL/min; 40 °C; t_R is given in min. Purity of all target compounds was checked by an additional LC−MS analysis on a Waters Acquity UPLC system equipped with an ACQ-PDA detector, an ACQ-ESLD detector, and an ACQ-SQD detector; column, ACQUITY UPLC CSH C18 1.7 μm, 2.1 mm × 50 mm or ACQUITY UPLC HSS T3 C18 1.8 μm, 2.1 mm × 50 mm; gradient, 2−98% acetonitrile containing 0.045% formic acid in water containing 0.05% formic acid over 2 min; flow, 1.0 mL/min; 60 °C. Chiral HPLC (chiral stationary phase):

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

hardware from UltiMate instrument series (Dionex), HPG-3200SD binary pump, WPS-3000 autosampler, TCC-3200 thermostated column compartment, DAD-3000 detector, SRD-3400 degasser. Column, solvent, and retention time (t_R) are as indicated, at 25 °C, flow 1 mL/min. High resolution LC−MS (LC−HRMS): analytical pump, Waters Acquity Binary; MS, SYNAPT G2 MS; source temperature, 150 °C; desolvation temperature, 400 °C; desolvatation gas flow, 700 L/h; cone gas flow, 10 L/h; extraction cone, 4; RF lens, 0.1 V; sampling cone, 30; capillary, 3 kV; high-resolution mode; gain, 1.0; MS function, 0.2 s per scan, 120−1000 amu in full scan, centroid mode; lock spray, leucine enkephalin 2 ng/mL (556.2771 Da) scan time 0.2 s with interval of 10 s and average of five scans; DAD, Acquity UPLC PDA detector; column, Acquity UPLC CSH C18 1.7 μm, 2.1 mm × 50 mm from Waters; gradient, 2−98% acetonitrile containing 0.05% formic acid in water containing 0.05% formic acid over 2 min; flow, 0.6 mL/min; 60 °C; detection, UV 214 nm and MS, t_R is given in min. NMR spectroscopy: Bruker Avance II 400 MHz Ultrashield or Bruker Ascend 500. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), quint (quint), or m (multiplet), br = broad, and coupling constants are given in Hz. Compound purification: compounds were purified by either flash column chromatography (CC) on silica gel 60 (Fluka Sigma−Aldrich, Switzerland) or preparative LC−MS (column, solvent and retention time (t_R) as indicated). Purity of all target compounds was assessed using the two independent LC−MS methods described above: (1) a Zorbax SB-AQ, 5 μm, 120 Å, 4.6 mm × 50 mm (Agilent) column, eluting with a gradient of 5−95% acetonitrile in water containing 0.04% of TFA, within 1 min, flow of 4.5 mL/min at 40 °C; (2) an ACQUITY UPLC CSH C18 1.7 μm, 2.1 mm × 50 mm column or an ACQUITY UPLC HSS T3 C18 1.8 μm, 2.1 mm × 50 mm column, eluting with a gradient of 2−98% acetonitrile containing 0.045% formic acid in water containing 0.05% formic acid over 2 min, flow of 1.0 mL/min at 60 °C. In addition, target compounds were analyzed by LC−HRMS as described above. Purity and identity of the target compounds were further corroborated by NMR spectroscopy, and chiral integrity was proven by HPLC using chiral stationary phases. According to these LC−MS analyses, target compounds showed a purity of greater than or equal to 95% (UV at 230 and at 214 nm).

Clopidogrel.[54] Plavix 75 mg (6.3 g, 84 tablets) was grounded in a mortar, and the powder was suspended in Et_2O (160 mL) and sat. aq Na_2CO_3 (160 mL). The resulting mixture was vigorously stirred for 30 min, filtered off, and the phases were separated. The organic layer was washed with sat. aq Na_2CO_3, dried (Na_2SO_4), and evaporated in vacuo. The obtained colorless paste was dissolved in Et_2O (137 mL), and HCl (2 M, 16 mL) was added. The resulting suspension was stirred at rt for 5 min and filtered off. The white powder was washed with Et_2O and dried in high vacuum to afford 5.1 g of clopidogrel as HCl salt. LC−MS: t_R = 0.72 min, $[M + H]^+$ = 321.94. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.37 (d, J = 7.8 Hz, 1 H), 7.55−7.44 (m, 3 H), 7.30 (m, 1 H), 6.78 (br, 1 H), 5.56 (s, 1 H), 4.44 (br, 1 H), 3.54 (s, 3 H), 3.85 (s, 3 H), 3.80 (m, 1 H), 3.19 (m, 1 H).** $[\alpha]_D^{24.5}$ +57 (c 1, MeOH).

(*S*)-3-Methoxypyrrolidine Hydrochloride (7). (a) Di-*tert*-butyl-dicarbonate (27.5 g) was added portionwise to a solution of (*S*)-3-hydroxypyrrolidine (**6**) (10.0 g) and Et_3N (32.0 mL) in DCM (240 mL). The reaction mixture was stirred overnight at rt. Water was added, and the phases were separated. The organic layer was washed with water and saturated aq NaCl, dried over Na_2SO_4, and evaporated off to give (*S*)-3-hydroxypyrrolidine-1-carboxylic acid *tert*-butyl ester (23.0 g, 100%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.45 (s, 1 H), 3.49−3.32 (m, 4 H), 2.17 (s, 1 H), 2.07−1.81 (m, 2 H), 1.47 (s, 9 H).

(b) To an ice-cold solution of (*S*)-3-hydroxypyrrolidine-1-carboxylic acid *tert*-butyl ester (23.0 g) in THF (230 mL), NaH (7.37 g, 60% dispersion in mineral oil) was added portionwise. The reaction mixture was stirred for 30 min at rt, and MeI (11.5 mL) was added dropwise. Stirring was continued for additional 20 h at rt. Water was added to the reaction mixture. The layers were separated, and the aq phase was extracted with DCM three times. The combined organic phases were dried over Na_2SO_4 and evaporated off to give (*S*)-3-methoxypyrrolidine-1-carboxylic acid *tert*-butyl ester (23.0 g, 93%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.93 (s, 1 H), 3.50−3.35 (m, 4 H), 3.34 (s, 3 H), 2.04−1.88 (m, 2 H), 1.47 (s, 9 H).

(c) (*S*)-3-Methoxypyrrolidine-1-carboxylic acid *tert*-butyl ester (23.0 g) was dissolved in EA (100 mL), and 3 M HCl in EA (115 mL) was added. The reaction mixture was stirred at rt for 20 h, and the solvent was evaporated off. The residue was taken up in Et_2O and the compound precipitated out. The suspension was filtered off and the powder washed with Et_2O. High vacuum drying afforded title compound **7** (15.2 g, 95%) as hydrochloride salt, brown solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.71 (br, 1 H), 9.37 (br, 1 H), 4.07 (s, 1 H), 3.23 (s, 3 H), 3.18−3.04 (m, 4 H), 3.34 (s, 3 H), 2.05−1.99 (m, 1 H), 1.93−1.84 (m, 1 H).

6-((*S*)-3-Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carboxylic Acid (8). (a) Benzamidine hydrochloride (**4**) (6.00 g), ethyl 4-methoxy-3-oxobutanoate (**3**) (7.30 g), and NaOMe (30% in MeOH, 18.0 g) were suspended in EtOH (30.0 mL). The reaction mixture was refluxed overnight, cooled down, and water (50.0 mL) was added. The resulting suspension was filtered off and the solid was washed with Et_2O and dried to give 6-methoxymethyl-2-phenylpyrimidin-4-ol (3.81 g). The remaining aq liquors were extracted three times, and the combined organic layers were dried over $MgSO_4$, filtered, and evaporated off. The resulting crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 0:1) to give additional 2.20 g. An amount of 6.01 g (65%) of 6-methoxymethyl-2-phenylpyrimidin-4-ol was obtained as a brown solid. LC−MS: t_R = 0.68 min, $[M + H]^+$ = 217.11. ^{1}H NMR (400 MHz, $CDCl_3$): δ 13.1 (br, 1 H), 8.24 (m, 2 H), 7.58−7.54 (m, 3 H), 6.60 (s, 1 H), 4.46 (s, 2 H), 3.54 (s, 3 H).

(b) $POCl_3$ (40.0 mL) was slowly added to a stirred powder of 6-methoxymethyl-2-phenylpyrimidin-4-ol (8.64 g). The resulting mixture was refluxed for 2 h, cooled down, and carefully added onto crushed ice. After 30 min of stirring, the obtained suspension was extracted twice with EA. The organic layers were washed twice with aq $NaHCO_3$, dried over Na_2SO_4, and evaporated off to afford 4-chloro-6-methoxymethyl-2-phenylpyrimidine (8.81 g, 94%) as a brown oil. LC−MS: t_R = 1.00 min, $[M + H]^+$ = 235.08. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.30 (m, 2 H), 7.56−7.49 (m, 3 H), 7.45 (s, 1 H), 4.57 (s, 2 H), 3.42 (s, 3 H).

(c) A solution of BBr_3 (2.51 mL) in DCM (20.0 mL) was syringed into a solution of the above material (4.85 g) in DCM (100 mL) under argon at 0 °C. After 30 min of stirring at 0 °C, the reaction was complete. It was quenched by the addition of Et_2O (100 mL), water (100 mL), and 1 M NaOH (100 mL). After 1 h of stirring at rt, the mixture was extracted three times with DCM and the organic layers were washed with water, dried over Na_2SO_4, and evaporated off. The resulting oil crushed out and the solid was washed with heptane to give (6-chloro-2-phenylpyrimidin-4-yl)methanol (4.58 g, 100%) as a brown oil. LC−MS: t_R = 0.88 min, $[M + H]^+$ = 221.29. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.38 (m, 2 H), 7.43 (m, 3 H), 7.20 (s, 1 H), 4.74 (d, J = 5.1 Hz, 2 H), 3.08 (t, J = 5.3 Hz, 1 H).

(d) The above alcohol (4.58 g) was dissolved in dioxane (170 mL), and a solution of NaOH (0.829 g) in water (400 mL) was added, followed by $KMnO_4$ (9.82 g). The mixture was stirred at rt for 3 h. Then 2 M HCl (100 mL) was added and the resulting mixture was stirred for 1 h and filtered off. The solution was extracted twice with EA. The organic phases were dried over Na_2SO_4 and evaporated off to afford **5** (4.04 g, 83%) as a white solid. LC−MS: t_R = 0.87 min, $[M + H]^+$ = 235.28. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.37 (m, 2 H), 7.95 (s, 1 H), 7.55 (m, 3 H).

(e) A solution of intermediates **5** (3.20 g) and **7** (2.25 g) and DIPEA (5.14 mL) in THF (40.0 mL) was stirred at 60 °C for 20 h. Intermediate **7** (0.375 g) and DIPEA (0.467 mL) were added, and the reaction mixture was further stirred at 60 °C for 72 h. Water and DCM were added, and the phases were separated. The aq phase was extracted three times with DCM and the combined organic layers were dried over Na_2SO_4 and evaporated off to give title compound **8** (3.78 g, 93%) as a beige solid. LC−MS: t_R = 0.74 min, $[M + H]^+$ = 300.42. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.39 (m, 2 H), 7.56−7.48 (m, 3 H),

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

7.09 (d, 1 H), 4.22−4.01 (m, 2 H), 3.86−3.66 (m, 1 H), 3.62 (br, 2 H), 3.41 (s, 3 H), 2.37−2.06 (m, 2 H).

Piperazine-1-carboxylic Acid Butyl Ester (9b). (a) To a solution of 1-benzylpiperazine (1.97 mL) and Et_3N (1.90 mL) in DCM (100 mL), butyl chloroformate (1.47 mL) was added. The mixture was stirred at rt for 2 h. Water was added, and the organic layer was separated, dried (Na_2SO_4), and evaporated off to give 4-benzylpiperazine-1-carboxylic acid butyl ester (3.13 g, 100%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35−7.25 (m, 5 H), 4.10 (t, J = 6.5 Hz, 2 H), 3.53 (s, 2 H), 3.49 (br, 4 H), 2.41 (br, 4 H), 1.63 (m, 2 H), 1.40 (m, 2 H), 0.95 (t, J = 7.2 Hz, 3 H).

(b) The above material (3.13 g) was hydrogenated in EtOH (100 mL) with Pd/C (wet, 5%, 0.480 g) for 24 h. The mixture was filtered through Celite and evaporated off to give title compound **9b** (2.04 g, 96%) as a pale yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.54 (s, 1 H), 4.06 (t, J = 6.6 Hz, 2 H), 3.45 (m, 4 H), 2.83 (m, 4 H), 1.78 (s, 1 H), 1.66−1.59 (quint, J = 6.7 Hz, 2 H), 1.45−1.35 (m, 2 H), 0.95 (t, J = 7.4 Hz, 3 H).

4-((*S*)-3-Methoxy-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic Acid Butyl Ester (19). (a) A solution of *N*-Boc-L-serine (2.00 g), HOBt (1.97 g), and EDC-Cl (2.24 g) in DCM (20.0 mL) was stirred for 15 min at rt. **9b** (1.81 g) was added, and the reaction mixture was stirred overnight at rt. Precipitation occurred upon addition of 1 M $NaHSO_4$. After 10 min of stirring, the suspension was filtered off. The layers were separated, and the aq phase was extracted three times with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The resulting crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:1 to heptane/EA 0:1) to give 4-((*S*)-2-*tert*-butoxycarbonylamino-3-hydroxypropionyl)piperazine-1-carboxylic acid butyl ester (0.500 mg, 14%) as a colorless oil. LC−MS: t_R = 0.82 min, $[M + H]^+$ = 374.34. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.64 (d, J = 8.0 Hz, 1 H), 4.64 (m, 1 H), 4.13 (t, J = 6.7 Hz, 2 H), 3.87 (m, 1 H), 3.77−3.65 (m, 3 H), 3.63−3.40 (m, 6 H), 3.26 (br, 1 H), 1.63 (m, 2 H), 1.46 (s, 9 H), 1.39 (quint, J = 7.5 Hz, 2 H), 0.96 (t, J = 7.4 Hz, 3 H).

(b) To a solution of the above compound (0.900 g) in THF (10.0 mL), NaH (0.174 g, 60% dispersion in mineral oil) was added at rt. After stirring at rt for 15 min, MeI (0.150 mL) was added and the stirring was continued for 4 h. Water was added, the organic layer was separated, dried (Na_2SO_4), and evaporated off to give 4-((*S*)-2-*tert*-butoxycarbonylamino-3-methoxypropionyl)piperazine-1-carboxylic acid butyl ester (0.500 g, 54%) as a yellow oil. LC−MS: t_R = 0.91 min, $[M + H]^+$ = 388.35.

(c) A mixture of the above material (0.500 g) in DCM/TFA (6.00 mL, 5:1) was stirred at rt for 4 h and concentrated to dryness. The residue was taken up in DCM and washed with aq Na_2CO_3. The layers were separated, and the aq phase was extracted three times with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness to give 4-((*S*)-2-amino-3-methoxypropionyl)-piperazine-1-carboxylic acid butyl ester (0.120 g, 32%) as a yellow oil. LC−MS: t_R = 0.62 min, $[M + H]^+$ = 288.23. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.13 (t, J = 6.7 Hz, 2 H), 3.95 (m, 1 H), 3.76 (br, 1 H), 3.64−3.40 (m, 8 H), 3.36 (s, 3 H), 2.86 (br, 1 H), 1.90 (br, NH_2), 1.64 (m, 2 H), 1.46 (s, 9 H), 1.41 (m, 2 H), 0.97 (t, J = 7.4 Hz, 3 H).

(d) A solution of **8** (0.120 g), HOBt (0.081 g), and EDC-Cl (0.092 g) was stirred for 15 min at rt. The above material (0.115 g) was added, and the reaction mixture was stirred overnight at rt. Addition of 1 M $NaHSO_4$ was followed by 20 min of stirring and filtration of the resulting suspension. The layers were separated, and the aq phase was extracted three times with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The resulting crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:1 to heptane/EA 0:1) to give title compound **19** (0.074 g, 32%) as a pale yellow foam. LC−MS: t_R = 1.3 min, $[M + H]^+$ = 569.5. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.97 (d, J = 8.3 Hz, 1 H), 8.48 (s, 2 H), 7.47 (s, 3 H), 7.05 (br, 1 H), 5.33 (m, 1 H), 4.15 (m, 1 H), 4.12 (t, J = 6.7 Hz, 2 H), 4.01 (br, 1 H), 3.88−3.54 (m, 11 H), 3.47 (m, 2 H), 3.39 (s, 6 H), 2.32−2.05 (m, 2 H), 1.64 (quint, J = 6.8 Hz, 2 H), 1.40 (m, 2 H), 0.96 (t, J = 7.4 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.2, 163.1, 162.5, 161.4, 155.1, 137.6, 131.2, 128.9, 128.4, 99.8, 79.6, 78.8, 72.4, 65.2, 59.1, 56.4, 52.0, 51.7, 49.4, 45.3, 45.1, 44.8, 44.0, 43.6, 42.1, 31.1, 30.6, 30.1, 19.1, 14.1. LC−HRMS: t_R = 1.42 min, $[M + H]/z$ = 569.3087, found 569.3087.

4-((*S*)-3-Carbamoyloxy-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic Acid Butyl Ester (28). To a solution of **22** (0.160 g) in THF (2.00 mL) at 0 °C, trichloroacetyl isocyanate (0.089 g) was added. After 20 min of stirring at rt, EtOH (0.200 mL) was added and the reaction mixture was concentrated to dryness. The residue was dissolved in EtOH (10.0 mL), cooled to 0 °C, and treated with $NaBH_4$ (0.123 g). The reaction mixture was stirred at rt overnight. Aqueous Na_2CO_3 was added, and the mixture was stirred for 3 h and extracted with DCM three times. The combined organic phases were dried over $MgSO_4$ and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 0:1) and by preparative TLC (DCM/MeOH, 97:3) to give title compound **28** (0.006 g, 5%) as a white solid. LC−MS: t_R = 1.18 min, $[M + H]^+$ = 598.5. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.07 (m, 1 H), 8.49 (m, 2 H), 7.48 (m, 3 H), 7.05 (s, 1 H), 5.43 (m, 1 H), 4.80 (br, 2 H), 4.48 (m, 1 H), 4.28 (m, 1 H), 4.15 (m, 1 H), 4.12 (t, J = 6.3 Hz, 2 H), 4.02 (m, 1 H), 3.84−3.46 (m, 11 H), 3.40 (s, 3 H), 2.31−2.04 (m, 2 H), 1.64 (m, 2 H), 1.40 (m, 2 H), 0.96 (t, J = 7.3 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 167.5, 163.4, 162.5, 161.3, 156.9, 155.1, 137.7, 131.2, 128.9, 128.5, 99.9, 99.8, 79.6, 78.8, 65.2, 63.2, 56.4, 52.0, 51.7, 49.7, 45.2, 44.8, 43.8, 43.5, 42.2, 31.1, 30.6, 30.1, 19.1, 14.1. LC−HRMS: t_R = 1.29 min, $[M + H]/z$ = 598.2989, found 598.2991.

4-((*R*)-3-Carboxy-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic Acid Ethyl Ester (29c). (a) A suspension of Cbz-D-Asp(O^{t}Bu)-OH (3.00 g), HOBt (0.500 g), EDC-Cl (0.356 g), and DIPEA (0.291 mL) in THF/DCM (6.00 mL, 5:1) was stirred for 15 min at rt. 1-(Ethoxycarbonyl)piperazine (0.244 g) was added, and the reaction mixture was stirred for 24 h at rt. The reaction mixture was partitioned between water and EA. The organic layer was washed with 2 M Na_2CO_3, with 1 M $NaHSO_4$, dried over $MgSO_4$, and concentrated to dryness to give 4-((*R*)-2-benzyloxycarbonylamino-3-*tert*-butoxycarbonylpropionyl)piperazine-1-carboxylic acid ethyl ester (0.800 g, 100%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (m, 5 H), 5.70 (m, 1 H), 5.11 (s, 2 H), 5.00 (m, 1 H), 4.18 (q, J = 7.1 Hz, 2 H), 3.70−3.38 (m, 8 H), 2.76 (dd, J = 7.2 and 15.8 Hz, 1 H), 2.55 (dd, J = 5.6 and 15.8 Hz, 1 H), 1.43 (s, 9 H), 1.29 (t, J = 7.1 Hz, 3 H).

(b) The above material (0.800 g) was hydrogenated in EtOH (20.0 mL) with Pd/C (wet, 5%, 0.080 g) for 24 h. The mixture was filtered through Celite and evaporated off to give 4-((*R*)-2-amino-3-*tert*-butoxycarbonylpropionyl)piperazine-1-carboxylic acid ethyl ester (0.600 g, 100%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.18 (q, J = 7.0 Hz, 2 H), 3.68−3.46 (m, 8 H), 2.63 (dd, J = 4.9 and 16.2 Hz, 1 H), 2.49 (dd, J = 8.0 and 16.3 Hz, 1 H), 1.46 (s, 9 H), 1.29 (t, J = 7.1 Hz, 3 H).

(c) A solution of **8** (0.363 g) and PyBOP (0.695 g) in DCM (10.0 mL) was stirred at rt for 10 min. DIPEA (0.173 mL) and the above material (0.400 g) were added. After 24 h of stirring at rt, the reaction mixture was diluted with DCM and 2 M Na_2CO_3. The organic layer was washed with 1 M $NaHSO_4$, brine, dried over Na_2SO_4, and concentrated to dryness. The resulting crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 0:1) to give 4-((*R*)-3-*tert*-butoxycarbonyl-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)-piperazine-1-carboxylic acid ethyl ester (**23c**) (0.250 g, 34%) as a colorless oil. LC−MS: t_R = 1.09 min, $[M + H]^+$ = 611.12. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.91 (d, J = 9.4 Hz, 1 H), 8.47 (m, 2 H), 7.48 (m, 3 H), 7.05 (br, 1 H), 5.47 (m, 1 H), 4.16 (m, 2 H), 4.08−3.97 (m, 1 H), 3.84−3.44 (m, 12 H), 3.41 (s, 3 H), 2.99 (dd, J = 7.8 and 16.0 Hz, 1 H), 2.71 (dd, J = 5.0 and 16.0 Hz, 1 H), 2.32−2.08 (m, 2 H), 1.46 (s, 9 H), 1.28 (t, J = 7.1 Hz, 3 H).

(d) A solution of the above compound (0.250 g) in DCM/TFA (2.00 mL/1.20 mL) was stirred at rt for 4 h and concentrated to dryness. The crude product was purified by preparative LC−MS

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

(Gemini C18 110A Ax, 10 μm, 21.2 mm × 50 mm, 38.8−95% MeCN in water containing 0.2% formic acid, 45 mL/min) to afford the title compound **29c** (0.090 g, 40%) as a white powder. LC−MS: t_R = 1.07 min, $[M + H]^+$ = 555.4. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.11 (m, 1 H), 8.44 (m, 2 H), 7.46 (m, 3 H), 7.02 (br, 1 H), 5.52 (m, 1 H), 4.15 (q, *J* = 7.0 Hz, 3 H), 4.01 (m, 1 H), 3.84−3.44 (m, 11 H), 3.40 (s, 3 H), 3.09 (dd, *J* = 7.2 and 16.5 Hz, 1 H), 2.86 (dd, *J* = 4.6 and 16.5 Hz, 1 H), 2.30−2.07 (m, 2 H), 1.26 (t, *J* = 7.1 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.6, 169.8, 163.3, 162.5, 161.3, 155.2, 155.0, 137.7, 131.2, 128.8, 128.7, 100.0, 79.6, 78.8, 61.4, 56.4, 51.9, 51.7, 46.6, 45.2, 45.1, 44.8, 43.6, 42.2, 36.8, 30.6, 30.1, 15.0. LC−HRMS: t_R = 1.19 min, $[M + H]/z$ = 555.2567, found 555.2569.

4-((*S*)-2-{[6-((*S*)-3-Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-4-oxo-4-trifluoromethanesulfonylaminobutyryl)piperazine-1-carboxylic Acid Ethyl Ester (35). A solution of **29a** (0.153 g) in $SOCl_2$ (1.00 mL) was stirred at rt for 1 h and concentrated to dryness. The residue was taken up in DCM (4.00 mL), and Et_3N (0.115 mL) was added, followed by $CF_3SO_2NH_2$ (0.041 g). The reaction mixture was stirred overnight at rt and poured onto aq $NaHCO_3$. The layers were separated, and the aq phase was acidified to pH 1 with 2 M HCl and extracted with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The crude product was purified by preparative LC−MS (Zorbax PrepHT SB.Aq, 5 μm, 21.2 mm × 50 mm, 31.5−100% MeCN in water containing 0.2% formic acid, 100 mL) and by preparative TLC (DCM/MeOH, 97:3) to afford title compound **35** (0.012 g, 6%) as a white foam. LC−MS: t_R = 1.61 min, $[M + H]^+$ = 686.4. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.17 (br, 1 H), 8.44 (m, 2 H), 7.46 (m, 3 H), 7.01 (br, 1 H), 5.52 (s, 1 H), 4.13 (m, 2 H), 3.95 (m, 1 H), 3.82−3.42 (m, 11 H), 3.39 (s, 3 H), 3.04 (m, 1 H), 2.83 (m, 1 H), 2.30−2.00 (m, 2 H), 1.25 (m, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.9, 169.8, 163.1, 162.5, 161.4, 155.3, 155.1, 137.7, 131.1, 128.8, 128.6, 122.1, 119.5, 99.8, 79.6, 78.8, 61.4, 56.4, 46.8, 45.4, 45.1, 44.8, 43.6, 42.1, 30.6, 30.1, 29.5, 29.2, 22.6, 15.0, 14.5. LC−HRMS: t_R = 1.20 min, $[M + H]/z$ = 686.2220, found 686.2231.

4-((S)-5-Methanesulfonylamino-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-5-oxopentanoyl)piperazine-1-carboxylic Acid Ethyl Ester (36). To a solution of **29b** (0.325 g) in DCM (10.0 mL), DMAP (0.076 g) and $MeSO_2NH_2$ (0.059 g) were added, followed by DCC (0.128 g) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and at rt for 20 h and was filtered off. The filtrate was concentrated to dryness and the crude product was purified by CC on silica gel (eluting with DCM/MeOH 10:1) and preparative TLC (DCM/acetone, 1:5) to give title compound **36** (0.111 g, 36%) as a white solid. LC−MS: t_R = 1.1 min, $[M + H]^+$ = 646.4. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.6 (s, 1 H), 9.8 (d, *J* = 7.8 Hz, 1 H), 8.49 (m, 2 H), 7.54 (m, 3 H), 6.97 (s, 1 H), 5.01 (m, 1 H), 4.14 (m, 1 H), 4.07 (q, *J* = 7.0 Hz, 2 H), 3.88 (m, 1 H), 3.74−3.34 (m, 11 H), 3.30 (s, 3 H), 3.21 (s, 3 H), 2.18−2.06 (m, 3 H), 1.93 (m, 1 H), 1.20 (t, *J* = 7.1 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.6, 169.6, 163.5, 162.5, 161.4, 155.2, 155.1, 137.7, 131.2, 128.9, 128.4, 99.8, 99.7, 79.6, 78.8, 61.5, 56.4, 52.0, 51.7, 48.7, 45.1, 44.8, 43.9, 43.5, 42.0, 41.4, 40.9, 31.6, 30.6, 30.1, 26.3, 15.0. LC−HRMS: t_R = 1.33 min, $[M + H]/z$ = 646.2659, found 646.2659.

4-((*S*)-3-Amino-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic Acid Butyl Ester (31a). A mixture of 4-((*S*)-3-*tert*-butoxycarbonylamino-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic acid butyl ester (**25a**) (0.305 g) in 4 M HCl in dioxane (1.50 mL) was stirred at rt for 30 min and concentrated to dryness to give title compound **31a** (0.250 g, 99%) as hydrochloride salt, as a beige solid. LC−MS: t_R = 0.86 min, $[M + H]^+$ = 554.5. ^{1}H NMR (400 MHz, $CDCl_3$ + drop of D_2O): δ 8.48 (m, 2 H), 7.51 (m, 3 H), 6.96 (s, 1 H), 5.20 (m, 1 H), 4.13 (m, 1 H), 3.94 (t, *J* = 5.8 Hz, 2 H), 3.86 (m, 1 H), 3.69−3.35 (m, 10 H), 3.30−3.15 (m, 3 H), 3.26 (s, 3 H), 2.12 (m, 2 H), 1.47 (m, 2 H), 1.25 (m, 2 H), 0.81 (m, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 167.3, 164.4, 162.5, 161.3, 155.1, 155.0, 137.6, 131.2, 128.7, 100.1, 79.6, 78.8, 65.2, 56.4, 52.0, 51.7, 48.0, 45.2, 44.8, 43.6, 42.3, 31.0, 30.6, 30.1, 19.1, 14.1. LC−HRMS: t_R = 0.93 min, $[M + H]/z$ = 554.3091, found 554.3094.

4-((*S*)-2-{[6-((*S*)-3-Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-4-trifluoromethanesulfonylaminobutyryl)piperazine-1-carboxylic Acid Butyl Ester (37). To a suspension of **31b** (0.053 g) in DCM (1.00 mL) at 0 °C, Et_3N (0.368 mL) was added, followed by trifluorosulfonyl chloride (0.112 mL). The reaction mixture was stirred at rt and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from DCM/MeOH 1:0 to DCM/MeOH 9:1) to give title compound **37** (0.007 g, 11%) as a white solid. LC−MS: t_R = 1.38 min, $[M + H]^+$ = 700.5. ^{1}H NMR (400 MHz, CD_3OD): δ 8.50 (m, 2 H), 7.48 (m, 3 H), 7.01 (s, 1 H), 5.22 (m, 1 H), 4.58 (br, 1 H), 4.19 (m, 1 H), 4.11 (t, *J* = 6.5 Hz, 2 H), 3.99 (br, 1 H), 3.80−3.45 (m, 11 H), 3.40 (s, 3 H), 3.38 (m, 2 H), 2.23 (m, 2 H), 2.16−2.00 (m, 2 H), 1.64 (m, 2 H), 1.41 (m, 2 H), 0.96 (t, *J* = 7.4 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.1, 163.7, 162.5, 161.4, 155.1, 137.7, 131.2, 128.8, 128.5, 123.9, 121.4, 118.8, 99.8, 79.6, 78.8, 65.2, 56.4, 52.0, 51.7, 47.3, 45.1, 44.8, 43.9, 43.6, 42.1, 40.9, 33.0, 31.0, 30.7, 30.1, 19.1, 14.1. LC−HRMS: t_R = 1.49 min, $[M + H]/z$ = 700.2740, found 700.2742.

4-((*S*)-3-(2-Hydroxy-3,4-dioxocyclobut-1-enylamino)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic Acid Butyl Ester (38a). (a) To a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (0.022 g) in EtOH (0.500 mL), a solution of **31a** (0.075 g) and Et_3N (0.188 mL) in EtOH (0.500 mL) was added dropwise. The reaction mixture was stirred overnight at rt and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 0:1) to give 4-((*S*)-3-(2-ethoxy-3,4-dioxocyclobut-1-enylamino)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic acid butyl ester (0.045 g, 52%) as a white foam. LC−MS: t_R = 1.01 min, $[M + H]^+$ = 678.25.

(b) 4 M HCl in dioxane (0.500 mL) was added to a solution of the above compound (0.045 g) in THF (1.00 mL). The reaction mixture was stirred at 50 °C for 48 h and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 0:1) to give title compound **38a** (0.024 g, 56%) as a gray solid. LC−MS: t_R = 1.38 min, $[M + H]^+$ = 650.5. ^{1}H NMR (400 MHz, CD_3OD): δ 8.44 (m, 2 H), 7.46 (m, 3 H), 6.91 (s, 1 H), 5.36 (m, 1 H), 4.17−3.99 (m, 2 H), 4.10 (t, *J* = 6.4 Hz, 2 H), 3.89 (m, 2 H), 3.78 (m, 2 H), 3.68−3.45 (m, 9 H), 3.37 (s, 3 H), 2.25−2.02 (m, 2 H), 1.64 (m, 2 H), 1.41 (m, 2 H), 0.96 (t, *J* = 7.4 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 200.1, 189.1, 168.4, 163.7, 162.5, 161.3, 155.4, 155.1, 137.7, 131.1, 128.9, 128.6, 99.7, 79.6, 78.8, 65.2, 56.4, 51.8, 45.2, 44.1, 42.0, 31.1, 19.2, 14.1. LC−HRMS: t_R = 1.15 min, $[M + H]/z$ = 650.2938, found 650.2939.

4-[(*S*)-2-{[6-((*S*)-3-Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-4-(1*H*-tetrazol-5-yl)butyryl]-piperazine-1-carboxylic Acid Ethyl Ester (32b). (a) A solution of Cbz-Gln-OH (25.0 g), HOBt (14.5 g), and EDC-Cl (20.5 g) in THF/DCM (750 mL, 4:1) was stirred for 5 min at rt. 1-(Ethoxycarbonyl)-piperazine (14.1 g) was added, and the reaction mixture was stirred at rt for 24 h. Water and EA were added, and the layers were separated. The organic phase was washed with 2 M Na_2CO_3 and 1 M $NaHSO_4$, was dried (Na_2SO_4) and evaporated off to give 4-((*S*)-2-benzyloxycarbonylamino-4-carbamoylbutyryl)piperazine-1-carboxylic acid ethyl ester (37.5 g, 100%) as a yellow oil. LC−MS: t_R = 0.74 min, $[M + H]^+$ = 421.49.

(b) Benzenesulfonyl chloride (13.8 mL) was added to a solution of the above compound (37.4 g) in pyridine (29.6 mL), and the resulting reaction mixture was stirred at 50 °C for 1 h. After cooling down, 2 N HCl was added to pH 7 and the mixture was extracted three times with EA. The combined organic layers were washed with 1 N HCl, aq $NaHCO_3$, and water, dried (Na_2SO_4), and concentrated to dryness to give 4-((*S*)-2-benzyloxycarbonylamino-4-cyanobutyryl)piperazine-1-carboxylic acid ethyl ester (30.0 g, 84%) as a yellow oil. LC−MS: t_R = 0.85 min, $[M + H]^+$ = 403.48.

(c) The above material (10.0 g) was hydrogenated in EtOH (40.0 mL) with Pd/C (wet, 5%, 1.22 g) for 24 h. The mixture was filtered

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

through Celite and evaporated off to give 4-((*S*)-2-amino-4-cyanobutyryl)piperazine-1-carboxylic acid ethyl ester (6.99 g, 100%) as a brown oil. LC−MS: t_R = 0.51 min, $[M + H]^+$ = 269.39.

(d) A solution of **8** (0.200 g), HOBt (0.108 g), and EDC-Cl (0.154 g) in THF/DCM (5.00 mL, 4:1) was stirred for 5 min at rt. The above product (0.179 g) was added, and the reaction mixture was stirred at rt for 24 h. Water and EA were added, and the layers were separated. The organic phase was washed with 2 M Na_2CO_3 and 1 M $NaHSO_4$, was dried (Na_2SO_4) and evaporated off to give 4-((*S*)-4-cyano-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-butyryl)piperazine-1-carboxylic acid ethyl ester (**26b**) (0.290 g, 79%) as a brown foam. LC−MS: t_R = 0.97 min, $[M + H]^+$ = 550.56.

(e) A mixture of **26b** (0.250 g), $ZnBr_2$ (0.102 g), and NaN_3 (0.032 g) in water (2.00 mL) was stirred at 100 °C in a sealed tube overnight. After cooling down, 2 N HCl was added to pH 1, followed by DCM (100 mL), EA (50.0 mL), and water (30.0 mL). The layers were separated, and the aq phase was extracted with DCM. The combined organic layers were dried (Na_2SO_4) and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from DCM/MeOH 1:0 to DCM/MeOH 7:3) to give title compound **32b** (0.060 g, 22%) as a beige solid. LC−MS: t_R = 1.04 min, $[M + H]^+$ = 593.4. ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.15 (d, J = 7.9 Hz, 1 H), 8.49 (m, 2 H), 7.53 (m, 3 H), 6.96 (s, 1 H), 5.11 (m, 1 H), 4.13 (m, 1 H), 4.06 (q, J = 7.0 Hz, 2 H), 3.89 (m, 1 H), 3.73−3.33 (m, 11 H), 3.30 (s, 3 H), 3.02−2.89 (m, 2 H), 2.36 (m, 1 H), 2.20−2.04 (m, 3 H), 1.20 (t, J = 7.1 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.4, 163.6, 162.5, 161.4, 156.1, 155.2, 155.1, 137.7, 131.2, 128.9, 128.4, 99.8, 79.6, 78.8, 61.5, 56.4, 52.0, 51.7, 48.9, 45.1, 44.8, 44.0, 43.5, 42.0, 30.6, 30.1, 30.0, 19.8, 15.1. LC−HRMS: t_R = 1.19 min, $[M + H]/z$ = 593.2948, found 593.2959.

4-[(*S*)-2-{[6-((*S*)-3-Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-3-(5-oxo-4,5-dihydro[1,2,4]oxadiazol-3-yl)propionyl]piperazine-1-carboxylic Acid Ethyl Ester (33). (a) Hydroxylamine hydrochloride (0.233 g) and $NaHCO_3$ (0.279 g) were added to a solution of 4-((*S*)-3-cyano-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)-piperazine-1-carboxylic acid ethyl ester (**26a**) (1.60 g) in MeOH (5.50 mL), and the reaction mixture was stirred at reflux for 20 h. Hydroxylamine hydrochloride (0.062 g) and $NaHCO_3$ (0.076 g) were added, the reflux was continued for 3 h, and the reaction mixture was concentrated to dryness. The residue was taken up in EA/water, and the resulting suspension was filtered off. The filtrate was decanted, and the organic layer was washed with water, dried ($MgSO_4$), and evaporated off. The crude product was purified by preparative LC−MS (Zorbax PrepHT SB.Aq, 5 μm, 21.2 mm × 50 mm, 21−100% MeCN in water containing 0.2% formic acid, 100 mL/min) to afford 4-((*S*,*Z*)-4-amino-4-(hydroxyimino)-2-(6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carboxamido)butanoyl)piperazine-1-carboxylic acid ethyl ester (0.046 g, 3%) as a white solid. LC−MS: t_R = 0.47 min, $[M + H]^+$ = 569.32. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.07 (m, 1 H), 8.47 (m, 2 H), 7.48 (m, 3 H), 7.03 (s, 1 H), 5.32 (m, 1 H), 5.17 (br, 1 H), 4.17 (q, J = 7.0 Hz, 2 H), 4.08−3.95 (m, 1 H), 3.84−3.70 (m, 2 H), 3.67−3.47 (m, 8 H), 3.41 (s, 3 H), 3.02−2.85 (m, 2 H), 2.82−2.75 (m, 1 H), 2.70−2.63 (m, 1 H), 2.30−2.05 (m, 2 H), 1.28 (t, J = 7.1 Hz, 3 H).

(b) 1,1′-Carbonyldiimidazole (0.016 g) and DBU (0.013 mL) were added to a solution of the above material (0.045 g) in dioxane (0.100 mL). The reaction mixture was refluxed for 2 h and poured onto DCM/water. pH was adjusted to 2 with 1 N HCl, and the layers were separated. The organic phase was dried over Na_2SO_4 and concentrated to dryness. The crude product was purified by preparative TLC (DCM/acetone/acetic acid, 10:4:0.1) to give title compound **33** (0.026 g, 55%) as a yellow oil. LC−MS: t_R = 1.1 min, $[M + H]^+$ = 595.4. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.3 (br, 1 H), 9.34 (m, 1 H), 8.52 (m, 2 H), 7.52 (m, 3 H), 6.94 (s, 1 H), 5.29 (m, 1 H), 4.13 (m, 1 H), 4.05 (m, 2 H), 3.88 (m, 1 H), 3.72 (m, 1 H), 3.65−3.40 (m, 10 H), 3.30 (s, 3 H), 3.10 (m, 1 H), 2.90 (m, 1 H), 2.19−2.05 (m, 2 H), 1.17 (t, J = 7.2 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 167.9, 163.6, 162.5, 161.4, 155.0, 137.7, 131.2, 128.8, 128.6, 99.9, 79.6, 78.8, 62.5, 61.5, 56.4, 52.0, 51.7, 47.1, 45.2, 44.8, 43.5, 42.3, 30.6, 30.1, 29.5, 28.5, 28.2, 26.0, 15.0. LC−HRMS: t_R = 1.21 min, $[M + H]/z$ = 595.2628, found 595.2635.

4-((*S*)-2-{[6-((*S*)-3-Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-4-sulfobutyryl)piperazine-1-carboxylic Acid Ethyl Ester (34). A mixture of **27** (0.404 g) and Me_4NCl (0.402 g) in DMF (6.00 mL) was heated at 150 °C overnight and partitioned in DCM/water. The aq phase was extracted with DCM, and the combined organic layers were dried over Na_2SO_4 and concentrated to dryness. The crude product was purified by preparative LC−MS (XBridge Prep C18, 10 μm, OBD 30 mm × 75 mm, 5−95% MeCN in water containing 0.5% concentrated ammonia, 75 mL/min) to afford title compound **34** (0.082 g, 23%) as a beige powder. LC−MS: t_R = 1.43 min, $[M + H]^+$ = 605.30. ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.01 (m, 1 H), 8.49 (m, 2 H), 7.54 (m, 3 H), 7.20 (s, 1 H), 7.09 (s, 1 H), 6.99 (s, 1 H), 6.98 (s, 1 H), 5.14 (m, 1 H), 4.17−4.10 (m, 1 H), 4.07 (q, J = 7.0 Hz, 2 H), 3.91−3.86 (m, 1 H), 3.74−3.35 (m, 11 H), 3.30 (s, 3 H), 2.53 (m, 1 H), 2.40 (m, 1 H), 2.18−1.95 (m, 4 H), 1.20 (t, J = 7.1 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.9, 163.2, 162.4, 161.4, 155.3, 155.1, 137.7, 131.2, 128.9, 128.4, 99.8, 99.7, 79.6, 78.8, 61.4, 56.4, 51.9, 51.7, 48.9, 47.7, 45.1, 44.8, 44.0, 43.6, 42.0, 30.6, 30.1, 29.0, 15.0. LC−HRMS: t_R = 1.19 min, $[M + H]/z$ = 605.2393, found 605.2394.

(*R*)-2-*tert*-Butoxycarbonylamino-3-(diethoxyphosphoryl)-propionic Acid Methyl Ester (39b). A mixture of Boc-3-iodo-L-Ala-OMe (**40b**) (4.00 g) in $P(OEt)_3$ (40.4 g) was stirred at 150 °C overnight and was concentrated to dryness to give title compound **39b** (3.95 g, 96%) as crude, as a yellow oil. LC−MS: t_R = 0.85 min, $[M + H]^+$ = 340.09. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (m, 5 H), 5.41 (m, 1 H), 5.14 (s, 2 H), 4.53 (m, 1 H), 3.78 (s, 3 H), 3.45 (t, J = 6.9 Hz, 2 H), 2.46 (m, 1 H), 2.27 (m, 1 H).

(*R*)-2-*tert*-Butoxycarbonylamino-3-(diethoxyphosphoryl)-propionic Acid (10b). A solution of LiOH (monohydrate, 0.418 g) in water (11.8 mL) was added to a solution of **39b** (3.95 g) in THF (35.0 mL). After stirring at rt for 2 h, LiOH (monohydrate, 0.139 g) was added and the stirring was continued for 2 h. Water and Et_2O were added, and the layers were separated. The aq phase was acidified with 2 M HCl to pH 1 and extracted with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness to give title compound **10b** (2.03 g, 53%) as a yellow solid. LC−MS: t_R = 0.77 min, $[M + H]^+$ = 326.13. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.75 (br, 1 H), 5.75 (d, J = 7.2 Hz, 1 H), 4.51 (dd, J = 6.4 and 27.8 Hz, 1 H), 4.12 (m, 4 H), 2.47 (m, 2 H), 1.32 (t, J = 7.1 Hz, 6 H).

4-((*R*)-2-{[6-((*S*)-3-Methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-3-phosphonopropionyl)piperazine-1-carboxylic Acid Butyl Ester (30d). A solution of 4-((*R*)-3-(diethoxyphosphoryl)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic acid butyl ester (**24d**) (2.66 g) in MeCN (11.8 mL) was cooled down to 0 °C, and TMSBr (10.2 mL) was added dropwise. The reaction mixture was stirred at rt overnight. Water (20.0 mL) was added, and the reaction mixture was stirred at rt for 2 h and extracted with DCM five times. The combined organic phases were dried over Na_2SO_4 and concentrated to dryness. The crude product was purified by CC on reverse phase (using a gradient from water/MeCN/TFA 95:5:1 to water/MeCN/TFA 10:90:1) to give title compound **30d** (1.54 g, 63%) as a white powder. LC−MS: t_R = 1.37 min, $[M + H]^+$ = 619.4. ^{1}H NMR (400 MHz, CD_3OD): δ 8.38 (d, J = 7.1 Hz, 2 H), 7.55 (m, 3 H), 7.16 (s, 1 H), 5.39 (m, 1 H), 4.27−4.19 (m, 1 H), 4.13 (t, J = 6.5 Hz, 2 H), 3.89−3.67 (m, 7 H), 3.63−3.55 (m, 3 H), 3.53−3.47 (m, 2 H), 3.41 (s, 3 H), 2.45−2.17 (m, 4 H), 1.66 (m, 2 H), 1.43 (m, 2 H), 0.98 (t, J = 7.3 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 170.9, 163.3, 162.4, 161.4, 155.1, 137.7, 130.9, 128.7, 128.4, 99.5, 79.6, 78.8, 65.1, 56.4, 51.8, 51.6, 47.1, 45.4, 44.9, 44.7, 43.7, 42.1, 31.0, 30.6, 30.1, 19.1, 14.1. LC−HRMS: t_R = 1.14 min, $[M + H]/z$ = 619.2645, found 647.2651. HPLC with chiral stationary phase (ChiralCel OZ-H 4.6 mm × 250 mm, 5 μm; 60% heptane/TFA 100:0.5, 40% EtOH/MeOH/TFA 50:50:0.5): t_R = 14.1 min, 94% de.

(*S*)-2-Benzyloxycarbonylamino-4-hydroxybutyric Acid Methyl Ester (41d). (a) To an ice-cold solution of H-Hse-OH (5.00 g) in dioxane (168 mL) and 2 M NaOH (42.0 mL), Cbz-Cl

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

(8.12 g) was added portionwise. The reaction mixture was stirred at rt overnight, and dioxane was removed in vacuo. The resulting aq solution was extracted with Et_2O, acidified with 2 M HCl to pH 1, and extracted with DCM. The combined organic layers were dried over Na_2SO_4 and concentrated to dryness to give (*S*)-2-benzyloxycarbonylamino-4-hydroxybutyric acid (8.90 g, 84%) as a beige solid. LC−MS: t_R = 0.71 min, $[M + H]^+$ = 254.41. ^1H NMR (400 MHz, CD_3OD): δ 7.34 (m, 5 H), 5.09 (s, 2 H), 4.34 (m, 1 H), 3.66 (m, 2 H), 2.09 (m, 1 H), 1.88 (m, 1 H).

(b) Dicyclohexylamine (6.37 g) was added portionwise to a solution of the above material (8.90 g) in EtOH (77.0 mL). The mixture was concentrated leading to a white suspension which was filtered off. The white solid was suspended in Et_2O, filtered off, and dried under vacuum to give (*S*)-2-benzyloxycarbonylamino-4-hydroxybutyric acid as dicyclohexylamine salt (12.2 g, 80%), as a white powder. ^1H NMR (400 MHz, CD_3OD): δ 7.35 (m, 5 H), 5.10 (s, 2 H), 4.12 (m, 1 H), 3.65 (m, 2 H), 2.07 (m, 5 H), 1.88 (m, 5 H), 1.74 (m, 2 H), 1.45−1.18 (m, 12 H).

(c) MeI (2.10 mL) was added dropwise at rt to a suspension of the above material (12.2 g) in DMF (196 mL). The reaction mixture was stirred overnight at rt. MeI (1.75 mL) was added, and the stirring was continued at rt for 6 h. MeI (3.50 mL) was added, and the stirring was continued at rt overnight. The solvent was removed in vacuo, and the residue was taken up in water and EA. The layers were separated, the organic phase was washed with saturated NaCl, dried over Na_2SO_4, and concentrated to dryness. The crude product was purified by CC on silica gel (eluting with Et_2O) to give title compound **41d** (4.82 g, 64%) as a colorless resin. LC−MS: t_R = 0.79 min, $[M + H]^+$ = 268.30. ^1H NMR (400 MHz, $CDCl_3$): δ 7.38 (m, 5 H), 5.66 (m, 1 H), 5.15 (m, 2 H), 4.58 (m, 1 H), 4.45 (br, 0.5 H), 4.29 (br, 0.5 H), 3.79 (s, 3 H), 3.73 (m, 1 H), 2.19 (m, 1 H), 1.74 (m, 1 H).

(*S*)-2-Benzyloxycarbonylamino-4-bromobutyric Acid Methyl Ester (40d). To an ice-cold solution of **41d** (2.41 g) and CBr_4 (6.75 g) in DCM (120 mL), PPh_3 on resin (12.4 g, 1.60 mmol/g) was added. The ice bath was removed, the reaction mixture was stirred at rt for 2.5 h, filtered off, and concentrated to dryness. The crude product was purified by CC on silica gel (eluting with heptane/EA 3:1) to give title compound **40d** (1.54 g, 52%) as a colorless resin. LC−MS: t_R = 0.98 min, $[M + H]^+$ = 330.33. ^1H NMR (400 MHz, $CDCl_3$): δ 7.37 (m, 5 H), 5.41 (m, 1 H), 5.14 (s, 2 H), 4.53 (m, 1 H), 3.78 (s, 3 H), 3.45 (t, J = 6.9 Hz, 2 H), 2.46 (m, 1 H), 2.27 (m, 1 H).

(*R*)-2-Benzyloxycarbonylamino-5-hydroxypentanoic Acid Methyl Ester (41g). To a −15 °C solution of Cbz-D-Glu-OMe (**42g**, 0.700 g) in THF (35.0 mL), NMM (0.264 g) was added followed by dropwise addition of isobutyl chloroformate (0.341 mL). The reaction mixture was stirred at −15 °C for 14 h. $NaBH_4$ (0.269 g) was added followed by dropwise addition of MeOH (23.0 mL). The reaction mixture was stirred at 0 °C for 50 min and quenched by adding 1 M $KHSO_4$. The mixture was extracted with EA three times, the combined organic layers were washed with water, dried over Na_2SO_4 and concentrated to dryness to give title compound **41g** (0.647 g, 100%) as a colorless gel. LC−MS: t_R = 0.82 min, $[M + H]^+$ = 282.11. ^1H NMR (400 MHz, $CDCl_3$): δ 7.37 (m, 5 H), 5.46 (m, 1 H), 5.13 (m, 2 H), 4.44 (m, 1 H), 3.80 (m, 2 H), 3.76 (s, 3 H), 1.96 (m, 1 H), 1.78 (m, 1 H), 1.63 (m, 2 H).

(*R*)-2-Benzyloxycarbonylamino-5-iodopentanoic Acid Methyl Ester (40g). PPh_3 (0.905 g) and imidazole (0.251 g) were added to a solution of **41g** (0.647 g) in THF (25.0 mL). At 0 °C iodine (0.876 g) was added portionwise. The reaction mixture was stirred at rt for 3 h. Aqueous $Na_2S_2O_3$ was added to quench the excess iodine, followed by Et_2O and water. The layers were separated, and the organic phase was dried over $MgSO_4$ and concentrated in vacuo. Et_2O (20.0 mL) was added to the residue. Precipitation occurred upon standing. The suspension was decanted, the solution was separated and concentrated to dryness to give title compound **40g** (0.586 g, 65%) as a brown oil. LC−MS: t_R = 1.04 min, $[M + H]^+$ = 391.84.

(*R*)-2-Benzyloxycarbonylamino-5-(diethoxyphosphoryl)-pentanoic Acid (10g). A mixture of **39g** (0.602 g) and LiOH (0.126 g) in water/EtOH/MeOH (5.00 mL, 1:2:2) was stirred at 0 °C for 1 h and at rt for 2 h. Water and Et_2O were added. The layers were separated. The aq phase was acidified with 2 M HCl to pH 3 and extracted with Et_2O. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness to give title compound **10g** (0.277 g, 48%) as a colorless oil. LC−MS: t_R = 0.80 min, $[M + H]^+$ = 388.09. ^1H NMR (400 MHz, $CDCl_3$): δ 7.36 (m, 5 H), 5.64−5.51 (m, 1 H), 5.12 (br, 2 H), 4.43 (m, 2 H), 4.12 (m, 4 H), 2.03 (m, 1 H), 1.90−1.66 (m, 3 H), 1.35 (t, J = 7.1 Hz, 6 H).

4-((2*S*,3*R*)-3,4-Dihydroxy-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}butyryl)piperazine-1-carboxylic Acid Ethyl Ester (43). (a) Imidazole (1.83 g) was added to a solution of (*R*)-3,4-dihydroxybutyric acid methyl ester[55] (2.40 g) in DMF (40.0 mL) at rt. After 15 min of stirring, chlorotriisopropylsilane (3.79 mL) was added and the stirring was pursued overnight at rt. Additional chlorotriisopropylsilane (0.758 mL) was added, and the mixture was stirred at 35 °C for 2 days and concentrated to dryness. The crude product was purified by CC on silica gel (eluting with heptane/EA 95:5) to give (*R*)-3-hydroxy-4-triisopropylsilanyloxybutyric acid methyl ester (2.61 g, 50%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ 4.13 (m, 1 H), 3.80−3.67 (m, 2 H), 3.73 (s, 3 H), 2.89 (m, 1 H), 2.63−2.51 (m, 2 H), 1.14 (m, 3 H), 1.08 (d, 18 H).

(b) Reference 56. $ZnBr_2$ (0.733 g) was charged into a heat gun dried flask. The solid was heated to 120 °C under vacuum for 2 h and filled with argon. The flask was cooled down to −50 °C, and THF (3.00 mL) was added, followed by dropwise addition of MeLi (1.6 M in Et_2O, 2.00 mL). The reaction mixture was allowed to warm up to 0 °C. A flask was charged with THF (6.00 mL) and diisopropylamine (0.963 mL) under argon. The solution was cooled to −78 °C, and BuLi (2.5 M in hexane, 2.80 mL) was added dropwise. The resulting solution was allowed to warm up to 0 °C over 15 min and was cooled down to −78 °C. To a third flask containing (*R*)-3-hydroxy-4-triisopropylsilanyloxybutyric acid methyl ester (0.900 g) in THF (3.00 mL) under argon at 0 °C was added the MeZnBr solution. After stirring at 0 °C for 1 h, the mixture was cooled down to −78 °C and the −78 °C LDA solution was added. The dark blue reaction mixture was stirred at −78 °C for 1 h, and a solution of dibenzylazocarboxylate (1.85 g) in THF (3.00 mL) was added dropwise. The reaction mixture was stirred for 40 min at −78 °C, was quenched with saturated aq NH_4Cl and allowed to warm up to rt and extracted with Et_2O three times. The combined organic layers were dried over $MgSO_4$, filtered off, and concentrated to dryness. The crude product was purified by CC on silica gel (eluting with heptane/EA 1:1) to give dibenzyl 1-((2*S*,3*R*)-3-hydroxy-1-methoxy-1-oxo-4-((triisopropylsilyl)oxy)butan-2-yl)hydrazine-1,2-dicarboxylate (0.480 g, 26%) as a yellow oil. LC−MS: t_R = 1.19 min, $[M + H]^+$ = 589.37.

(c) 2,6-Lutidine (0.178 mL) was added to a solution of the above material (0.450 g) in DCM (1.00 mL) at 0 °C. After 15 min of stirring at 0 °C, *tert*-butyldimethylsilyltrifluoromethanesulfonate (1.15 mL) was added dropwise and the reaction mixture was stirred at 0 °C for 2 h. Aqueous saturated NH_4Cl was added, the aqueous phase was extracted with EA three times, the combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 9:1) to give dibenzyl 1-((2*S*,3*R*)-3-((*tert*-butyldimethylsilyl)oxy)-1-methoxy-1-oxo-4-((triisopropylsilyl)oxy)-butan-2-yl)hydrazine-1,2-dicarboxylate (0.490 g, 91%) as a colorless oil. LC−MS: t_R = 1.33 min, $[M + H]^+$ = 703.64.

(d) To a solution of the above material (0.220 g) in EtOH (5.00 mL), Pd/C (wet, 5%, 0.033 g) was added. The flask was evacuated and backfilled with argon, evacuated, and backfilled with hydrogen. The reaction mixture was stirred for 1 h, and Raney nickel (suspension in water, 5.00 mL) was added. The reaction mixture was stirred at rt under hydrogen overnight and filtered off over Celite. The Celite was washed with DCM/MeOH/Et_3N 9:1:0.1 and the filtrate was concentrated to dryness to give (2*S*,3*R*)-2-amino-3-(*tert*-butyldimethylsilanyloxy)-4-triisopropylsilanyloxybutyric acid methyl ester (0.280 g, 100%) as crude, as a colorless oil. LC−MS: t_R = 1.05 min, $[M + H]^+$ = 420.56.

(e) PyBOP (0.287 g) was added to a solution of **8** (0.150 g) in DCM (2.00 mL). The reaction mixture was stirred at rt for 15 min,

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

and the above material (0.210 g) and DIPEA (0.094 mL) were added. The resulting mixture was stirred at rt overnight and partitioned in saturated aq NH_4Cl and EA. The aq phase was extracted with EA, and the combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 4:6) to give (2*S*,3*R*)-3-(*tert*-butyldimethylsilanyloxy)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-4-triisopropylsilanyloxybutyric acid methyl ester (0.170 g, 48%) as a colorless oil. LC−MS: t_R = 1.37 min, $[M + H]^+$ = 701.30. ^1H NMR (400 MHz, $CDCl_3$): δ 9.02 (d, J = 8.7 Hz, 1 H), 8.51 (m, 2 H), 7.46 (m, 3 H), 7.11 (br, 1 H), 5.18 (dd, J = 2.0 and 8.7 Hz, 1 H), 4.22 (m, 1 H), 4.16−3.98 (m, 2 H), 3.93 (m, 1 H), 3.80 (s, 3 H), 3.79 (m, 2 H), 3.58 (m, 2 H), 3.38 (s, 3 H), 2.29−2.03 (m, 2 H), 1.15 (m, 3 H), 1.13 (s, 12 H), 1.12 (s, 6 H), 0.90 (s, 9 H), 0.09 (s, 3 H), 0.07 (s, 3 H).

(f) 2 M LiOH (0.350 mL) was added to a solution of the above material (0.170 g) in THF (5.00 mL) and water (0.700 mL). The reaction mixture was stirred overnight at rt and then quenched with aq NH_4Cl followed by addition of 2 M HCl until pH 3. The mixture was extracted with EA three times, and the combined organic layers were dried over $MgSO_4$ and concentrated to dryness to give (2*S*,3*R*)-3-(*tert*-butyldimethylsilanyloxy)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-4-triisopropylsilanyloxybutyric acid (0.160 g, 96%) as a white foam. LC−MS: t_R = 1.30 min, $[M + H]^+$ = 687.63. ^1H NMR (400 MHz, $CDCl_3$): δ 9.24 (m, 1 H), 8.51 (m, 2 H), 7.47 (m, 3 H), 7.07 (br, 1 H), 5.04 (m, 1 H), 4.32 (m, 1 H), 4.19−4.00 (m, 2 H), 3.97 (dd, J = 4.5 and 10.3 Hz, 1 H), 3.87−3.55 (m, 4 H), 3.40 (s, 3 H), 2.30−2.08 (m, 2 H), 1.23 (m, 3 H), 1.15 (s, 12 H), 1.14 (s, 6 H), 0.89 (s, 9 H), 0.05 (s, 3 H), 0.04 (s, 3 H).

(g) A solution of the above material (0.160 g), EDC-Cl (0.536 g), and HOBt (0.472 g) in DCM (2.00 mL) was stirred at rt for 15 min, and **9a** (0.368 g) was added. The reaction mixture was stirred at rt overnight, and aq NH_4Cl was added. The resulting mixture was extracted three times with EA. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 1:1) to give 4-((2*S*,3*R*)-3-(*tert*-butyldimethylsilanyloxy)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]-amino}-4-triisopropylsilanyloxybutyryl)piperazine-1-carboxylic acid ethyl ester (0.130 g, 67%) as a white foam. LC−MS: t_R = 1.35 min, $[M + H]^+$ = 827.80. ^1H NMR (400 MHz, $CDCl_3$): δ 8.77 (d, J = 9.5 Hz, 1 H), 8.47 (m, 2 H), 7.48 (m, 3 H), 7.04 (br, 1 H), 5.40 (t, J = 8.8 Hz, 1 H), 4.19−4.00 (m, 4 H), 4.17 (q, J = 7.0 Hz, 2 H), 3.85−3.71 (m, 2 H), 3.83 (d, J = 3.8 Hz, 2 H), 3.63−3.52 (m, 3 H), 3.41 (s, 3 H), 3.37−3.23 (m, 3 H), 2.30−2.09 (m, 2 H), 1.29 (m, 6 H), 1.02 (br, 18 H), 0.90 (s, 9 H), 0.13 (s, 3 H), 0.08 (s, 3 H).

(h) TBAF (1 M in THF, 0.363 mL) was added to a solution of the above material (0.120 g) in THF (3.00 mL). The reaction mixture was stirred at rt for 4 h and concentrated to dryness. The crude product was purified by CC on silica gel (using a gradient from DCM/MeOH 1:0 to DCM/MeOH 9:1) to give title compound **43** (0.061 g, 76%) as a beige foam. LC−MS: t_R = 1.39 min, $[M + H]^+$ = 557.5. ^1H NMR (400 MHz, DMSO-d_6): δ 9.27 (d, J = 7.7 Hz, 1 H), 8.47 (m, 2 H), 7.53 (dd, J = 1.8 and 5.2 Hz, 3 H), 6.96 (s, 1 H), 5.29 (d, J = 5.6 Hz, 1 H), 5.01 (t, J = 7.6 Hz, 1 H), 4.96 (t, J = 4.9 Hz, 1 H), 4.13 (d, J = 23.6 Hz, 1 H), 4.07 (q, J = 7.1 Hz, 2 H), 3.88 (m, 1 H), 3.80 (m, 1 H), 3.72−3.41 (m, 11 H), 3.34 (m, 2 H), 3.30 (s, 3 H), 2.16−2.01 (m, 2 H), 1.21 (t, J = 7.1 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.4, 163.3, 162.4, 161.4, 155.1, 137.6, 131.2, 128.9, 128.4, 99.7, 79.6, 78.8, 72.3, 63.9, 61.4, 56.4, 52.1, 52.0, 51.7, 45.7, 45.1, 44.8, 43.9, 43.6, 42.1, 30.6, 30.1, 15.1. LC−HRMS: t_R = 1.14 min, $[M + H]/z$ = 557.2723, found 557.2728.

4-((*S*)-3-(3-Hydroxyisoxazol-5-yl)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)-piperazine-1-carboxylic Acid Ethyl Ester (44). (a) Reference 57. A suspension of methyl 3-hydroxyisoxazole-5-carboxylate (1.00 g) and K_2CO_3 (1.97 g) in acetone (20.0 mL) was heated at 70 °C for 1 h. Benzyl bromide (1.27 mL) was added, and the reaction mixture was stirred at 70 °C for 3 h and at rt for 2 h. The mixture was filtered off and concentrated to dryness. The crude product was purified by CC on silica gel (eluting with heptane/EA 5:1) to give 3-benzyloxyisoxazole-5-carboxylic acid methyl ester (1.60 g, 100%) as a beige oil. LC−MS: t_R = 0.97 min, $[M + H]^+$ = 234.24.

(b) $NaBH_4$ (0.353 g) was added to a solution of the above material (1.60 g) in MeOH (49.0 mL) at 0 °C, and the reaction mixture was stirred at 0 °C for 4 h. MeOH (100 mL) and 1 M HCl (200 mL) were added, and the resulting mixture was extracted three times with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The crude product was purified by CC on silica gel (eluting with heptane/EA 3:1) to give (3-benzyloxyisoxazol-5-yl)methanol (1.23 g, 87%) as a yellow oil. LC−MS: t_R = 0.81 min, $[M + H]^+$ = 206.22.

(c) Reference 58. A solution of the above material (1.23 g) and pyridine (0.989 mL) in DCM (22.5 mL) was added dropwise to a 0 °C suspension of triphenylphosphine dibromide (4.10 g) in DCM (30.0 mL). The reaction mixture was stirred at 0 °C for 1 h and washed twice with 10% aq sodium bisulfite. The aq layers were extracted with Et_2O. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The resulting solid was triturated with Et_2O, the suspension was filtered off, and the solution was concentrated to dryness. The crude product was purified by CC on silica gel (eluting with heptane/EA 2:1) to give 3-benzyloxy-5-bromomethylisoxazole (1.54 g, 96%) as a yellow oil. LC−MS: t_R = 0.99 min, $[M + H]^+$ = 268.03.

(d) Reference 59. BuLi (1.6 M in hexane, 2.53 mL) was added to a solution of (*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine (1.06 mL) in THF (53.0 mL) cooled at −78 °C, and the reaction mixture was stirred at −78 °C for 30 min. A solution of 3-benzyloxy-5-bromomethylisoxazole (1.54 g) in THF (42.0 mL) was added, and the reaction mixture was stirred at −78 °C for 4 h. A solution of (*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine (1.06 mL) and BuLi (1.6 M in hexane, 2.53 mL) in THF (53.0 mL) prepared as before was added three more times at 1 h intervals to the reaction mixture stirred at −78 °C. The reaction mixture was allowed to warm to 0 °C, aq NH_4Cl was added, and the mixture was concentrated in vacuo. The residue was extracted with Et_2O three times. The combined organic layers were dried over $MgSO_4$ and concentrated to dryness. The crude product was purified by CC on silica gel (eluting with heptane/EA 9:1) to give (2*S*,5*R*)-2-(3-benzyloxyisoxazol-5-ylmethyl)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine (2.14 g, 100%) as a yellow oil. LC−MS: t_R = 1.06 min, $[M + H]^+$ = 372.24. ^1H NMR (400 MHz, $CDCl_3$): δ 7.45−7.32 (m, 6 H), 5.66 (s, 1 H), 5.23 (s, 2 H), 4.30 (m, 1 H), 4.03−3.98 (m, 2 H), 3.88 (t, J = 3.5 Hz, 1 H), 3.72 (s, 5 H), 3.69 (s, 2 H), 3.66 (s, 3 H), 3.22 (dd, J = 4.3 and 15 Hz, 1 H), 3.10 (dd, J = 6.3 and 15 Hz, 1 H), 2.24 (m, 2 H), 3.88 (t, J = 6.4 Hz, 6 H).

(e) A mixture of the above compound (2.14 g) and 1 M HCl (11.5 mL) in MeCN (57.5 mL) was stirred at rt for 2 h and evaporated in vacuo. Concentrated ammonia was added to the residue until pH 9, and the mixture was extracted with DCM. The organic phase was dried ($MgSO_4$) and concentrated to dryness to give (*S*)-2-amino-3-(3-benzyloxyisoxazol-5-yl)propionic acid methyl ester (1.85 g, 100%) as a beige solid. LC−MS: t_R = 0.69 min, $[M + H]^+$ = 277.28.

(f) A solution of **8** (0.861 g), HOBt (0.497 g), and EDC-Cl (0.619 g) in DCM (16.0 mL) was stirred for 5 min at rt. The above product (0.794 g) was added, and the reaction mixture was stirred at rt overnight. Aqueous $NaHSO_4$ was added, and the layers were separated. The organic phase was washed with aq NH_4Cl, dried (Na_2SO_4), and evaporated off. The crude product was purified by CC on silica gel (eluting with heptane/EA 1:1) to give (*S*)-3-(3-benzyloxyisoxazol-5-yl)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionic acid methyl ester (0.447 g, 28%) as a beige powder. LC−MS: t_R = 1.10 min, $[M + H]^+$ = 558.20. ^1H NMR (400 MHz, $CDCl_3$): δ 8.89 (s, 1 H), 8.46 (s, 2 H), 7.49−7.34 (m, 8 H), 7.05 (s, 1 H), 5.84 (s, 1 H), 5.25 (s, 2 H), 5.09 (m, 1 H), 4.20−3.94 (m, 2 H), 3.84 (br, 3 H), 3.78−3.53 (m, 3 H), 3.49−3.36 (m, 2 H), 3.41 (br, 3H), 2.33−2.07 (m, 2 H).

(g) A solution of the above compound (0.447 g) and $LiOH·H_2O$ (0.168 g) in THF/H_2O (10.0 mL, 3:1) was stirred at rt for 1 h. The solvent was removed in vacuo, and 1 M HCl and EA were added to the residue. The layers were separated, and the aq phase was further

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

extracted with EA. The combined organic layers were dried (Na_2SO_4) and evaporated off to give (*S*)-3-(3-benzyloxyisoxazol-5-yl)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-propionic acid (0.430 g, 98%) as a beige foam which was used without further purification. LC−MS: t_R = 1.03 min, $[M + H]^+$ = 544.21.

(h) A solution of the above material (0.430 g), HOBt (0.139 g), EDC-Cl (0.164 g), and DIPEA (0.282 mL) in DCM (9.00 mL) was stirred for 15 min at rt. **9a** (0.129 g) was added, and the reaction mixture was stirred at rt overnight. Aqueous $NaHSO_4$ was added, and the layers were separated. The aq phase was extracted with EA, and the combined organic layers were dried (Na_2SO_4) and evaporated off. The crude product was purified by CC on silica gel (eluting with DCM/MeOH 9:1) to give 4-((*S*)-3-(3-benzyloxyisoxazol-5-yl)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}-propionyl)piperazine-1-carboxylic acid ethyl ester (0.542 g, 99%) as a beige powder. LC−MS: t_R = 1.09 min, $[M + H]^+$ = 684.28. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.90 (m, 1 H), 8.45 (br, 2 H), 7.50−7.34 (m, 8 H), 7.04 (s, 1 H), 5.84 (s, 1 H), 5.48 (m, 1 H), 5.25 (s, 2 H), 4.15 (m, 2 H), 4.03 (m, 1 H), 3.86−3.43 (m, 12 H), 3.41 (s, 3 H), 3.34 (m, 1 H), 3.20 (m, 1 H), 2.32−2.08 (m, 2 H), 1.28 (m, 3 H).

(i) Pd on barium sulfate (5%, 0.098 g) was added to a solution of the above material (0.393 g) in MeOH (13.0 mL). The flask was evacuated and backfilled with argon, evacuated, and backfilled with hydrogen. The reaction mixture was stirred for 3.5 h, and additional Pd on barium sulfate (5%, 0.098 g) was added. The reaction mixture was stirred at rt under hydrogen overnight and filtered off over Celite. The Celite was washed with MeOH, and the filtrate was concentrated to dryness. The crude was purified by preparative LC−MS (Gemini C18 110A Ax, 10 μm, 21.2 mm × 50 mm, 27.7−95% MeCN in water containing 0.2% formic acid, 45 mL/min) to give title compound **44** (0.070 g, 21%) as a white powder. LC−MS: t_R = 1.10 min, $[M + H]^+$ = 594.4. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.91 (d, J = 8.6 Hz, 1 H), 8.45 (m, 2 H), 7.48 (m, 3 H), 7.25 (m, 0.5 H), 7.19 (m, 0.5 H), 7.05 (s, 1 H), 5.83 (s, 1 H), 5.49 (m, 1 H), 4.19−4.10 (m, 3 H), 4.02 (m, 1 H), 3.85−3.70 (m, 2 H), 3.68−3.43 (m, 9 H), 3.40 (s, 3 H), 3.35−3.27 (m, 1 H), 3.22−3.14 (m, 1 H), 2.31−2.10 (m, 2 H), 1.27 (m, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 170.7, 169.8, 168.4, 163.4, 162.5, 161.3, 155.0, 137.7, 131.2, 128.8, 128.5, 99.8, 95.0, 79.6, 78.8, 61.5, 56.4, 52.0, 51.7, 48.0, 45.2, 44.8, 43.6, 42.2, 30.6, 29.9, 15.0. LC−HRMS: t_R = 1.21 min, $[M + H]/z$ = 594.2676, found 594.2671.

4-((*R*)-3-(Bis-isopropoxycarbonyloxymethoxyphosphoryl)-2-{[6-((*S*)-3-methoxypyrrolidin-1-yl)-2-phenylpyrimidine-4-carbonyl]amino}propionyl)piperazine-1-carboxylic Acid Butyl Ester (45). (a) Reference 60. Chloromethyl chloroformate (2.07 mL) and isopropyl alcohol (1.75 mL) were dissolved in Et_2O (34.0 mL). The solution was cooled down to 0 °C, and pyridine was added dropwise. The ice bath was removed, and the reaction mixture was stirred at rt overnight. The suspension was filtered off, and the filtrate was washed with 10% aq citric acid, water, sat. aq $NaHCO_3$, and brine. The organic phase was dried (Na_2SO_4) and concentrated in vacuo to give carbonic acid chloromethyl ester isopropyl ester (3.03 g, 87%) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.74 (s, 2 H), 4.98 (m, 1 H), 1.35 (d, J = 6.3 Hz, 6 H).

(b) Reference 61. Et_3N (0.101 mL) was added to a solution of **30d** (0.150 g) in anhydrous DMPU (0.44 mL). The mixture was stirred at rt for 10 min, and carbonic acid chloromethyl ester isopropyl ester (0.416 mL) followed by NaI (0.0441 g) was added. The reaction mixture was stirred at 45 °C overnight. TEA (0.101 mL) and carbonic acid chloromethyl ester isopropyl ester (0.416 mL) were added, and the mixture was stirred for 3.5 h at 45 °C and partitioned between toluene and water/brine. The layers were separated, the aq phase was extracted with toluene, and the combined organic layers were dried (Na_2SO_4), filtered off, and concentrated to dryness. The resulting crude product was purified by CC on silica gel (using a gradient from heptane/EA 1:0 to heptane/EA 0:1) to give a yellow oil contaminated with residual DMPU. The oil was lyophilized twice to give title compound **45** (0.094 g, 45%) as a white powder. LC−MS: t_R = 1.38 min, $[M + H]^+$ = 851.1. ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.27 (m, 1 H), 8.54 (m, 2 H), 7.52 (m, 3 H), 6.96 (s, 1 H), 5.63−5.52 (m, 4 H), 5.24 (m, 1 H), 4.79 (m, 2 H), 4.19−4.09 (m, 1 H), 4.00 (t, J = 6.5 Hz, 2 H), 3.89 (m, 1 H), 3.80−3.37 (m, 11 H), 3.30 (s, 3 H), 2.66−2.54 (m, 1 H), 2.21−2.04 (m, 2 H), 1.54 (m, 2 H), 2.33 (m, 2 H), 1.23 (m, 12 H), 1.04 (d, J = 6.1 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.4, 163.4, 162.6, 161.4, 155.1, 153.1, 137.7, 131.2, 128.8, 128.6, 99.9, 84.6, 79.6, 78.8, 73.3, 65.2, 62.5, 56.4, 51.9, 51.7, 47.9, 45.3, 45.1, 44.8, 44.6, 43.7, 43.3, 42.3, 35.6, 31.0, 30.6, 30.1, 29.5, 28.3, 26.0, 22.3, 21.8, 19.1, 14.1. LC−HRMS: t_R = 1.29 min, $[M + H]/z$ = 851.3514, found 851.3585.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00933.

Molecular formula strings (CSV)

Characterization of all target compounds not described in the main text, synthetic schemes for analogs **43** and **44**, and SDs and ranges for the data in Table 4 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +41 61 565 62 31. Fax: +41 61 565 65 00. E-mail: eva.caroff@actelion.com.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Raimund Brauner, Simon Buetikofer, Rémy Castro, Dominik Fosshag, Yannick Gallin, Marie Idoux, Petar Maric, Valérie Siefken, and Gina Wintenberger for the synthetic chemistry work, Martine Baumann, Brigitte Butscha, Isabelle Guillaumat, and Benoit Lack for biological testing, Markus Kramberg for technical assistance for in vivo experiments, Fabienne Drouet, Rolf Wuest, Isabelle Weber, Aude Weigel, and Nathalie Jaouen for generating DMPK data, Eric Ertel for providing hERG data, Bruno Capeleto for early formulation, and Henri Ramuz for stimulating discussions.

■ ABBREVIATIONS USED

CDI, 1,1′-carbonyldiimidazole; DIPEA, diisopropylethylamine; DMPU, 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone; EA, ethyl acetate; EDC-Cl, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; HATU, 1-[bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide hexafluorophosphate; HOBt, 1-hydroxybenzotriazole hydrate; NMM, *N*-methylmorpholine; PS, polymer-supported; PyBOP, (benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate; [^{33}P]-2Me-SADP, ^{33}P-labeled 2-methylthioadenosine 5′-diphosphate

■ REFERENCES

(1) Davies, M. J.; Thomas, A. C.; Knapman, P. A.; Hangartner, J. R. Intramyocardial platelet aggregation in patients with unstable angina suffering sudden ischemic cardiac death. *Circulation* **1986**, *73*, 418−427.

(2) Davì, G.; Patrono, C. Platelet activation and atherothrombosis. *N. Engl. J. Med.* **2007**, *357*, 2482−2494.

(3) Michelson, A. D. Antiplatelet therapies for the treatment of cardiovascular disease. *Nat. Rev. Drug Discovery* **2010**, *9*, 154−169.

(4) Andre, P.; Delaney, S. M.; LaRocca, T.; Vincent, D.; DeGuzman, F.; Jurek, M.; Koller, B.; Phillips, D. R.; Conley, P. B. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J. Clin. Invest.* **2003**, *112*, 398−406.

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

(5) Dorsam, R. T.; Kunapuli, S. P. Central role of the P2Y12 receptor in platelet activation. *J. Clin. Invest.* **2004**, *113*, 340−345.
(6) Gachet, C. Antiplatelet drugs: which targets for which treatments? *J. Thromb. Haemostasis* **2015**, *13* (Suppl. 1), S313−S322.
(7) Steg, P. G.; James, S. K.; Atar, D.; Badano, L. P.; Blomstrom Lundqvist, C.; Borger, M. A.; Di Mario, C.; Dickstein, K.; Ducrocq, G.; Fernandez-Aviles, F.; Gershlick, A. H.; Giannuzzi, P.; Halvorsen, S.; Huber, K.; Juni, P.; Kastrati, A.; Knuuti, J.; Lenzen, M. J.; Mahaffey, K. W.; Valgimigli, M.; van't Hof, A.; Widimsky, P.; Zahger, D.; Bax, J. J.; Baumgartner, H.; Ceconi, C.; Dean, V.; Deaton, C.; Fagard, R.; Funck-Brentano, C.; Hasdai, D.; Hoes, A.; Kirchhof, P.; Knuuti, J.; Kolh, P.; McDonagh, T.; Moulin, C.; Popescu, B. A.; Reiner, Ž.; Sechtem, U.; Sirnes, P. A.; Tendera, M.; Torbicki, A.; Vahanian, A.; Windecker, S.; Hasdai, D.; Astin, F.; Åström-Olsson, K.; Budaj, A.; Clemmensen, P.; Collet, J.-P.; Fox, K. A.; Fuat, A.; Gustiene, O.; Hamm, C. W.; Kala, P.; Lancellotti, P.; Maggioni, A. P.; Merkely, B.; Neumann, F.-J.; Piepoli, M. F.; Van de Werf, F.; Verheugt, F.; Wallentin, L. ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force on the management of ST-segment elevation acute myocardial infarction of the European Society of Cardiology (ESC). *Eur. Heart J.* **2012**, *33*, 2569−2619.
(8) O'Gara, P. T.; Kushner, F. G.; Ascheim, D. D.; Casey, D. E., Jr; Chung, M. K.; de Lemos, J. A.; Ettinger, S. M.; Fang, J. C.; Fesmire, F. M.; Franklin, B. A.; Granger, C. B.; Krumholz, H. M.; Linderbaum, J. A.; Morrow, D. A.; Newby, L. K.; Ornato, J. P.; Ou, N.; Radford, M. J.; Tamis-Holland, J. E.; Tommaso, C. L.; Tracy, C. M.; Woo, Y. J.; Zhao, D. X. 2013 ACCF/AHA Guideline for the Management of ST-Elevation Myocardial Infarction: A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* **2013**, *61*, e78−140.
(9) King, S. B., III; Smith, S. C., Jr; Hirshfeld, J. W., Jr; Jacobs, A. K.; Morrison, D. A.; Williams, D. O. 2007 Focused Update of the ACC/AHA/SCAI 2005 Guideline Update for Percutaneous Coronary Intervention. A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines: 2007 Writing Group to Review New Evidence and Update the ACC/AHA/SCAI 2005 Guideline Update for Percutaneous Coronary Intervention, Writing on Behalf of the 2005 Writing Committee. *Circulation* **2008**, *117*, 261−295.
(10) Yusuf, S.; Zhao, F.; Mehta, S. R.; Chrolavicius, S.; Tognoni, G.; Fox, K. K. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N. Engl. J. Med.* **2001**, *345*, 494−502.
(11) Fox, K. A.; Mehta, S. R.; Peters, R.; Zhao, F.; Lakkis, N.; Gersh, B. J.; Yusuf, S. Benefits and risks of the combination of clopidogrel and aspirin in patients undergoing surgical revascularization for non-ST-elevation acute coronary syndrome: the Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE) Trial. *Circulation* **2004**, *110*, 1202−1208.
(12) Snoep, J. D.; Hovens, M. M.; Eikenboom, J. C.; van der Bom, J. G.; Jukema, J. W.; Huisman, M. V. Clopidogrel nonresponsiveness in patients undergoing percutaneous coronary intervention with stenting: a systematic review and meta-analysis. *Am. Heart J.* **2007**, *154*, 221−231.
(13) O'Connor, S. A.; Hulot, J. S.; Silvain, J.; Cayla, G.; Montalescot, G.; Collet, J. P. Pharmacogenetics of Clopidogrel. *Curr. Pharm. Des.* **2012**, *18*, 5309−5327.
(14) Schrör, K.; Siller-Matula, J. M.; Huber, K. Pharmacokinetic basis of the antiplatelet action of prasugrel. *Fundam. Clin. Pharmacol.* **2012**, *26*, 39−46.
(15) Jernberg, T.; Payne, C. D.; Winters, K. J.; Darstein, C.; Brandt, J. T.; Jakubowski, J. A.; Naganuma, H.; Siegbahn, A.; Wallentin, L. Prasugrel achieves greater inhibition of platelet aggregation and a lower rate of non-responders compared with clopidogrel in aspirin-treated patients with stable coronary artery disease. *Eur. Heart J.* **2006**, *27*, 1166−1173.
(16) Wallentin, L.; Varenhorst, C.; James, S.; Erlinge, D.; Braun, O. O.; Jakubowski, J. A.; Sugidachi, A.; Winters, K. J.; Siegbahn, A. Prasugrel achieves greater and faster P2Y12 receptor-mediated platelet inhibition than clopidogrel due to more efficient generation of its active metabolite in aspirin-treated patients with coronary artery disease. *Eur. Heart J.* **2008**, *29*, 21−30.
(17) Wiviott, S. D.; Braunwald, E.; McCabe, C. H.; Montalescot, G.; Ruzyllo, W.; Gottlieb, S.; Neumann, F. J.; Ardissino, D.; De Servi, S.; Murphy, S. A.; Riesmeyer, J.; Weerakkody, G.; Gibson, C. M.; Antman, E. M. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N. Engl. J. Med.* **2007**, *357*, 2001−2015.
(18) Wallentin, L.; Becker, R. C.; Budaj, A.; Cannon, C. P.; Emanuelsson, H.; Held, C.; Horrow, J.; Husted, S.; James, S.; Katus, H.; Mahaffey, K. W.; Scirica, B. M.; Skene, A.; Steg, P. G.; Storey, R. F.; Harrington, R. A.; Freij, A.; Thorsén, M.; PLATO Investigators.. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N. Engl. J. Med.* **2009**, *361*, 1045−1057.
(19) Reny, J. L.; Fontana, P. Antiplatelet drugs and platelet reactivity: is it time to halt clinical research on tailored strategies? *Expert Opin. Pharmacother.* **2015**, *16*, 449−452.
(20) (a) Parlow, J. J.; Burney, m. W.; Case, B. L.; Girard, T. J.; Hall, K. A.; Harris, P. K.; Hiebsch, R. R.; Huff, R. M.; Lachance, R. M.; Mischke, D. A.; Rapp, S. R.; Woerndle, R. S.; Ennis, M. D. Piperazinyl Glutamate Pyridines as Potent Orally Bioavailable P2Y12 Antagonists for Inhibition of Platelet Aggregation. *J. Med. Chem.* **2010**, *53*, 2010−2037. (b) Zech, G.; Hessler, G.; Evers, A.; Weiss, T.; Florian, P.; Just, M.; Czech, J.; Czechtizky, W.; Görlitzer, J.; Ruf, S.; Kohlmann, M.; Nazaré, M. Identification of High-Affinity P2Y12 Antagonists Based on a Phenylpyrazole Glutamic Acid Piperazine Backbone. *J. Med. Chem.* **2012**, *55*, 8615−8629. (c) Bach, P.; Antonsson, T.; Bylund, R.; Björkman, J.-A.; Österlund, K.; Giordanetto, F.; van Giezen, J. J. J.; Andersen, S. M.; Zachrisson, H.; Zetterberg, F. Lead Optimization of Ethyl 6-Aminonicotinate Acyl Sulfonamides as Antagonists of the P2Y12 Receptor. Separation of the Antithrombotic Effect and Bleeding for Candidate Drug AZD1283. *J. Med. Chem.* **2013**, *56*, 7015−7024. (d) Boldron, C.; Besse, A.; Bordes, M.-F.; Tissandié, S.; Yvon, X.; Gau, B.; Badorc, A.; Rousseaux, T.; Barré, G.; Meneyrol, J.; Zech, G.; Nazare, M.; Fossey, V.; Pflieger, A.-M.; Bonnet-Lignon, S.; Millet, L.; Briot, C.; Dol, F.; Hérault, J.-P.; Savi, P.; Lassalle, G.; Delesque, N.; Herbert, J.-M.; Bono, F. N-[6-(4-Butanoyl-5-methyl-1H-pyrazol-1-yl)pyridazin-3-yl]-5-chloro-1-[2-(4-methylpiperazin-1-yl)-2-oxoethyl]-1H-indole-3-carboxamide (SAR216471), a Novel Intravenous and Oral, Reversible, and Directly Acting P2Y12 Antagonist. *J. Med. Chem.* **2014**, *57*, 7293−7316.
(21) Zhang, K.; Zhang, J.; Gao, Z. G.; Zhang, D.; Zhu, L.; Han, G. W.; Moss, S. M.; Paoletta, S.; Kiselev, E.; Lu, W.; Fenalti, G.; Zhang, W.; Müller, C. E.; Yang, H.; Jiang, H.; Cherezov, V.; Katritch, V.; Jacobson, K. A.; Stevens, R. C.; Wu, B.; Zhao, Q. Structure of the human P2Y12 receptor in complex with an antithrombotic drug. *Nature* **2014**, *509*, 115−118.
(22) Zhang, J.; Zhang, K.; Gao, Z. G.; Paoletta, S.; Zhang, D.; Han, G. W.; Li, T.; Ma, L.; Zhang, W.; Müller, C. E.; Yang, H.; Jiang, H.; Cherezov, V.; Katritch, V.; Jacobson, K. A.; Stevens, R. C.; Wu, B.; Zhao, Q. Agonist-bound structure of the human P2Y12 receptor. *Nature* **2014**, *509*, 119−122.
(23) Paoletta, S.; Sabbadin, S.; von Kügelgen, I.; Hinz, S.; Katritch, V.; Hoffmann, K.; Abdelrahman, A.; Straßburger, J.; Baqi, Y.; Zhao, Q.; Stevens, R. C.; Moro, S.; Müller, C. E.; Jacobson, K. A. Modeling ligand recognition at the P2Y12 receptor in light of X-ray structural information. *J. Comput.-Aided Mol. Des.* **2015**, *29*, 737−756.
(24) Tantry, U. S.; Gesheff, M.; Liu, F.; Bliden, K. P.; Gurbel, P. A. Resistance to antiplatelet drugs: what progress has been made? *Expert Opin. Pharmacother.* **2014**, *15*, 2553−2564.
(25) van Giezen, J. J.; Berntsson, P.; Zachrisson, H.; Bjorkman, J. A. Comparison of ticagrelor and thienopyridine P2Y(12) binding characteristics and antithrombotic and bleeding effects in rat and dog models of thrombosis/hemostasis. *Thromb. Res.* **2009**, *124*, 565−571.
(26) Becker, R. C.; Gurbel, P. A. Platelet P2Y12 receptor antagonist pharmacokinetics and pharmacodynamics: A foundation for distinguishing mechanisms of bleeding and anticipated risk for platelet-directed therapies. *Thromb. Haemostasis* **2010**, *103*, 535−544.

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

(27) André, P.; DeGuzman, F.; Haberstock-Debic, H.; Mills, S.; Pak, Y.; Inagaki, M.; Pandey, A.; Hollenbach, S.; Phillips, D. R.; Conley, P. B. Thienopyridines, but not elinogrel, result in off-target effects at the vessel wall that contribute to bleeding. *J. Pharmacol. Exp. Ther.* **2011**, *338*, 22−30.
(28) Caroff, E.; Meyer, E.; Treiber, A.; Hilpert, K.; Riederer, M. A. Optimization of 2-phenyl-pyrimidine-4-carboxamides towards potent, orally bioavailable and selective P2Y12 antagonists for inhibition of platelet aggregation. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 4323−4331.
(29) Paleo, M. R.; Aurrecoechea, N.; Jung, K.-Y.; Rapoport, H. Formal Enantiospecific Synthesis of (+)-FR900482. *J. Org. Chem.* **2003**, *68*, 130−138.
(30) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M.-C. The facile dealkylation of phosphonic acid dialkyl esters by bromotrimethylsilane. *Tetrahedron Lett.* **1977**, *18* (2), 155−158.
(31) Demko, Z. P.; Sharpless, K. B. Preparation of 5-Substituted 1*H*-Tetrazoles from Nitriles in Water. *J. Org. Chem.* **2001**, *66*, 7945−7950.
(32) Kohara, Y.; Kubo, K.; Imamiya, E.; Wada, T.; Inada, Y.; Naka, T. Synthesis and Angiotensin II Receptor Antagonistic Activities of Benzimidazole Derivatives Bearing Acidic Heterocycles as Novel Tetrazole Bioisosteres. *J. Med. Chem.* **1996**, *39*, 5228−5235.
(33) Tachibana, K.; Sato, H.; Ohta, M.; Nakamura, M.; Shiraishi, T.; Imaoka, I.; Yoshino, H.; Nagamuta, M.; Kawata, H. Imidazoline Derivative. EP1634874, March 15, 2006; 22 pp.
(34) Smet, C.; Duckert, J.-F.; Wieruszeski, J.-M.; Landrieu, I.; Buée, L.; Lippens, G.; Déprez, B. Control of Protein−Protein Interactions: Structure-Based Discovery of Low Molecular Weight Inhibitors of the Interactions between Pin1 WW Domain and Phosphopeptides. *J. Med. Chem.* **2005**, *48*, 4815−4823.
(35) Storer, R. I.; Aciro, C.; Jones, L. H. Squaramides: physical properties, synthesis and applications. *Chem. Soc. Rev.* **2011**, *40*, 2330−2346.
(36) Malachowski, W. P.; Coward, J. K. The Chemistry of Phosphapeptides: Investigations on the Synthesis of Phosphonamidate, Phosphonate, and Phosphinate Analogs of Glutamyl-.gamma.-glutamate. *J. Org. Chem.* **1994**, *59*, 7625−7634.
(37) Salituro, G. M.; Townsend, C. A. Total syntheses of (−)-nocardicins A-G: a biogenetic approach. *J. Am. Chem. Soc.* **1990**, *112*, 760−770.
(38) Yamaguchi, J.-I.; Ueki, M. Facile New Method for Preparation of Optically Active Protected Proline. *Chem. Lett.* **1996**, 621−622.
(39) Meanwell, N. A. Synopsis of Some Recent Tactical Application of Bioisosteres in Drug Design. *J. Med. Chem.* **2011**, *54*, 2529−2591.
(40) Ballatore, C.; Huryn, D. M.; Smith, A. B., III Carboxylic Acid (Bio)Isosteres in Drug Design. *ChemMedChem* **2013**, *8*, 385−395.
(41) pK_a values were predicted using ACD/pKa DB software.
(42) Predicted pK_a values of phosphonate-containing analogs are the following: **30c** and **30d**, 1.38 and 7.39; **30h**, 2.23 and 7.81.
(43) Baldoni, D.; Bruderer, S.; Krause, A.; Gutierrez, M.; Gueret, P.; Astruc, B.; Dingemanse, J. A New Reversible and Potent P2Y12 Receptor Antagonist (ACT-246475): Tolerability, Pharmacokinetics, and Pharmacodynamics in a First-in-Man Trial. *Clin. Drug Invest.* **2014**, *34*, 807−818.
(44) The effect of the compounds on the hERG K^+ channel was evaluated using manual patch-clamp of HEK-hERG cells at 37 °C. Compounds were first tested near their limit of solubility in the assay solution (150 mM NaCl; 4 mM KCl; 1.2 mM $CaCl_2$; 1 mM $MgCl_2$; 10 mM HEPES; pH 7.4), and concentration−response curves were determined if current block exceeded 20%.
(45) Inhibition of the major human cytochrome P450 enzymes was investigated using human liver microsomes and P450 isoform-specific marker reactions. Diclofenac 4′-hydroxylation and dextromethorphan O-demethylation were used for CYP2C9 and CYP2D6, respectively. CYP3A4 inhibition was tested using midazolam 1′-hydroxylation and testosterone 6β-hydroxylation as markers. Inhibition experiments were performed at a single substrate concentration around the respective K_m value of each P450 marker. Eight inhibitor concentrations up to 50 μM were used and all experiments performed in duplicate under linear enzyme kinetic conditions. Organic solvent content in none of the experiments exceeded 1.1%. Sulfaphenazole, fluoxetine, and nicardipine were use as positive controls with the test compounds. Metabolite formation was monitored using liquid chromatography (Shimadzu LC-10AD VP pumps, SCL-10A VP system controller, and an HTS PAL autosampler, Shimadzu AG, Reinach, Switzerland) coupled to triple stage mass spectrometer (API2000 and API4000, AB SCIEX, Concord, Ontario, Canada, or TSQ Quantum, Thermo Electron Corporation, Waltham, MA, U.S.). Data acquisition was done using the Analyst (version 1.5 for API2000 and version 1.5.1 for API4000) or Xcalibur (TSQ Quantum, version 1.3) software packages. Data of the inhibition experiments were evaluated by plotting the inhibitor concentration (logarithmic scale) against the metabolite formation rates. IC_{50} values were then determined from the plot by nonlinear regression using the following equation:

$$y = \frac{\text{Top}}{1 + \left(\frac{x}{IC_{50}}\right)^s} + \text{Bottom}$$

where y is the rate of metabolite formation, x is the inhibitor concentration, s is the slope at the point of inversion, and Top and Bottom are the maximum and minimum rates of metabolite formation.
(46) Krise, J. P.; Stella, V. J. Prodrugs of Phosphates, Phosphonates and Phosphinates. *Adv. Drug Delivery Rev.* **1996**, *19*, 287−310.
(47) He, G.-X.; Krise, J. P.; Oliyai, R. Prodrugs of Phosphonates, Phosphinates, and Phosphates. In *Prodrugs: Challenges and Rewards Part 2*; Stella, V. J., Borchardt, R. T., Hageman, M. J., Oliyai, R., Maag, H., Tilley, J. W., Eds.; Springer: New York, 2007; pp 223−264.
(48) Figure 5A and Figure 6A: Rey, M.; Kramberg, M.; Cattaneo, C.; Hess, P.; Weber, E.; Ernst, R.; Haag, F.; Morrison, K.; Clozel, M.; Baumann, M.; Caroff, E.; Hubler, F.; Riederer, M. A.; Steiner, B. Unpublished results.
(49) Born, G. V. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* **1962**, *194*, 927−929.
(50) Gast, A.; Tschopp, T. B.; Schmid, G.; Hilpert, K.; Ackermann, J. Inhibition of clot-bound and free (fluid-phase thrombin) by a novel synthetic thrombin inhibitor (Ro 46-6240), recombinant hirudin and heparin in human plasma. *Blood Coagulation Fibrinolysis* **1994**, *5*, 879−887.
(51) Teng, R.; Butler, K. Pharmacokinetics, pharmacodynamics, tolerability and safety of single ascending doses of ticagrelor, a reversibly binding oral P2Y(12) receptor antagonist, in healthy subjects. *Eur. J. Clin. Pharmacol.* **2010**, *66*, 487−496.
(52) Kurz, K. D.; Main, B. W.; Sandusky, G. E. Rat model of arterial thrombosis induced by ferric chloride. *Thromb. Res.* **1990**, *60*, 269−280.
(53) Liu, A.-C.; Zhao, L.-X.; Yu, S.-W.; Lou, H.-X. Pre-treatment with puerarin affects pharmacokinetics of warfarin, but not clopidogrel, in experimental rats. *Chin. J. Nat. Med.* **2015**, *13*, 257−263.
(54) Descamps, M.; Radisson, J. Process for the preparation of an N-phenylacetic derivative of tetrahydrothieno(3,2-c)pyridine and its chemical intermediate. US5204469, 1993.
(55) Fox, M. E.; Jackson, M.; Lennon, I. C.; McCague, R. A Convergent Synthesis of the 11-Oxa Prostaglandin Analogue AL-12182. *J. Org. Chem.* **2005**, *70*, 1227−1236.
(56) (a) Greck, C.; Bischoff, L.; Ferreira, F.; Pinel, C.; Piveteau, E.; Genet, J.-P. Asymmetric synthesis of anti N-Boc-α-hydrazino-β-hydroxyesters from β-ketoesters by sequential catalytic hydrogenation and electrophilic amination. *Synlett* **1993**, *1993* (7), 475−477. (b) Bourdreux, Y.; Drouillat, B.; Greck, C. Enantio- and diastereoselective syntheses of orthogonally protected anti-2-amino-1,3,4-butanetriols from an achiral β-keto ester. *Synlett* **2005**, *13*, 2086−2088.
(57) Riess, R.; Schoen, M.; Laschat, S.; Jaeger, V. Evaluation of protecting groups for 3-hydroxyisoxazoles. Short access to 3-alkoxyisoxazole-5-carbaldehydes and 3-hydroxyisoxazole-5-carbaldehyde, the putative toxic metabolite of muscimol. *Eur. J. Org. Chem.* **1998**, *1998* (3), 473−479.
(58) Ornstein, P. L.; Arnold, M. B.; Allen, N. K.; Bleisch, T.; Borromeo, P. S.; Lugar, C. W.; leander, J. D.; Lodge, D.; Schoepp, D.

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX

D. Structure−Activity Studies of 6-Substituted Decahydroisoquinoline-3-carboxylic Acid AMPA Receptor Antagonists. 2. Effects of Distal Acid Bioisosteric Substitution, Absolute Stereochemical Preferences, and in Vivo Activity. *J. Med. Chem.* **1996**, *39*, 2232−2244.

(59) Croce, P. D.; La Rosa, C.; Pizzatti, E. Stereoselective synthesis of 3-heteroaromatic-substituted alanines. *Tetrahedron: Asymmetry* **2000**, *11*, 2635−2642.

(60) Baudy, R. B.; Butera, J. A. Derivatives of 2-(8,9-dioxo-2,6-diazabicyclo(5.2.0)non-1(7)-en-2-yl)alkyl phosphonic acid and their use as N-methyl-D-aspartate (NMDA) receptor antagonists. WO 2004/092189, 2004; 21 pp.

(61) Serafinowska, H. T.; Ashton, R. J.; Bailey, S.; Harnden, M. R.; Jackson, S. M.; Sutton, D. Synthesis and in Vivo Evaluation of Prodrugs of 9-[2-(Phosphonomethoxy) ethoxy]adenine. *J. Med. Chem.* **1995**, *38*, 1372−1379.

DOI: 10.1021/acs.jmedchem.5b00933
J. Med. Chem. XXXX, XXX, XXX−XXX