Covalent Guanosine Mimetic Inhibitors of G12C KRAS

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S Supporting Information

ABSTRACT: Ras proteins are members of a large family of GTPase enzymes that are commonly mutated in cancer where they act as dominant oncogenes. We previously developed an irreversible guanosine-derived inhibitor, SML-8-73-1, of mutant G12C RAS that forms a covalent bond with cysteine 12. Here we report exploration of the structure–activity relationships (SAR) of hydrolytically stable analogues of SML-8-73-1 as covalent G12C KRAS inhibitors. We report the discovery of difluoromethylene bisphosphonate analogues such as compound 11, which, despite exhibiting reduced efficiency as covalent G12C KRAS inhibitors, remove the liability of the hydrolytic instability of the diphosphate moiety present in SML-8-73-1 and provide the foundation for development of prodrugs to facilitate cellular uptake. The SAR and crystallographic results reaffirm the exquisite molecular recognition that exists in the diphosphate region of RAS for guanosine nucleotides which must be considered in the design of nucleotide-competitive inhibitors.

KEYWORDS: KRAS G12C, drug design, covalent inhibitor, GDP mimetic, bisphosphonate, bioisostere, CPM, ActivX

RAS mutations are frequently observed in malignant tumors and support diverse hallmarks of cancer including genomic instability,^{1,2} cell proliferation,³ suppression of apoptosis,⁴ reprogramming of metabolism,^{5,6} alteration of the microenvironment,^{7,8} evasion of immune responses,^{9,10} and promotion of metastases.^{11,12} Consistent with its pervasive influence on cancer cell function, extinction of oncogenic KRAS in many established tumor models results in tumor regression.^{6,13,14} Targeting RAS as a therapeutic measure is therefore a high priority.¹⁵

RAS mutations appear to fall into functional categories and will likely require tailored strategies to target each functional class.^{16–18} KRAS G12C is the most commonly found RAS mutation in non-small cell lung cancer, the leading cause of cancer death in the United States.¹⁹ Previously we hypothesized that the activating cysteine mutation in KRAS G12C might allow development of covalent GTP-competitive inhibitors. We reasoned that targeting the active site of KRAS would likely perturb KRAS G12C-mediated signaling based on the observation that RAS signaling is predicated on the identity of its bound ligand.^{20,21}

We developed a GDP mimetic inhibitor SML-8-73-1, which contains an alpha-chloroacetamide electrophile and reacts with Cys12 upon inhibitor binding to KRAS G12C (Scheme 1). SML-8-73-1 is also able to compete *in vitro* with high concentration of GDP and GTP and decreases the affinity of KRAS G12C for the

Scheme 1. Analogue Design Based on Diphosphate Bioisosteres

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RAS binding domain (RBD) of BRAF.²² Furthermore, unbiased proteomics based profiling showed that SML-8-73-1 is highly selective for KRAS G12C among other GTP binding proteins.²³ Nevertheless, SML-8-73-1 contains multiple charged phosphate groups and cannot pass through the cell membrane. Caging strategies to shield the charged phosphates were hampered by compound instability.²²

To enable caging strategies and to explore the possibility of other chemical moieties that might improve properties of the diphosphate pharmacophore for covalent targeting of KRAS G12C, we investigated structure–activity relationships (SAR) on a series of analogues of SML-8-73-1 that varied the diphosphate group as well as the linker moiety. Here we report biochemical characterization of these compounds including an illustrative X-ray crystal structure demonstrating key difficulties inherent to this approach.

A diphosphate compound, SML-8-73-1 suffers from chemical and enzymatic instability, given that the phosphate anhydride bond is prone to hydrolysis.²⁴ This is problematic from two perspectives: first, unstable compounds are inherently disadvantageous from a pharmacokinetic perspective; and second, a caging strategy will likely be required to shield charged atoms in the phosphate pharmacophore to enhance cell permeability, and the resulting steric bulk may further destabilize the phosphate anhydride bond. To address these potential issues, the central oxygen was substituted with a methylene group. The corresponding bisphosphonate is considered to be resistant to acidic and enzymatic hydrolysis at the P–C–P bond,^{25,26} and has been broadly used as an isostere of the diphosphate.

Synthesis of the bisphosphonates utilizes a one-pot reaction sequence, starting with nucleophilic addition–elimination reaction between methylenebis(phosphonic dichloride) and acetonide-protected guanosine **3** affording phosphonic chloride **5**, which was subsequently reacted with ethanolamine **6** to give phosphoric ester **7** after aqueous quench (Scheme 2).

Scheme 2. Synthesis of Bisphosphonate Analogues^a

^a(a) PO(OEt)₃, 0 °C; (b) **6**, NEt₃, PO(OEt)₃, 0 °C to rt; (c) TFA/DCM, rt; (d) **9**, DIEA, DMF, 0 °C.

Deprotection under acidic conditions followed by selective acylation using activated NHS-ester **9** gave rise to desired bisphosphonate diester **10** (XY-01-103). This synthetic sequence allows facile and quick access to bisphosphonate analogues **10** to **20** with various linkers and different guanine analogues, by utilizing different alcohol reaction partners.

Different routes were employed to synthesize various bisphosphonate isosteres. Synthesis of phosphonate intermediate **25** commenced with DCC coupling of diethylphosphonoacetic acid **21** and ethanolamine **22**, and the resulting phosphoryl acetate **23** was deprotected and coupled to guanosine **3** to yield phosphoryl acetate **25** (Scheme 3). Similarly, phosphoryl acetate **29** was obtained by an altered sequence. Deprotection and selective acylation of intermediates **25** and **29** gave rise to

Scheme 3. Synthesis of Bisphosphonate Isosteres^a

^a(a) **22**, DCC, cat. DMAP, DCM, rt; (b) TMSBr, MeCN, 0 °C to rt; (c) **3**, DCC, cat. DMAP, DMF, 60 °C; (d) **3** or **6**, DCC, cat. DMAP, DMF, rt; (e) H₂, Pd/C, MeOH, rt; then PTSA, DCM, rt; (f) **9**, DIEA, DMF, 0 °C; (g) TFA/DCM, rt; (h) **33**, NEt₃, DCM, 0 °C; (i) **35**, *n*BuLi, THF, −78 °C; (j) TMSBr, collidine, MeCN, rt; (k) **22**, NEt₃, DCM, 0 °C; (l) **41**, NEt₃, DCM, 0 °C to rt; (m) NaOH aq, EtOH, rt; (n) **3**, MeCN/pyridine, rt; (o) H₂, Pd/C, MeOH, rt; then 4 N HCl in dioxane, DCM, rt.

phosphoryl acetate compounds **30** and **31**, respectively. The synthesis of **47** started from sulfonylation of ethylenediamine **32**. The resulting sulfonamide **34** was deprotonated and reacted with diethyl chlorophosphate **35**. Following deprotection, the collidine salt **37** was coupled with guanosine **3** to give sulfamoyl phosphonate **44**, which was deprotected and acylated to provide **47** (XY-02-075). For **48**, sequential addition of ethanolamine **22** and guanosine **3** to chlorosulfonyl isocyanate **38** gave rise to sulfamate **45**, which after deprotection was acylated to yield **48**. Alternatively, the synthesis of **49** started from reaction of ethylenediamine **40** with chlorosulfonyl acetate **41**. Subsequent saponification and DCC coupling with guanosine **3** yielded the requisite sulfamoylacetate **46**, which was deprotected and acylated to give **49**.

To measure GDP displacement we utilized a competitive “chemosensor assay”, wherein purified recombinant GDP-loaded KRAS G12C is incubated with compound, then probed at various time points with a probe compound that detects the presence of free (unreacted) G12C thiol. This assay provides a composite measurement of kinetic displacement of GDP and covalent inactivation of KRAS G12C (Figure S1).²³

To determine the relative affinities of compounds we measured k_{inact}/K_i using GMP-stabilized KRAS G12C (Figure S2). To obtain these values we used the strategy advocated by Copeland²⁷ wherein reaction rates are plotted vs compound concentrations and fit to the curve described by the equation $k_{\text{obs}} = k_{\text{inact}}[I]/(K_i + [I])$. Using the fit, estimates for k_{inact} and K_i can be extracted (Figure S2C,D). For this assay purified nucleotide free KRAS G12C was prepared and stabilized with an excess of GMP, which has a low affinity of $3.5 \times 10^4 \text{ M}^{-1}$ for RAS.²⁸ GMP-stabilized RAS

is incubated with compound then probed with a GTP-desthiobiotin probe which, similarly to widely used ATP-biotin (ActivX) probes,^{29,30} contains a reactive acyl phosphate anhydride that reacts with lysine 16 of KRAS. Desthiobiotinylated protein is detected using AlphaScreen (PerkinElmer) reagents (Figure S2A). Because the ActivX and AlphaScreen reagents are used in combination, we call this assay ActivAlpha (Figure S2D).

A third assay consists of incubating recombinant G12C KRAS with new inhibitors and then performing electrospray ionization mass spectrometry as reported previously.²² The percent labeling of the protein can be detected as shown in Figure S3.

To meet our overarching goal of developing GTP-competitive inhibitors that have anticancer effects in cells, compounds will likely need to achieve adequate RAS inhibition well within the typical time scale of the RAS protein turnover; the half-life has been estimated at 12–24 h.^{31,32} We previously performed rudimentary kinetics simulations³³ showing that G12C KRAS inactivators with a k_{inact} of 0.6 min⁻¹ and K_i of 10 nM will yield 50% inhibition of KRAS G12C in 5 h. We therefore adopt these as preliminary standards for inhibitor development. Nevertheless it should be noted that the ActivAlpha assay utilizes an excess of GMP to stabilize the KRAS protein and, unlike the *in vivo* situation, does not require displacement of GDP or GTP.

In the ActivAlpha assay diphosphate **1** (SML-8-73-1) shows an excellent K_i of 9 nM and a relatively fast k_{inact} of 0.86 min⁻¹, which is consistent with the reactive chloroacetamide electrophile of SML-8-73-1. However, the corresponding methylenebisphosphonate analogue **10** loses 300-fold in binding affinity (K_i), and labels KRAS at a rate that is 11-fold slower (Table 1). This highlights the importance of interactions between the oxygen atom of the diphosphate and the various residues in the P-loop of KRAS. By substituting the central oxygen with a methylene group in **10**, both the pK_a of the resulting phosphoric acid and bond lengths and bond angles of the P–X–P linkage (X = O, CH₂) are altered, which may result in lower affinity.³⁴ To regain the electronic and conformational properties as in SML-8-73-1, difluoromethylene and monofluoromethylene groups were incorporated in compounds **11** and **12**, respectively. Fluorinated phosphonates are widely used in the pharmaceutical industry as phosphate isosteres and are found to be better phosphate mimetics than phosphonates.^{35,36} In this case, difluoromethylene bisphosphonate analogue **11** improved the affinity for KRAS by 7-fold, compared to bisphosphonate analogue **10**, while the monofluoromethylene bisphosphonate analogue **12** showed a lower affinity and slower labeling rate.³⁷

The acrylamide analogue of SML-8-73-1 (**2**) exhibits a higher rate constant k_{inact} than α -chloroacetamide-containing SML-8-73-1, although it is generally believed that the α -chloroacetamide warhead is more reactive toward cysteine group than acrylamide.³⁸ We postulated that longer length between the β -phosphate and the reactive site of the acrylamide in **2** may be optimal for the requisite covalent bond formation. Analogues with a propyl linker (**13**, **14**) improved the affinity to KRAS G12C, possibly due to a combinatory effect of optimal spacing, and better trajectory of the electrophile warhead toward nucleophilic substitution of Cys12. On the contrary, analogue **20**, with an internal electrophile and shorter distance between the β -phosphate and the reactive site for Cys12, showed complete loss of activity.

We hypothesized that a rigid linker with a preferred trajectory may further improve labeling efficiency, and a series of analogues with cyclic linkers were synthesized. In the case of **16** with a pyrrolidine linker, a larger k_{inact} of 2.2 min⁻¹ was observed, along

Table 1. SAR of Bisphosphonate Analogues and Diphosphate Isosteres

Cmpds	substitution	chemo-sensor	K_i	k_{inact}	k_{inact}/K_i
		$t_{1/2}$ (h)	(μM)	(min ⁻¹)	(min ⁻¹ • μM^{-1})
1		0.68	0.009	0.86	95
2		0.6	0.28	2.124	7.6
10		2.1	2.7	0.06	0.02
11		4.2	0.38	0.365	0.9
12		6.6	8	0.21	0.03
13		2.5	0.374	0.07	0.2
14		1.06	0.382	0.3	0.8
15		20	5	0.14	0.028
16		1.1	0.92	2.2	2.4
17		12	3.5	0.55	0.16
18		>36 (42)	50	1	0.02
19		>36 (64)	36	2.3	0.06
20		>36 (120)	>20	--	--
30		>36 (84)	15.2	0.055	0.003
31		>36 (47)	2.2	0.053	0.02
47		2.3	1.6	0.33	0.2
48		9.5	5	0.24	0.05
49		24	>10	--	--
50		13	>25	--	--

with fast labeling of G12C KRAS in the chemosensor assay ($t_{1/2}$ of 1.1 h). Cyclopentane-containing analogues **18** and **19** which place the nitrogen in the exocyclic position also showed larger k_{inact} , however the affinities for these two compounds are significantly lower. A piperidine linker in **17** did not improve affinity or labeling efficiency.

We also examined substitution of one or both phosphates with carbonyl or sulfonyl groups. The results showed that both phosphates are crucial in achieving high binding affinity to KRAS; substituting one or both phosphates significantly lowered affinity

Figure 1. Comparison between G12C in complex with XY-02-075 (cyan/blue) and SML 8-73-1 (gray/orange). (A) Structural superposition of nucleotide binding pocket: switch I is highlighted in XY-02-075-bound structure in yellow and in SML-8-73-1 in salmon. Tyr32 and Asp33 are in sticks as labeled. (B, C) Schematic representation comparing interactions between SML-8-73-1 (B) or XY-02-075 (C) and Kras G12C. Dashed lines represent the hydrogen bonding interactions.

and resulted in longer labeling time. Among these, phosphonyl sulfonamide analogue **47** (XY-02-075) had the lowest K_i of 1.6 μ M and a $t_{1/2}$ of 2.3 h. A cocrystal structure of XY-02-075 with KRAS G12C showed an intramolecular hydrogen bonding between the sulfonamide hydrogen and the phosphonate oxygen (see below). Phosphoryl acetate analogue **31**, in which the α -phosphate was replaced with an acetate group, showed a 7-fold lower K_i than the corresponding reversed phosphoryl acetate **30**. This observation is in line with previous reports that interactions between the β -phosphate in GDP and Ras or magnesium are stronger than that of the α -phosphate.²⁸ Further replacement of the remaining phosphonate with sulfonamide in compounds **48** and **49** did not significantly improve affinity. Incorporation of a squaryldiamide group, a common isostere for diphosphates and bisphosphonates,³⁹ in **50** resulted in poor affinity.

New GDP analogues did not achieve the comparable labeling efficiency observed for SML-8-73-1. To gain insight into why our analogues were inferior to SML-8-73-1, we determined the X-ray crystal structure of XY-02-075 bound to KRAS G12C. Complete labeling of KRAS G12C with XY-02-075 was confirmed prior to crystallization using mass spectrometry (Figure S3). Crystals were in the monoclinic space group C_2 with a unit cell similar to other KRAS structures obtained previously.²³ Molecular replacement using WT KRAS GDP-bound structure as a search model (PDB 4OBE) was used to obtain phase information, and the final model was refined to a resolution of 2.70 Å with R -work of 28.0%, R -free of 33.5%, and average B -factor of 89.0 Å² (Table S2).

The structure of the G domain is similar to previously solved RAS family protein structures including the previously reported SML-8-73-1-bound structure (PDB 4NMM) (RMSD = 0.43 Å, 166 atoms aligned). Continuous positive density connecting the terminal carbon atom of XY-02-075 to Cys12 confirmed a covalent link between the compound and protein (Figure S4). The conformation of residues surrounding the guanosine binding site, including P-loop (residue 10–17),⁵⁷ DXXG,¹¹⁶ NKXD, and ¹⁴⁶SAK motifs, were similar to the SML-8-73-1-bound structure with several differences. Importantly there is a lack of density where a magnesium ion and coordinated water molecules have been observed in nearly all previous structures of HRAS and KRAS (Figure 1B,C). Also, in the XY-02-075-bound structure we observed a hydrogen bond between the amide carbonyl oxygen atom and the backbone nitrogen atom of Gly13, whereas the linker amide group formed a hydrogen bond with the ϵ -nitrogen group of Lys16 in the SML-8-73-1 structure. We also noted an outward displacement of switch I residues Tyr32 and Asp33 (Figure 1A). Finally, due to poor electron density and a high B -factor, switch II residues 63 and 64 could not be unambiguously assigned in our model.

Replacement of β -phosphate with sulfonamide apparently leads to dissociation of the magnesium ion and its coordinated water network. Compared to the SML-8-73-1 structure, the magnesium-bridged interaction between the β -phosphate and Tyr 32 and Asp 33 is lost when bound to XY-02-075. Additionally, an intramolecular hydrogen bond forms within XY-02-075 between the amide hydrogen of the sulfonamide and the oxygen atom of the α -phosphonate resulting in a kink in the inhibitor conformation relative to SML-8-73-1 (Figure 1B,C). This kink contributes to displacement of switch I residues Tyr32 and Asp33 by 3.3 Å relative to the position observed in the SML-8-73-1-bound structure. The folded conformation of XY-02-075 also does not allow the sulfonamide oxygens to form hydrogen bonds with the backbone nitrogen atoms of P-loop residues Gly15 and Lys16 as observed with SML-8-73-1. Consequently, the orientation of the linker amide group of XY-02-075 is shifted in comparison to SML-8-73-1.

To confirm that our compounds do not coordinate efficiently with Mg^{2+} , we performed a ³¹P NMR $MgCl_2$ titration study⁴⁰ against fixed concentrations of GDP, GTP, and several of our compounds. These showed a Mg^{2+} -dependent shift in the ³¹P NMR signal with the half-maximal effect at ~0.5 equiv for GDP and GTP and a plateau by 2 equiv. However, with the same concentration of our phosphonate compounds, up to 20 equiv of Mg^{2+} was required before the shift in signal began to plateau, demonstrating that our phosphonate analogues bind Mg^{2+} with much lower affinity (Figures S5, S6).

Mono-, di-, and triphosphate esters are ubiquitously found in biological molecules including small molecules such as nucleotides, acetyl CoA, inositides, and phospholipids and as post-translational modifications to serine, threonine, and tyrosine on many proteins. Therefore, it is not surprising that a host of protein catalytic and binding pockets have evolved to use these molecules as substrates, cofactors, and recognition motifs. While phosphates are powerful binding elements and vastly improve the water solubility of compounds to which they are attached, they typically impair passive diffusion across a lipid bilayer and therefore are typically not found in small molecule drugs. Medicinal chemists have found a number of phosphate bioisosteres that are recognized by these pockets, but typically there is a considerable loss of binding affinity.³⁶ An alternative approach is to create phosphate prodrugs in which the phosphate is unveiled following intracellular metabolism by a suitable enzyme.

In stark contrast to the relative success in finding mono-phosphate mimetics, there has been much more limited effort and success in developing diphosphate mimetics. For example, squaryldiamides have been studied as diphosphate surrogates in mannosyltransferase targeting sugar-nucleotide mimics;³⁹ phos-

phenylformate has been used as diphosphate isostere in squalene synthetase inhibitors;⁴¹ furthermore, sulfonylbenzoyl-nitrostyrenes were explored as bisubstrate type inhibitors of EGFR, in which the sulfonylbenzoyl moiety served as a diphosphate mimic.⁴² However, these bioisosteres suffer from lowered bioactivity when compared with their diphosphate counterparts. We faced this challenge when considering how to generate a cell penetrant version of our prototype G12C KRAS labeling compound SML-8-73-1. We envisioned two possible approaches: to find noncharged phosphate mimetics or to create diphosphate prodrugs. Here we described the synthesis and biological characterization of 18 analogues where we explored phosphate replacements and “linker” variation. We discovered that relatively subtle changes to the diphosphate group resulted in dramatic reduction in affinity and labeling efficiency.

Based on our structural analysis the loss of affinity appears to be, at least in some cases, driven by disruption of the coordination between our compounds and magnesium resulting in loss of numerous interactions seen in native ligand-bound structures. Future efforts to optimize the phosphate pharmacophore will likely benefit from analysis of coordination between compounds and Mg²⁺. This structure also adds evidence to the already extensive data supporting the highly dynamic nature of the RAS switches and their dependence on bound ligand for sampling certain conformations. In the structure reported here, switch I (especially residues 32 and 33) is shifted outward to accommodate the folded XY-02-075, which does not coordinate magnesium and water molecules. This suggests that even small alterations to a ligand bound to KRAS will result in conformational changes that will likely disrupt interactions with RAS effectors that use the switch I or switch II interfaces. The list of such proteins includes important proteins that transduce major signaling cascades from RAS including PI3K and B-RAF. This suggests that development of GTP-competitive inhibitors, if possible, will be highly likely to perturb RAS-dependent cellular processes such as oncogenesis in the case of activated RAS.

A promising compound obtained in this study, **11**, possesses a difluoromethylene bisphosphonate moiety and exhibits a 40-fold reduced affinity relative to SML-8-73-1 but has the potential advantage of more facile prodrug creation due to not having the chemical instability that results from the phosphate anhydride bond. Current efforts focus on development of prodrugs of difluoromethylene bisphosphonate analogues as well as replacement of the guanine heterocycle with moieties that possess better binding affinity in order to regain affinity lost in the phosphate binding pocket.

■ ASSOCIATED CONTENT

Supporting Information

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Experimental methods, X-ray data, and Figures S1–S6 (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DMAP, 4-dimethylaminopyridine; DCC, *N,N'*-dicyclohexylcarbodiimide; PTSA, *p*-toluenesulfonic acid; DCM, dichloromethane; TFA, trifluoroacetic acid; DIEA, *N,N*-diisopropylethylamine; TMSBr, bromotrimethylsilane

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