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FULL PAPER

A Guanidyl-based Bivalent Peptidomimetic Inhibits K-Ras Prenylation and Association with c-Raf

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Abstract: Unusual lipid modification of K-Ras makes Ras-directed cancer therapy a challenging task. Aiming to disrupt electrostatic-driven protein-protein interactions (PPIs) of K-Ras with FTase and GGTase I, a series of bivalent dual inhibitors that recognize the active pocket and the common acidic surface of FTase and GGTase I were designed. The structure-activity-relationship study resulted in **8b**, in which a biphenyl-based peptidomimetic FTI-277 was attached to a guanidyl-containing gallate moiety through an alkyl linker. Cell-based evaluation demonstrated that **8b** exhibited substantial inhibition of K-Ras processing without apparent interference with Rap-1A processing. Fluorescent imaging showed that **8b** disrupts localization of K-Ras to the plasma membrane and impairs interaction with c-Raf, whereas only FTI-277 was found to be inactive. These results suggest that targeting the PPI interface of K-Ras may provide an alternative method of inhibiting K-Ras.

Introduction

Members of the Ras family of 21-kDa GTPases play essential roles as molecular switches of signalling pathways that regulate many cellular events, including cell survival and proliferation. K-Ras is the most frequently mutated Ras isoform in human cancers, and it promotes aggressive tumorigenesis, leading to drug resistance and poor clinical outcomes.[1,2] Thus, synthetic molecules that suppress K-Ras-mediated signals are urgently needed.[3] Recent success in library screening-based approaches have identified low-molecular-weight agents[4–7] and cyclic peptides[8] that allosterically or irreversibly inhibit K-Ras function, highlighting the clinical promise of Ras-directed cancer therapy. However, because spherical structural features of Ras lack apparent binding cavities for small molecules, substantial medical efforts have largely relied on library screenings. Complementary structure-based approaches may therefore lead to the development and identification of K-Ras inhibitors.

Earlier efforts have focused on protein prenylation[9], as posttranslational modification followed by association with the plasma membrane is an essential step for Ras function. Many inhibitors[10] and chemical probes[11] for the protein farnesyltransferase (FTase) have been developed. However, the clinical trials of FTase inhibitors (FTIs) as cancer therapies eventually failed, shedding light on their ineffectiveness against K-Ras-mutated cancers.

This obstacle has been explained by the intrinsically higher affinity of K-Ras to FTase than H-Ras (20–50 times)[12,13] and the prenylation of K-Ras by the related Type I geranylgeranyltransferase (GGTase I) under FTI treatment conditions, after which the resulting geranylgeranylated K-Ras retains full biological function.[14,15] The unusual prenylation makes FTI-based approaches a difficult means of inactivating K-Ras. Studies have revealed that the alternative prenylation of K-Ras is triggered by electrostatic attraction between the basic C-terminal of K-Ras and the acidic surface of the common α-subunit of FTase and GGTase I.[12–16] This suggests that mimicking structural features of the cationic C-terminus of K-Ras may result in a dual inhibitor of K-Ras farnesylation and geranylgeranylation.

FTase and GGTase I are heterodimeric zinc metalloenzymes, and they transfer a prenyl group on the C-terminal cysteine residue of substrate proteins. In general, FTase and GGTase I recognize a consensus tetrapeptide sequence CAAX, where C is a cysteine, AA is any aliphatic dipeptide, and X is Ser, Met, Ala or Gln for FTase and hydrophobic Leu or Phe for GGTase I. Since K-Ras possesses a CVIM sequence, it normally receives farnesylation (Figure 1, path (i)). However, when FTase is inhibited by FTIs, it in turn undergoes geranylgeranylation (Figure 1, path (ii)).[14,15] Biological studies[12–15] and X-ray crystal structures of FTase bound to a K-Ras C-terminal peptide[16] have demonstrated that the electrostatic interaction between the oligo lysine domain of K-Ras and a local acidic surface area of the common α-subunit of FTase and GGTase I containing a number of acidic amino acids (i.e. Glu125, Glu160, Glu161, Glu187, Asp191, Glu229, Asp230) are responsible for increasing the affinity for both enzymes.

We envisioned that an agent capable of disrupting electrostatic-driven protein–protein interactions (PPIs) would be produced by covalent linking of a CVIM or its equivalent moiety and a compound mimicking the basic oligo lysine domain. The resulting hybrid compound would anchor to the active pocket and deliver the cationic moiety to the acidic PPI interfaces of FTase and GGTase I (Figure 1, (iii)), thereby acting as a dual inhibitor of K-Ras farnesylation and geranylgeranylation (Figure 1, (iv)).

Here, we report the first cell-permeable K-Ras C-terminal mimetics in which a conventional FTI is linked to a branched

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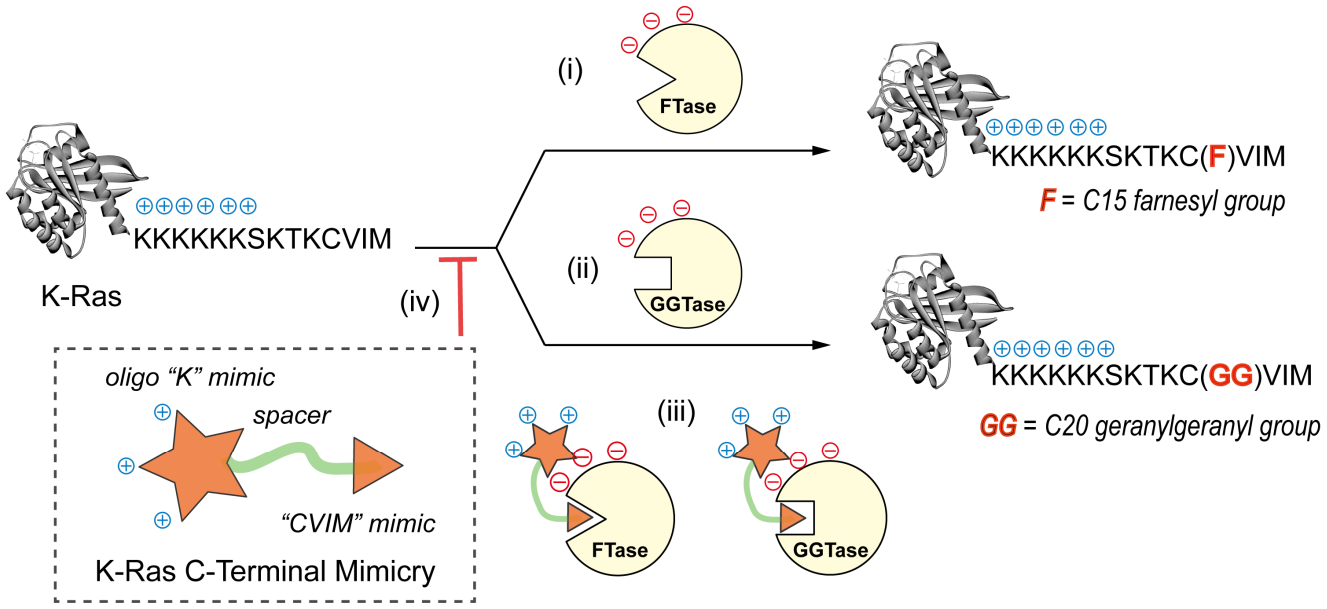


Figure 1. Schematic presentation of alternative prenylation of K-Ras in cells and the dual inhibition strategy for both farnesylation and geranylgeranylation of K-Ras using a bivalent agent mimicking the structure of the K-Ras C-terminus.

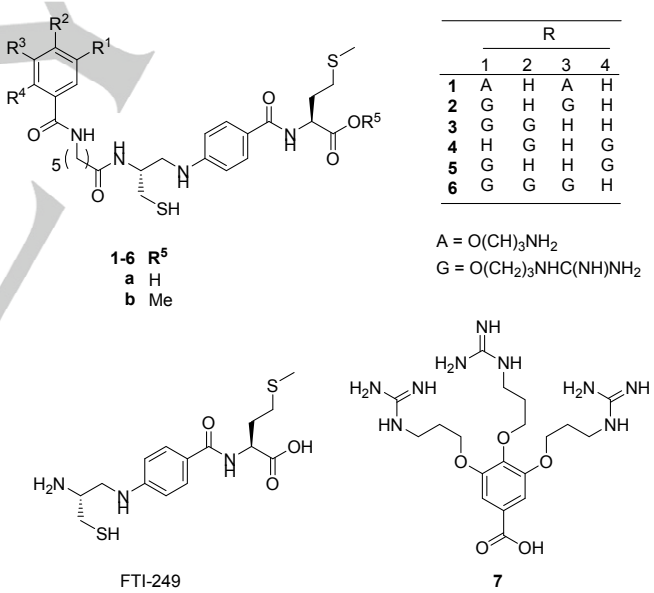
cationic element that binds to the acidic surface of these enzymes. The compound is shown to inhibit cellular processing of K-Ras more effectively than conventional FTIs and to disrupt localization of K-Ras to the plasma membrane, thereby interacting with c-Raf, a downstream effector.

Results and Discussion

Design and synthesis of 1st generation of bivalent compounds

We previously prepared a peptide-based bivalent agent, in which a CVIM tetrapeptide was covalently attached via an alkyl spacer to a gallate derivative containing six primary amino groups.[17] An *in vitro* enzyme assay demonstrated that the resulting hybrid compound shows substantially improved inhibition activity against farnesylation and geranylgeranylation of the C-terminal K-Ras peptide relative to the CVIM tetrapeptide alone, indicating the potential of the bivalent strategy for disrupting the PPIs.[17] However, the compound largely lacks cell permeability, hampering further cell-based evaluation.[17,18] These results prompted us to modify the chemical structure to improve its cell-based activity.

Arginine-rich peptides are widely used for intracellular delivery of bioactive compounds. The bidentate conjugated guanidinium cation in an arginine residue interacts with lipid phosphate anions and induces membrane curvature alteration,[19] while the C3 side chain is thought to interact with the hydrophobic portion of lipids and trigger membrane penetration of the peptide.[19,20] Thus, C3-alkylated guanidine moiety may be a useful building block for improving the compound's permeation of cells.



	R			
	1	2	3	4
1	A	H	A	H
2	G	H	G	H
3	G	G	H	H
4	H	G	H	G
5	G	H	H	G
6	G	G	G	H

Figure 2. Chemical structures of bivalent compounds (**1**–**6**) and module compounds, FTI-249 and guanidyl-containing gallate **7**.

Over the last few decades, Ras CAAX peptidomimetics have been intensively studied in development of pharmaceuticals for cancers[10] and parasitic diseases.[21] Hamilton and Sebti developed a series of 4-amino benzoic acid derivatives that mimic an extended conformation of a CVIM tetrapeptide.[22] Their early examples include FTI-249[23] (Figure 2), which selectively inhibits FTase over GGTase I *in vitro* (for FTase, IC_{50} = 0.03 μM; for

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GGTase I, IC_{50} = 4.4 μM),[23] and the corresponding methyl ester-prodrug, which suppressed H-Ras processing in cells.[23]

Thus, we first decided to incorporate C3-alkylated guanidyl groups and a FTI-249 unit into our new inhibitor design and then prepared a series of bivalent compounds **1a**–**6a**, in which guanidyl or amino groups were introduced to various positions of the benzoyl scaffold (Figure 2). The cationic benzoic acid was linked to FTI-249 through a C5 alkyl spacer, as this length was found to be optimal in previous work[17] (for synthesis, see SI). The corresponding methyl esters of each compound were also synthesized for cell-based evaluation (**1b–6b**).

In vitro inhibition of FTase and GGTase I activities

Inhibitory activities of **1a**–**6a** against recombinant FTase andGGTase I were performed with a previously reported fluorescent spectroscopic assay[18] using dansyl-labelled K-Ras C-terminal peptide $(K)_6$SK(Dans)TKCVIM (Dans = ω-*N*-dansyl) as a substrate, and the results are summarized in Table 1.

Table 1. Inhibition of prenylation of the K-Ras C-terminal peptide by FTase and GGTase I [a]

Compound	K_i (μM) [b]	
	Farnesylation	Geranylgeranylation
FTI-249[c]	0.785 ± 0.008	-
FTI-276[d]	0.00074	-
1a	0.805 ± 0.009	0.856 ± 0.037
2a	0.156 ± 0.014	0.573 ± 0.054
3a	0.768 ± 0.011	8.67 ± 0.077
4a	1.46 ± 0.011	8.13 ± 0.049
5a	1.35 ± 0.011	1.87 ± 0.045
6a	0.026 ± 0.001	2.50 ± 0.046
7	>500	-
8a	0.0006 ± 0.0002	0.710 ± 0.002

[a] Fluorescent *in vitro* assays were performed in a 96-well black plate using KKKKKK(Dans)TKCVIM (1 μM), farnesyl pyrophosphate (FPP, 5 μM), compounds (0–500 μM) and enzyme (50 nM) in 50 mM Tris HCl (pH 7.5) at 30°C. The reaction was monitored at 520 nm (ex. 340 nm) for 5 min with a plate reader. The standard deviation is given for n = 3. [b] K_i values were obtained by conversion of IC_{50} values using the Cheng–Prusoff equation (Ref. 24), $K_i = IC_{50}/\{1+([S]/K_m)\}$, where for FTase K_m = 0.006 ± 0.002 μM and for GGTase K_m = 0.033 ± 0.008 μM (Ref. 17). [c] Ref. 18. [d] Ref. 25.

Primary amino group–containing **1a** had similar activity for FTase and GGTase I, demonstrating that attachment of the cationic moiety to FTI-249 converts the selective FTI into a dual inhibitor, which is consistent with previously reported results.[17] Compound **2a**, in which the primary amino groups were replaced by guanidyl groups, was approximately 5-fold more active than **1a**, suggesting that a guanidyl group is more capable of association with acidic surfaces.

When an alkyl gruanidyl group in **2a** was repositioned from R^3 to R^2, the resulting **3a** exhibited significantly lower activity for GGTase I, indicating that R^2 substitution is unfavorble for binding GGTase I. This was further supported by **5a**, which has a substitutent at the R^1 position, showing better activity for GGTase I than **4a**, which is structually identical to **5a** except for the substituent occuring at R^2, not R^1. As seen in **2a** and **5a**, introduction of the substitutent at R^4 reduced activity for FTase approximately 9-fold, presumably owing to a steric repulsion with the protein.

We then introduced an additional substituent to obtain a trisubstituted compound, and the resulting **6a** showed substantially improved activity for FTase compared to **2a**, presumably owing to the gain in electrostatic force at the acidic surface of the protein. In contrast, gallate derivative **7** showed no inhibitory activity, validating that the strategy of anchoring and delivering the small cationic moiety onto the common acidic surface enables dual inhibition of FTase and GGTase I.

Antiproliferative activity against cancer cells

Next, we evaluated whether these compounds inhibit cell proliferation as reported for FTIs.[26–28] Previous studies have demonstrated that ester-prodrugs significantly improve cell-based activity.[29] Therefore, methyl esters **1b**, **2b**, **6b** and **7** were subjected to a trypan blue assay using oncogenic Ras-transformed T24 human bladder carcinoma cells (Figure 3). All tested compounds, except for **7**, exhibited antiproliferation activity in a concentration-dependent manner, in which their descending order of effects (**6b**, **2b** and **1b**) corresponds with the descending order of enzyme inhibition activities that were observed for their corresonding free acids (**6a**, **2a** and **1a**) (Table 1). These results demonstrated that the guanidyl-containing bivalent agents penetrate through cell membranes and exhibit inhibitory activity against cell growth. However, the activity remains moderate across a high micromolar range, and only weak inhibition against FTase was detected for **6b** in cells (Figure S1), prompting us to further modify the chemical structure of the agents.

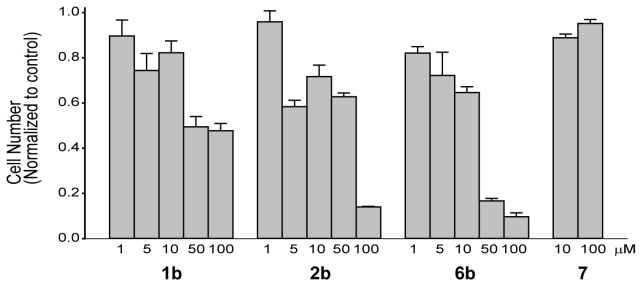

Figure 3. Antiproliferative activity for T24 cells (n = 3). Cells (1 × 10^4 cells) were treated with **1b**, **2b**, **6b**, **7** or DMSO and incubated at 37°C, 5% CO_2 for 5 days prior to a trypan blue assay. n ≥ 3.

Second generation guanidyl-containing bivalent compounds based on a biphenyl scaffold

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To this end, we decided to synthesize **8**, the second generation of guanidyl-containing bivalent compound, by exploiting biphenyl-based peptidomimetic FTI-276[30] (Figure 4). We envisioned that exploiting the potent peptidomimetic (for FTase, IC_{50} = 0.61 nM; for GGTase I, IC_{50} = 50 nM)[30,31] would increase *in vitro* and whole cell activity of the resulting bivalent compounds **8a** and **8b** (Figure 4), thus allowing for a detailed evaluation of their effects on cellular events.

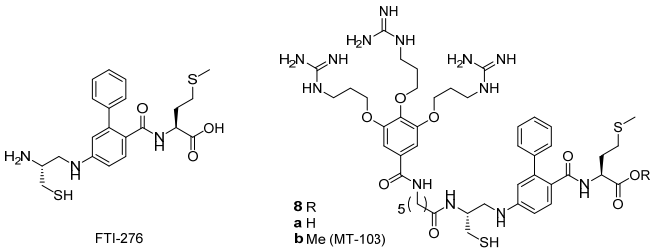

Figure 4. Chemical structures of the bivalent compound **8** and its module compound, FTI-276.

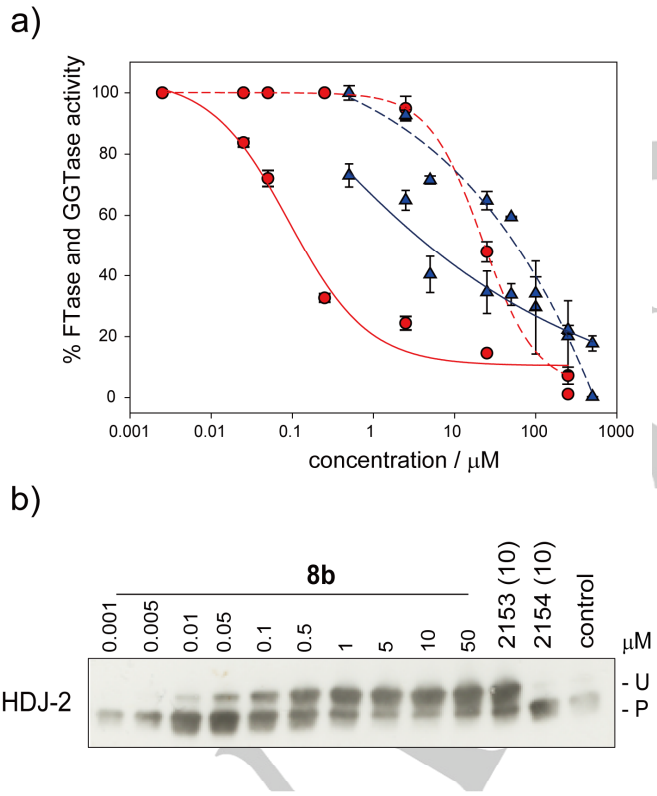

Figure 5. (a) Dose-response curves for the inhibition of FTase (solid) and GGTase I (dash) by **6a** (blue triangles) and **8a** (red circles). Fluorescent *in vitro* assays were conducted using KKKKKKSK(Dans)TKCVIM (1 μM) and FPP (5 μM) in 50 mM Tris HCl (pH 7.5) at 30°C. *n* = 3. (b) Effect of **8b** on processing of HDJ-2 in T24 cells. Inhibition of farnesylated HDJ-2 is seen by the band shift from processed (P) to unprocessed (U) protein. Imidazole-containing FTI-2153 (Ref. 33) and GGTI-2154 (Ref. 29) (10 μM) were used as positive controls (Ref. 34). Their chemical structures are shown in Figure S2.

First, we evaluated the enzyme inhibition activity of **8a** and compared it with that of FTI-249-based **6a** (Figure 5a). Thus, **8a** exhibited significant inhibition activity against FTase (K_i = 0.0006 ± 0.0002 μM, red solid line in Figure 5a), which is approximately 50-fold greater than that of **6a** (K_i = 0.026 ± 0.001 μM, blue solid line in Figure 5a). Notably, **8a** maintains submicromolar activity for GGTase I (K_i = 0.71 ± 0.002 μM), although the improvement remains only approximately 3-fold compared with that of **6a** (K_i = 2.5 ± 0.046 μM, red and blue dash lines in Figure 5a, respectively). This somewhat moderate effect of the introduction of FTI-276 on GGTase I inhibition might be explained by a possible conformational distortion of the peptidomimetic unit caused by the amide linkage to the gallate moiety, as previously discussed in the literature.[32] The presence of the substituent at the R^4 position in **8a** may also account for the relatively low activity for GGTase I, as discussed above.

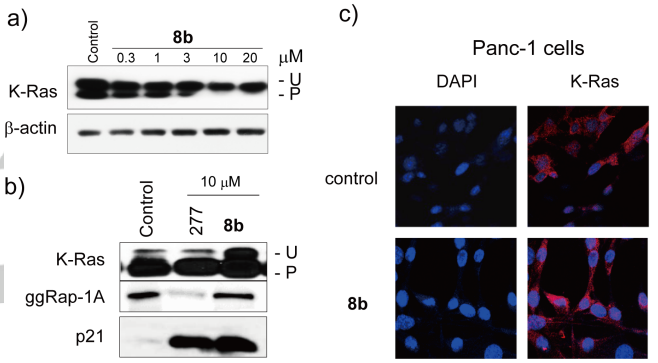

Figure 6. (a) Effect of **8b** on processing K-Ras (G12V) in NHH-3T3 cells stably expressing mutated K-Ras. U = unprocessed, P = processed. (b) Effect of FTI-277 and **8b** on processing K-Ras and Rap-1A and on induction of p21. Anti-K-Ras, -geranylgeranylated Rap-1A and -p21 were used, respectively. (c) Confocal fluorescent imaging using anti-K-Ras antibody and DAPI staining of Panc-1 cells treated with **8b** (10 μM).

Next, we evaluated the methyl ester form, **8b** (MT-103), against endogenous FTase in cells by monitoring the farnesylation level of the 40-kDa heat-shock protein HDJ-2 by a similar procedure to that reported in the literature.[29] Briefly, T24 cells were treated with various concentrations of **8b**, and cell lysates were analysed by western blot using anti-HDJ-2 antibody, allowing the differentiation of processed from unprocessed HDJ-2 protein[29] (Figure 5b). In contrast to **6b** (Figure S1), **8b** supressed HDJ-2 processing, with an IC_{50} value of approximately 0.5 μM, which is more than two orders of magnitude greater than that of **6b**, supporting the remarkable inhibition efficacy of **8b** against FTase in cells.

Bivalent compound inhibits prenylation processing of K-Ras

We then wanted to address a critical question: does the bivalent **8b** disrupt K-Ras processing in cells? Thus, we treated NIH-3T3 cells stably expressing G12V K-Ras with various concentrations of **8b** and performed western blot analysis using anti-K-Ras antibody. Notably, K-Ras processing was inhibited at low micromolar concentrations in cells (Figure 6a), whereas only FTI-277, the methyl ester form of FTI-276, was shown to be inactive

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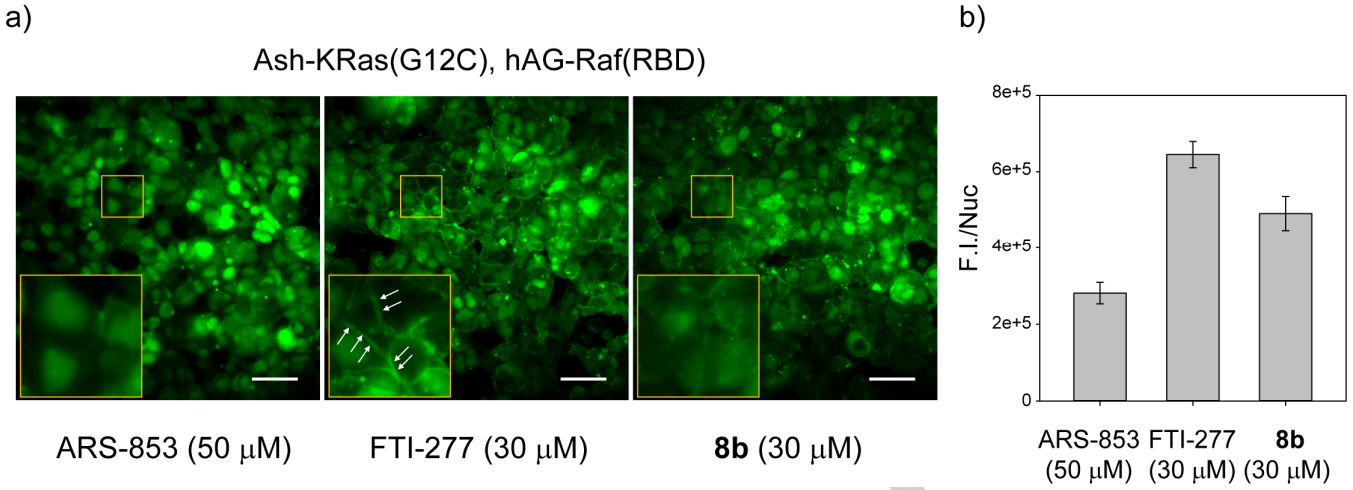

Figure 7. (a) Fluorescence images of cultured HEK293 cells stably expressing K-Ras (G12C) fused to Ash-tag and cRaf (RBD) fused to hAG (humanized Azami-Green). Cells were treated with 50 μM ARS-853, 30 μM FTI-277 and 30 μM **8b**, respectively, and incubated for 4.5 h prior to fixation with paraformaldehyde. Scale bars, 50 μm. Arrows in magnified window indicate puncta accumulated at the plasma membrane. (b) Fluorescence intensities of puncta were segmented and quantified using the ICY image analysis platform and then divided by the number of nuclei. At least 232 cells were analyzed for each image (n = 3).

at 10 μM, as previously reported[35] (Figure 6b, upper row). These results demonstrate that the ability to bind to the acidic surface of FTase and/or GGTase I accounts for the potency of **8b** against K-Ras processing.

Surprisingly, **8b** did not interfere the native GGTase I processing of Rap-1A (Figure 6, middle row). Rap-1A is a Ras-related protein with a C-terminal CLLL sequence and exclusively receives geranylgeranylation. The western blot analysis using anti-geranylgeranylated Rap-1A antibody showed that FTI-277 partially inhibited Rap-1A processing as previously reported at high concentrations (10–15 μM),[34] whereas **8b** did not show apparent activity (Figure 6b, middle row). This result suggests that covalent linking of the gallate moiety to FTI-277 reduced the affinity of **8b** for GGTase I, therefore **8b** failed to inhibit geranylgeranylation of the native substrate, which comprises the C-terminal leucine residue that has higher affinity to the active site of GGTase I than methionine residue does.

It has been reported that inhibition of FTase and GGTase I causes induction of cyclin-dependent kinase inhibitor p21 protein and thus G1 arrest.[33,36] As shown in Figure 6b (lower row), both FTI-277 and **8b** clearly induced p21 expression, further supporting that FTase and/or GGTase I are inhibited in cells. Furthermore, confocal fluorescent microscopy analysis of K-Ras-transformed pancreatic carcinoma Panc-cells using anti-K-Ras antibody showed that **8b** promotes cytosolic delocalization of endogenous K-Ras (Figure 6c), further supporting that K-Ras prenylation was disrupted by **8b**.

Bivalent compound inhibits K-Ras/c-Raf interaction on cytoplasmic membranes

Finally, we examined whether **8b** is capable of disrupting K-Ras-Raf PPIs on plasma membranes. To address this issue, we used a previously developed fluorescent-based PPI-visualisation system (Fluoppi)[37] to monitor interactions between the K-Ras GTP-bound form (i.e. active form) and c-Raf in cells. Briefly, K-Ras(G12C) fused to an assembly helper protein (Ash-K-Ras(G12C)) and the Ras-binding domain of c-Raf (c-Raf(RBD)) fused to fluorescent Azami-Green (hAG-cRaf(RBD)) were stably expressed in HEK239 cells, where Ash protein forms a oligomer and hAG protein forms a tetramer. As prenylation of clustered Ash-K-Ras(G12C) occurs and the tagged K-Ras proteins are promoted to localise near cytoplasmic membranes, tetrameric cRaf(RBD) are recruited to the membranes through PPIs with the tagged K-Ras to form a large protein assembly. This results in the formation of fluorescent puncta composed of these tagged proteins near the cell periphery.[37] A selective covalent inhibitor of K-Ras(G12C), ARS853,[38] induced no apparent formation of puncta at 50 μM (Figure 7a, left), indicating that AR853 inhibits K-Ras/c-Raf interactions. In contrast, when cells were treated with FTI-277 at 30 μM, puncta were clearly observed near membranes (Figure 7a, middle), indicating that K-Ras is recruited to the plasma membrane for interactions with c-Raf. In contrast, **8b** reduced the degree of puncta formation (Figure 7a, right), demonstrating that **8b** inhibits K-Ras-c-Raf interaction by approximately 25% compared to FTI-277 (Figure 7b). To our knowledge, this is the first example of a dual inhibitor of FTase and GGTase I disrupting K-Ras plasma membrane localization and interaction with c-Raf, one of its downstream effectors.

Conclusions

Based on the C-terminal structure of K-Ras, we developed a series of bivalent dual inhibitors of FTase and GGTase I by covalently linking a commercially available CVIM peptidomimetic and various guanidyl-containing benzoyl moieties. The biological evaluation demonstrated that **8b** is capable of inhibiting HDJ-2 and K-Ras processing in cells at submicromolar to low micromolar concentrations. Furthermore, fluorescent imaging revealed that

8b disrupts membrane localization of K-Ras and its association with c-Raf.

Because K-Ras must be localized to plasma membranes to be functional,[39,40] chemical disruption of the process of membrane localization remains an attractive therapeutic approach, and various inhibitors of Rce,[41] Icmt,[42,43] PDEδ[44] and others are under development. Although the activity of **8b** remains moderate, the results obtained in this study suggest that a molecular strategy for targeting PPI interfaces between K-Ras and FTase and GGTase I may provide a solution for overcoming the obstacles of FTIs in K-Ras-directed anticancer clinical applications. One limitation of **8b** is its sensitivity to oxidation caused by sulphur atoms in the peptidomimetic, and at present, it is unclear whether **8b** inhibits geranylgeranylation of K-Ras in cells or the degree to which downstream signals of K-Ras were perturbed by **8b**. These issues will need to be addressed in order to better understand the mode of action. To that end, more metabolically stable agents based on non-thiol-containing peptidomimetics are therefore desirable. Work is currently underway in our laboratory towards these goals.

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Keywords: K-Ras • prenylation • protein-protein interactions • bivalent inhibitors • c-Raf

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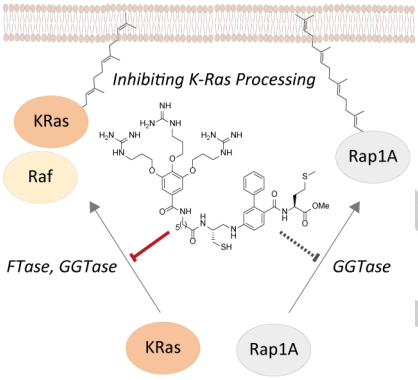
Layout 1:

FULL PAPER

Cell-permeable bivalent dual inhibitors for FTase and GGTase I were designed aiming to disrupt protein-protein interactions with K-Ras. Cell-based evaluation demonstrated that the compound inhibits K-Ras processing without apparent interference with Rap-1A processing. The compound was also shown to disrupt localization of K-Ras to the plasma membrane and impairs interaction with c-Raf, a downstream effector.



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A Guanidyl-based Bivalent Peptidomimetic Inhibits K-Ras Prenylation and Association with c-Raf