Development of a Nucleotide Exchange Inhibitor That Impairs Ras Oncogenic Signaling

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Abstract: Despite more than three decades of intense effort, no anti-Ras therapies have reached clinical application. Contributing to this failure has been an underestimation of Ras complexity and a dearth of structural information. In this regard, recent studies have revealed the highly dynamic character of the Ras surface and the existence of transient pockets suitable for small-molecule binding, opening up new possibilities for the development of Ras modulators. Herein, a novel Ras inhibitor (compound 12) is described that selectively impairs mutated Ras activity in a reversible manner without significantly affecting wild-type Ras, reduces the Ras–guanosine triphosphate (GTP) levels, inhibits the ac-

tivation of the mitogen-activated protein kinase (MAPK) pathway, and exhibits remarkable cytotoxic activity in Ras-driven cellular models. The use of molecular dynamics simulations and NMR spectroscopy experiments has enabled the molecular bases responsible for the interactions between compound 12 and Ras protein to be explored. The new Ras inhibitor binds partially to the GTP-binding region and extends into the adjacent hydrophobic pocket delimited by switch II. Hence, Ras inhibitor 12 could represent a new compound for the development of more efficacious drugs to target Ras-driven cancers; a currently unmet clinical need.

Introduction

Ras proteins are small GTPases (GTP = guanosine triphosphate) that regulate signaling pathways critical for proliferation and cell survival. Ras has three isoforms, H-Ras, N-Ras, and K-Ras. All of them oscillate between an active GTP-bound and an in-

active guanosine diphosphate (GDP)-bound state. This equilibrium must be tightly controlled because the signaling becomes oncogenic when it is deregulated.^[1] It is known that Ras over-activation in tumors is usually associated with a single mutation, typically at codons 12, 13, or 61. Mutation at these conserved sites favors GTP binding and produces constitutive activation of Ras. Aberrant Ras function is associated with around 20 to 30% of all human cancers;^[2] this makes Ras a therapeutic target of remarkable interest in oncology. However, after three decades of intense efforts in the pharmaceutical industry and academia, no direct inhibitors of Ras have been marketed, which has reinforced Ras as the paradigmatic example of an undruggable protein. Nonetheless, interest in Ras inhibition has not decreased with time and the last years have witnessed important advances in the field.^[3] In general, two approaches have been explored for inhibiting Ras activity: 1) the direct inhibition of Ras and 2) the indirect blockade of Ras activity by interfering with the enzymes involved in its post-translational modifications.^[4] In addition, direct Ras inhibitors can be further divided into compounds that act by impairing the Ras catalytic activity or molecules that block the activation of the Ras-induced signaling pathway by hampering Ras interaction with its effector proteins (protein–protein interaction inhibitors).^[5] During these years, the different strategies have raised hopes that have been, in general, followed by profound disappointments,^[3d] but they have undoubtedly contributed to increasing the understanding of Ras structure and function, and have set the basis for current research directions. In this context, it is

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generally considered that targeting Ras activity by interfering with the enzymes involved in its post-translational modifications (such as farnesyl transferase) or blocking the interaction between Ras and its effectors (such as the Raf serine/threonine kinases, phosphoinositide 3-kinase, Ral, or Rac proteins) can have drawbacks, such as the existence of alternative prenylation,^[6] a lack of selectivity, or the appearance of resistances.^[4a,7] On the contrary, the direct inhibition of Ras has generally faced a lack of efficacy due to the high affinity of Ras towards GDP and GTP and high intracellular nucleotide concentrations together with a lack of evident druggable hydrophobic pockets in the flat surface of Ras.^[3c,5a] However, recent results have challenged this idea. The Ras surface is highly dynamic and the protein exists in different conformational states in which transient pockets suitable for small-molecule ligands are created.^[8] These alternative sites, different from the canonical nucleotide-binding site, are being explored and structurally characterized, leading to the identification of new compounds that behave as direct Ras inhibitors in either a reversible^[9] or irreversible^[10] manner. These results clearly support the notion that small molecules can indeed successfully modulate Ras activity, although the compounds described to date still require further optimization in terms of activity, efficacy, and potential toxicity liabilities.^[9a] Hence, the ideal Ras direct inhibitor should selectively inactivate mutant Ras to minimize effects on wild-type Ras to reduce toxicity and decrease Ras-GTP levels to block the signaling pathways that promote cell proliferation. With the aim of contributing to such endeavors, herein we describe the identification of a new Ras direct inhibitor that selectively impairs Ras mutated activity in a reversible manner without significantly affecting wild-type Ras, reduces the Ras-GTP levels, inhibits the activation of the mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) pathways, and exhibits a remarkable cytotoxic activity in Ras-driven cellular models. This new compound adds to the scarce efficient directly reversible Ras inhibitors described to date and should help in the development of K-Ras inhibitors suitable for the treatment of specific tumors with current bad prognosis, either by themselves or in combination therapies to increase efficacy or to decrease the appearance of resistance.

Results and Discussion

Hit identification

The ExonHit proprietary library (1000 compounds) was screened for its capacity to induce cytotoxicity in a panel of several cancer cell lines characterized by the presence of mutated K-Ras, which is the most frequently mutated Ras isoform. The cell lines HCT-116 (G13D), H-460 (Q61H), and MDA-MB-231 (G13D) were selected as representative of colon, lung, and breast carcinomas with mutated K-Ras, as indicated by the American type cell collection (ATCC). Taking into account that up to 80 % of K-Ras mutations occur at codon 12 (G12X, in which X is different amino acids, such as aspartic acid, valine, or cysteine),^[2] NIH-3T3 cells transfected with the G12V mutant K-Ras were also included in the panel. These assays led to the

Table 1. Cytotoxicity of compounds **1** and **2** in four mutated K-Ras cancer cell lines.

R	Cytotoxicity (IC ₅₀ [μM]) ^[a]			
	HCT-116	H-460	MDA-MB-231	NIH-3T3 (G12V)
1 –OCOPh	43	44	40	47
2 –OH	23	14	18	32

[a] Cytotoxicity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see the Experimental Section for details). IC₅₀ values are the means of two to three independent experiments performed in triplicate. In all cases, the standard error of the mean (SEM) is within 10% of the mean value.

identification of compound **1**, with moderate values in the four cell lines, as an initial hit (Table 1). Aiming to improve the biological activity of **1**, we carried out some structural modifications to the ester group, including its replacement by hydroxyl, ether, carbamate, and amine groups (Table S1 in the Supporting Information). The new derivatives were synthesized and screened for cytotoxicity in the mutated K-Ras cellular panel (Scheme S1 and Table S1 in the Supporting Information). They were analyzed sequentially in the four cell lines and only compounds with IC₅₀ values below 25 μM proceeded along the assays. Among them, derivative **2** turned out to be the best one within the series regarding cytotoxicity (Table 1) and hydrophobicity (clogP=2.4). The fact that the rest of the analyzed indoles (see Table S1 in the Supporting Information) were poorly cytotoxic, led us to consider the disconnection approach shown in Figure 1 as a possibility to improve biological

Figure 1. Design of compound **3** by the disconnection carried out in indole **2**.

activity. In the initial design, the envisioned modification would yield an aniline, but taking into account that this moiety has raised structural alerts associated to potential side toxicities,^[11] we considered instead its isostere ether **3** (Figure 1, R'=H). This compound, synthesized as depicted in Scheme 1, showed good cytotoxicity values in all K-Ras mutated cells (IC₅₀ values between 22 and 15 μM, see Table 2). Hence, it was selected for further optimization.

Optimization of **3**

Through the optimization process, different substituents were included in the phenyl ring of compound **3**. We focused our attention on the introduction of halogen atoms, such as fluo-

Scheme 1. Synthesis of compounds 3–12: a) 1,5-dibromopentane, K₂CO₃, NaI, DMF, 80 °C, 24 h, 54%; b) K₂CO₃, NaI, 2-butanone, 80 °C, 24 h, 53–91%; c) microwave (MW), 210 °C, 45 min, 20–66%; d) H₂, 10% Pd/C (H-Cube reactor), EtOH, RT, 92–98%.

rine and chlorine. This decision was based on the positive role of fluorine and chlorine atoms on the pharmacokinetic properties, metabolic stability, and bioavailability.^{3,21} Hence, compounds 4–12 were synthesized as shown in Scheme 1. Williamson reaction of kojic acid with 1,5-dibromopentane, followed by alkylation of intermediate 13 with the corresponding phenol derivative afforded the target final compounds. Non-commercial intermediate phenols 14–17 were obtained by Claisen rearrangement under MW conditions of allyl aryl ethers 18–20 and subsequent catalytic hydrogenation of allyl derivatives 21–24. Compounds 3–12 were screened for cytotoxicity at the four mutated K-Ras cell lines (Table 2). Within this series, the best results were obtained for compound 12, with IC₅₀ values equal to or less than 15 μM in all analyzed cell lines. Table 2 also includes the corresponding data for DCAI^[22] one of the scarce Ras direct inhibitors described to date, as comparison. Finally, we assessed the importance of the two chlorine atoms and the influence of the spacer (see Table S2 and Scheme S2 in the Supporting Information), but none of these modifications improved the activity of compound 12, so it was selected for further biological studies focused on confirming whether 12 was, in fact, affecting Ras activity in a direct manner. To carry out these studies, the MDA-MB-231 cell line was chosen as an appropriate cellular model by considering that compound 12 showed the best IC₅₀ value in this cell line (Table 2).

Inhibition of Ras activity by 12

Oncogenic mutant Ras is locked in the active GTP-bound state, leading to constitutive stimulation of its downstream signaling pathways, which involve a plethora of pro-tumor effects, including sustained proliferative signaling and an increase in cell

Figure 2. Compound 12 significantly reduces the levels of the Ras-GTP complex (active form). MDA-MB-231 cells were treated with 12 (25 μM), lysed, and Ras-GTP complex was immunoprecipitated and then visualized by Western blot. The bar graph shows the ratio Ras-GTP/total Ras (T-Ras), expressed as percentage relative to DMSO after band intensity quantification. Images are representative of four independent experiments. ** = *P* < 0.01 versus DMSO (student *t* test).

Figure 3. Compound 12 significantly inhibits phosphorylation (activation) of the Ras effector kinases AKT, MEK, and ERK. Lysates were obtained from MDA-MB-231 cells treated with vehicle (DMSO) or compound 12 (25 μM). Representative Western blots of phosphorylated (p-AKT) and total AKT (T-AKT), phosphorylated (p-MEK1/2) and total MEK1/2 (T-MEK1/2), and phosphorylated (p-ERK1/2) and total ERK1/2 (T-ERK1/2) are shown. The bar graphs represent the optical density of the immunoreactive phosphorylated protein normalized to the total corresponding protein and expressed as the percentage relative to that of DMSO. Data correspond to the average ± SEM of three to four independent experiments performed in triplicate. ** = *P* < 0.01; *** = *P* < 0.001 versus DMSO (student *t* test).

migration. Hence, a Ras inhibitor should reduce all of these effects. Notably, compound 12 induced a remarkable decrease in the GTP-bound (active) form of Ras in MDA-MB-231 cells (Figure 2), which was significantly larger than that induced by DCAI, used for comparison (Figure S1 in the Supporting Information). As expected, this reduction in the active form of Ras translated into inhibition of the main Ras-induced signaling pathways, including the downstream kinases AKT, MEK, and ERK; all of them related to cellular proliferation (Figure 3).

Inhibitory effects of 12 on the growth of cancer cell cultures

We next tested the effect of compound 12 on migration, invasiveness, and anchorage-independent growth; these are hall-

	Ar	Cytotoxicity (IC ₅₀ [μM]) ^[a]			
		HCT-116	H-460	MDA-MB-231	NIH-3T3 (G12V)
3		22	19	15	15
4		50	ND ^[b]	ND	ND
5		>50	ND	ND	ND
6		20	15	12	27
7		>50	ND	ND	ND
8		>50	ND	ND	ND
9		10	10	20	25
10		27	ND	ND	ND
11		17	15	30	ND
12		8	15	7	14
DCAI ^[c]		14	ND	25	ND

[a] Cytotoxicity was evaluated by the MTT assay (see the Experimental Section for details). IC₅₀ values are the means from two to three independent experiments performed in triplicate. In all cases, the SEM is within 10% of the mean value. [b] ND = not determined. [c] DCAI = 2-(4,6-dichloro-2-methyl-1H-indol-3-yl)ethanamine.

marks of transformed cells related to their metastatic potential. Remarkably, compound **12** profoundly impaired migration (Figure 4) and growth of MDA-MB-231 cells in Matrigel (Figure 5A), along with the invasive capacity of these cells (Figure 5B). Our results show that under control conditions (Figure 4A) MDA-MB-231 cells can readily migrate, and only 37 and 6% of the initial wound area remains free of cells after 24 and 72 h, respectively. However, in the presence of 25 μM of compound **12**, cell migration was considerably blocked and, after 24 and 72 h, 71 and 59%, respectively, of the initial wound area still remained free of cells (Figure 4B). To rule out general cytotoxicity and study the reversibility of the effect, we performed an additional experiment in which the compound was removed after the first 24 h and the cells were incubated for an additional 48 h. Under these conditions (Figure 4C), the cells recovered their ability to migrate and only 14% of the initial wound area remained free of cells (Figure 4C, right). In fur-

ther support of these results, direct quantification of the number of migrated cells was also determined by using a Boyden chamber and, after 24 h, cell migration was inhibited by about 40% (Figure 4E). Importantly, compound **12** also affected the ability of MDA-MB-231 cells to grow in the absence of anchorage (Figure 5A) and their invasive capacity (Figure 5B) in a dose-dependent manner. In summary, all of these results suggest that the new Ras inhibitor **12** can efficiently prevent the metastatic activity induced by mutated Ras.

Specificity of Ras inhibition

Another important aspect was to confirm the generality of these results in other tumor cell lines, as well as the specificity of Ras inhibition. Hence, we selected a broader tumor cell panel (Table 3) that included cells with mutant K-Ras (such as the PANC-1 and MIA PaCa-2 pancreatic carcinoma cell lines, or

Figure 4. Compound 12 impairs cell migration without inducing general cytotoxicity. Compound 12 prevents cell migration, but cells recover their ability to migrate in the absence of compound. In vitro scratches (wounds) were made by scraping confluent MDA-MB-231 cell monolayers with a sterile pipet tip and were visualized by phase contrast microscopy. After the indicated times, remaining wound area was quantified in cells treated with: A) vehicle, B) compound 12 (25 μM) during 72 h, or C) compound 12 (25 μM) during 24 h and vehicle for additional 48 h. Bar, 250 μm. D) Percentage of the remaining wound area. E) Percentage of migrating cells quantified with a Boyden chamber. Bar graphs represent the mean ± SEM of the remaining wound area of three independent experiments performed in triplicate, in which three different fields were analyzed. ** = $P < 0.01$; *** = $P < 0.001$ (vs. DMSO-treated cells; student *t* test); ## = $P < 0.001$ (vs. cells treated with 25 μM of compound 12 during 72 h; student *t* test).

the colon SW-620 cell line), but also with wild-type K-Ras (BxPC-3 or MCF-7 cell lines). This is important because it is considered that compounds that selectively inhibit mutant K-Ras, but not its wild-type counterpart, are less prone to induce side cellular toxicity. Remarkably, compound 12 exhibited increased cytotoxicity toward human cancer cells that featured a mutated oncogenic K-Ras versus those that were not K-Ras mutated. For example, the IC_{50} values for PANC-1 and MIA PaCa-2 were 17.3 and 10.2 μM, respectively, whereas, in the case of BxPC-3 cells, the compound showed an $IC_{50} > 50$ μM (Table 3). Similarly,

Figure 5. Compound 12 inhibits the anchorage-independent proliferation and invasiveness of MDA-MB-231 cells in a dose-dependent manner. A) Cells were seeded and grown in Matrigel for 7 days in the presence of increasing concentrations of compound 12. B) Cells were treated, overnight, with the indicated concentration of the compound and placed in the upper chamber of a Boyden chamber, the membranes of which were previously coated with Matrigel. Medium containing 10% fetal bovine serum (FBS) plus the appropriate concentration of compound was placed in the lower chamber as a chemoattractant. After 6 h at 37 °C, the cells were fixed and stained and those on the underside of the filters were visualized and counted under a light microscope. Data represent the mean ± SEM of three independent experiments performed in triplicate. * = $P < 0.05$; *** = $P < 0.001$ (vs. DMSO-treated cells; student *t* test).

Table 3. Cytotoxicity of compound 12 and doxorubicin (Dox) in different cell lines.			
Ras status		IC ₅₀ [μM] ^a	Dox
		12	
pancreatic cells			
PANC-1	mutated K-Ras (G12D)	17.3	2.3
MIA PaCa-2	mutated K-Ras (G12C)	10.2	0.12
BxPC-3	wild-type Ras	>50	3
HPNE	non-tumor cell	>50	0.8
breast cells			
MDA-MB-231	mutated K-Ras (G13D)	7	0.3
MCF-7	wild-type Ras	50	0.17
MCF-10A	non-tumor cell	>50	ND ^b
lung cells			
H-460	mutated K-Ras (Q61H)	15	ND
MRC-5	non-tumor cell	>50	ND
colon cells			
SW-620	mutated K-Ras (G12V)	37.1	3.7
HCT-116	mutated K-Ras (G13D)	8	0.73
fibroblasts			
NIH-3T3	non-tumor cell	>50	ND

[a] Cytotoxicity was evaluated by the MTT assay, except for pancreatic cell lines. In this case, the (2,3)-bis-(2-methoxy-4-nitro-5-sulfonyl)-(2H)-tetrazolium-5-carboxanilide (XTT) assay was employed (see the Experimental Section for details). IC₅₀ values are the means from two to three independent experiments performed in triplicate. In all cases, the SEM is within 10% of the mean value. [b] ND = not determined.

for breast cancer, the IC_{50} value for MDA-MB-231 cells was 7 μM and for MCF-7 was 50 μM. To further confirm the mechanism of action, we checked that in highly aggressive PANC-1 the compound was inhibiting the activation of K-Ras (Figure S2 in the Supporting Information) and we also compared the cytotoxicity values shown by inhibitor 12 with those of Dox; a marketed anticancer drug used for treating different types of cancer with a different mechanism of action (Dox is an intercalating DNA agent). As expected, Dox shows similar

IC₅₀ values, regardless of the Ras status (Table 3). In addition, to further rule out general cytotoxicity, we evaluated the specificity of compound **12** in four different non-tumor cell lines: NIH-3T3 fibroblasts, lung MRC-5 cells, breast MCF-10A cells, and HPNE pancreatic cells. Importantly, Ras inhibitor **12** did not induce significant cytotoxicity (IC₅₀ > 50 μ M) in these cells. Furthermore, in agreement with the dependence on the presence of mutated Ras, compound **12** efficiently reduced colony formation of Ras^{G12V}-transfected NIH-3T3 cells in a dose-dependent manner without significantly affecting empty vector-transformed cells (Figure 6).

Figure 6. Inhibition colony formation in Ras^{G12V}-transfected cells by compound **12**. Empty vector (white columns) or Ras^{G12V}-transfected NIH-3T3 cells (black columns) were incubated with the indicated concentration of compound. Colonies were counted after staining with Fuchsin 2% under the microscope. *** = $P < 0.001$ versus DMSO (student t test).

In summary, compound **12** is a potent K-Ras inhibitor, as demonstrated by the cytotoxicity values and decrease in the Ras–GTP complex levels with selectivity for tumor cells versus non-tumor cells and specificity for mutant Ras versus wild-type Ras. Moreover, to rule out that the effects induced by compound **12** in Ras were due to regulation of other proteins upstream of Ras or that the decrease in Ras–GTP complex levels was due to an impairment of Ras localization in the membrane, we confirmed that compound **12** did not affect the activation of Shc (the main Ras upstream activator) or the membrane localization of Ras (Figure S3 in the Supporting Information).

Molecular basis for the interaction of **12** with Ras

Ras proteins have been extensively studied by high-resolution NMR spectroscopy and X-ray crystallography methods (see ref. [8c] for a revision). Comprehensive information at the structural level has been disclosed for H-Ras, K-Ras, and N-Ras, for several well-known mutations, as well as their complexes with small molecules and downstream effectors. Altogether, these studies underscore the structural resemblance among Ras isoforms; the dynamic nature of the functional regions of P-loop (residues 10–16), switch I (residues 30–38), and switch II (residues 60–68); and the presence of several accessible small ligand-binding sites (Figure 7).

Significant structural heterogeneity, together with the dynamic nature of accessible pockets on the Ras surface, which depend on the conformational state of the protein or are transiently induced by the interaction with ligands,^[8a,13] make the estimation of the binding modes of small molecules a challeng-

Figure 7. Cartoon representation of backbone superimposition of Ras proteins. Ras is characterized by a prototype “G domain fold”, consisting of a mixed six-stranded β sheets flanked by five α helices. The N-terminal effector (residues 1–86) and C-terminal allosteric (residues 87–166) lobes are indicated, as well as the functional P-loop (residues 10–16 in green), switch I (residues 30–38 in red), and switch II (residues 60–68 in blue). Three major sites (sites 1–3) for small-molecule binding are indicated as transparent surfaces in magenta, yellow, and cyan. Structures displayed correspond to GppNHp-bound H-Ras (PDB ID: 4EFL), GDP-bound H-Ras (PDB ID: 4Q21), GDP-bound K-Ras (PDB ID: 4OBE), GDP-bound K-Ras G12V (PDB ID: 4TQ9), GDP-bound K-Ras G13D (PDB ID: 4TQA), GppNHp-bound K-Ras Q61H (PDB ID: 3GTT), GppNHp-Kobe2601 H-Ras T35S complex (PDB ID: 2LW1), GppCp-DCAI K-Ras G12D complex (PDB ID: 4DST), GDP-HBLP K-Ras G12V complex (PDB ID: 4EPY), GDP-disulfide-based K-Ras G12C complexes (PDB IDs: 4LV6, 4LUC and 4M22), and GppNHp-Zn²⁺ cyclen H-Ras complex (PDB ID: 3LY8). Small molecule GDPs, GTP analogues, allosteric inhibitors, and Mg²⁺ are displayed as sticks.

ing task. To overcome this problem, we used molecular dynamics (MD) simulations to increase the structural ensembles of Ras for molecular docking experiments and, in a second step, to test the stability of the predicted protein–ligand complexes (see the Experimental Section). Twenty MD snapshots extracted from 100 ns trajectories of the apo forms of wild type K-Ras, G12V, G13D, and Q61H oncogenic mutants were considered as a flexible ensemble of protein coordinates (a total of 80 structures) for the docking of compound **12**.

These structures displayed a considerable conformational variability, as revealed by their C α root-mean-square deviations (RMSDs; Figure S4 in the Supporting Information). Clearly, the functional P-loop and switch regions I and II are more dynamic (average RMSD values of 2.9 Å) than the entire proteins (1.5 Å). In addition, the G12V, G13D, and Q61H mutants displayed increased fluctuations in the functional regions relative to that of wild-type K-Ras (Figure S4 in the Supporting Information), as previously stated by other computational studies.^[14]

Compound **12** was docked to each of the 80 structures obtained by MD simulations, by using two overlapping grid boxes centered at the N-terminal effector and C-terminal allosteric lobes (see Figure S5 in the Supporting Information and the Experimental Section). The obtained docking solutions (20 runs per structure, a total of 1600 poses) were clustered and ranked according to the predicted binding energy score. The best-scored docking poses locate compound **12** within the “effector lobe” in close proximity to the P-loop, switch I,

and switch II regions (Figure S5 in the Supporting Information). The 20 lower docking energy protein–ligand complexes were subjected to 100 ns of MD simulations. Analysis of the trajectories revealed that one of these predicted complexes was highly stable, with average ligand RMSD < 2.0 Å relative to the initial docking pose in the G12V, G13D, and Q61H K-Ras simulations (Figure S6 in the Supporting Information). In this conformation, compound **12** binds partially to the GTP-binding region and extends into the adjacent hydrophobic pocket delimited by switch II (site 2 in Figure 7). In particular, the pyranone ring of the ligand and its substituents overlap with the triphosphate moiety of GTP in such a manner that the hydroxymethyl group mimics an oxygen atom of the α -phosphate of GTP, the oxygen atom within the pyranone ring mimics an oxygen atom of the β -phosphate, and the carbonyl group of the pyranone and ether oxygen mimic two oxygen atoms of the γ -phosphate (Figure 8).

Accordingly, the pyranone system of compound **12** reproduces interactions with the backbone amide group of G13 in the P-loop, the protonated side chain of K16, and the side chains of T35 and Y32 in switch I (Figure 8), also observed for GTP. Furthermore, the alkyl chain expands through switch II to position the halobenzene ring into the hydrophobic/aromatic site II cavity near Q61. This hydrophobic pocket was first discovered as the binding site of K-Ras (G12C) disulfide-based allosteric inhibitors.^[10a] Clearly, the halobenzene rings of compound **12** and these allosteric inhibitors overlap (Figure 8). This mode of binding explains the experimental observation

Figure 8. Backbone superimposition of energy-minimized complexes between compound **12** (yellow) and wild-type K-Ras, G12V, G13D, and Q61H oncogenic mutants (highlighted as sticks). P-loop and switch I/II regions are colored in green, red, and blue, respectively. Inset: a detailed view of the interactions between compound **12** and wild-type K-Ras. Structural superimpositions with the GTP analogue-bound K-Ras Q61H (PDB ID: 3GFT) and GDP-6H05 analogue K-Ras G12C complex (PDB ID: 4LV6) reveal the spatial correspondence of the pyranone ring of compound **12** (in yellow) with the triphosphate group of GTP (purple) and between the halobenzene ring of compound **12** (yellow) and the disulfide irreversible inhibitor 6H05 (cyan).

(Table S2 in the Supporting Information) that the optimal spacer requires five methylene units ($n=5$), so that the halobenzene ring can reach, but not surpass, the site II cavity. Moreover, the hydrophobic propyl chain and chlorine atoms favor interactions with the hydrophobic/aromatic site II cavity. The chlorine atoms of compound **12** mimic the chlorine atoms of the irreversible inhibitor 6H05 analogue (Figure 8).^[10a]

Thus, compound **12** overlays both the binding region of the triphosphate group of GTP and the allosteric binding site of the irreversible inhibitors. MD simulations show that the more flexible part of the ligand corresponds to the halobenzene ring, whereas binding of the pyranone ring is highly stabilized by polar/hydrogen-bonding interactions in the triphosphate-binding region of the protein. Moreover, all oncogenic assayed mutations were located in close proximity to the ligand, which suggested a direct role of these positions in binding. V12 in the G12V mutant forms hydrophobic–hydrophobic interactions with the methylene chain of compound **12**, whereas H61 in the Q61H mutant forms aromatic–aromatic interactions with the halobenzene ring (Figure 8). The role of D13 in the G13D mutant is not straightforward, but its interaction with K117 probably modifies the shape of the binding cavity and the orientation of the backbone amide group involved in interactions with the ligand (see above).

Considering that all of the obtained results were consistent with the direct inhibition of Ras by compound **12**, we used NMR spectroscopy to obtain direct insights into the binding mode of **12** to the Ras protein. First, we studied the ^1H NMR signals of the compound upon addition of Ras protein (Figure 9). NMR transverse relaxation (described by the transverse relaxation time T_2) directly depends on the molecular size; an increase in T_2 is related to an increase in the size or apparent size of the system and can be seen in NMR spectra as intensity lost due to line broadening. Therefore, the enhancement of ligand relaxation is usually used to detect binding. In this case, clear line broadening of the ^1H NMR signals of compound **12** was detected upon addition of Ras, which indicated that the compound was bound to the protein. In particular, the intensity of the H6 signal, located in the less flexible pyranone ring, decreased to 33% of the initial intensity in the presence of Ras (Figure 9A and B). The effect is less pronounced in the more flexible propyl chain attached to the halobenzene ring, but can also be detected (CH_3 15 signal intensity decreases to 70% upon addition of Ras; Figure 9A and B, right panels). Addition of GTP to the NMR sample enables recovery of the signal intensity (Figure 9C), which confirms competition of both molecules for partially overlapped binding sites.

To further confirm the direct interaction of compound **12** with Ras, an analogous experiment with less potent derivative **5** (Table 2) was carried out. In this case, a significantly smaller perturbation of the NMR signals of the compound was observed (Figure S7 in the Supporting Information), which strongly suggested that **12** displayed a stronger interaction with Ras than that of compound **5**. In addition, we carried out a thermal shift assay for Ras in the presence of compound **12**, and a small increase in the melting temperature (ΔT_m) for Ras was observed ($\Delta T_m = 1^\circ\text{C}$).

Figure 9. Binding of compound 12 to Ras protein assessed by NMR spectroscopy. A) ^1H NMR spectrum of 12 acquired with 160 scans in a 700 MHz NMR spectrometer equipped with a cryo probe. Ligand concentration was $250\ \mu\text{M}$ in 20 mM Tris buffer, 150 mM NaCl, 2 mM MgCl_2 at pH 7.4 in D_2O . B) ^1H NMR spectrum of 12 acquired upon addition of RasG12V-GST protein (compound/protein ratio, 40:1). C) ^1H NMR spectrum of the same sample as that in B) upon addition of GTP to reach a final concentration of $250\ \mu\text{M}$.

Additional experiments were carried out to further confirm the direct interaction between compound 12 and Ras. In particular, affinity mass spectrometry methods were performed. RasG12V was incubated in the presence of 5 or 12 and the mixture was filtered through a size-exclusion chromatography unit. Thus, free compound is retained in the column, whereas the bound ligand elutes as a Ras–compound complex. After dissociation of the complex, the corresponding compounds are detected by liquid chromatography coupled to mass spectrometry (Figure 10). Again, the obtained results clearly support the direct interaction of compound 12 with Ras, unlike derivative 5, which does not bind Ras to a noticeable extent.

Conclusions

We described a new reversible Ras inhibitor that bound in an overlapping site that comprised the GTP-binding region and extended into the adjacent hydrophobic pocket delimited by switch II. Ras inhibitor 12 impaired the activity of mutated Ras isoforms selectively; this led to reduced toxicity towards non-tumor cells and decreased tumor hallmarks related to cellular proliferation, such as the MAPK signaling pathway, or to metastatic processes, such as cell migration and invasiveness. Inhibitor 12 is a new compound for the development of new anti-tumor agents suitable for the treatment of specific Ras-driven tumors with current bad prognosis, either by themselves or in combination therapies.

Experimental Section

Compound synthesis

Unless stated otherwise, starting materials, reagents, and solvents were purchased as high-grade commercial products from Sigma–

Figure 10. Binding of compound 12 to RasG12V determined by affinity mass spectrometry. A) RasG12V was incubated with either 5 or 12. The mixture was filtered in a micro-biospin chromatography column to isolate ligand-bound complexes. The eluted complexes were dissociated and the corresponding compound detected by liquid chromatography coupled to mass spectrometry. B) Compounds 5 and 12 (upper and lower chromatograms, respectively) added as internal standards for comparison. Signals shown correspond to the $[\text{M}+\text{H}]^+$ ion of each compound obtained by selected ion monitoring in positive mode. Chromatograms are representative of two independent experiments carried out in duplicate.

Aldrich, Acros, Fluka, Scharlab, or Panreac, and were used without further purification. All non-aqueous reactions were performed under an argon atmosphere in oven-dried glassware. THF and dichloromethane were dried by using a Pure SolvTM Micro 100 liter solvent purification system. Reactions under MW irradiation were performed in a Biotage Initiator 2.5 reactor. Hydrogenation reactions were carried out by using an H-Cube continuous-flow hydrogenation reactor. Full details regarding the synthetic procedures and characterization data of all compounds are given in the Supporting Information. Spectroscopic data of all described compounds were consistent with the proposed structures. Elemental analyses (C, H, N) were within 0.4 % of the calculated values, which confirmed a purity of at least 95 % for all tested compounds. DCAI was purchased from Aldrich.

General procedure for the synthesis of final compounds 3–12

A mixture of intermediate 13 (1.1 equiv, 1–2 mmol), the corresponding phenol (commercially available or 14–17, 1 equiv), anhydrous K_2CO_3 (1.2 equiv), and a catalytic amount of NaI in anhydrous 2-butanone ($3\ \text{mL}\cdot\text{mmol}^{-1}$) was stirred at 80°C for 24 h. After this time, the reaction mixture was filtered and concentrated under reduced pressure. The residue was dissolved in diethyl ether, washed with water, dried (anhydrous Na_2SO_4), and evaporated under reduced

pressure. The crude product was purified by column chromatography (hexane/ethyl acetate, 1:1) to afford the corresponding final compounds, which were crystallized in the appropriate solvent.

Compound 12

Yield: 73%; R_f (EtOAc)=0.2; m.p. 85–86°C (EtOAc/diethyl ether); ^1H NMR (300 MHz, CDCl_3): δ =0.95 (t, J =7.4 Hz, 3H; CH_3), 1.46–1.58 (m, 2H; CH_2), 1.59–1.70 (m, 2H; CH_2), 1.81–1.94 (m, 4H; 2 CH_2), 2.07 (brs, 1H; OH), 2.76–2.81 (m, 2H; CH_2Ar), 3.89 (t, J =6.4 Hz, 2H; CH_2O), 3.95 (t, J =6.2 Hz, 2H; CH_2O), 4.48 (s, 2H; CH_2OH), 6.52 (s, 1H; H^a), 6.67 (d, J =8.9 Hz, 1H; CH_a), 7.21 (d, J =8.9 Hz, 1H; CH_a), 7.56 ppm (s, 1H; H^b); ^{13}C NMR (75 MHz, CDCl_3): δ =14.3 (CH_3), 21.9, 22.7, 28.9, 29.1 (4CH_2), 30.2 (CH_2Ar), 60.9 (CH_2OH), 68.3, 69.7 (2 CH_2O), 110.4 (C^a), 111.9, 124.0 (2 CH_a), 127.5, 131.8, 132.0 (3 C_a), 139.6 (C^b), 147.9 (C^c), 156.2 ($\text{C}=\text{O}$), 169.0 (C^d), 175.1 ppm ($\text{C}=\text{O}$); IR (KBr): $\tilde{\nu}$ =3300, 2945, 1647, 1607, 1452, 1259, 1207, 1151, 1082 cm^{-1} ; MS (ESI): m/z : 414.1 [M^+]; elemental analysis calcd (%) for $\text{C}_{20}\text{H}_{24}\text{Cl}_2\text{O}_5$: C 57.84, H 5.82; found: C 57.96, H 5.72.

Cell culture and cytotoxicity assays

Human carcinoma cell lines from colon (HCT-116 and SW-620), lung (H-460), breast (MCF-7 and MDA-MB-231), and pancreas (PANC-1, MIA PaCa-2, and BxPC-3); immortalized but not tumorigenic cell lines NIH-3T3 mouse fibroblasts and human breast, lung, and pancreas-derived cell lines, MCF-10A, MRC-5, and HPNE, respectively, were purchased at ATCC and cultured according to their recommendations. Cell viability was determined after 48 h of incubation with the compound under study by using standard colorimetric assays based on the metabolism of the MTT or XTT salt by viable cells, as previously described.^[15]

Active Ras pulldown assay

MDA-MB-231 cells were plated at a density of 2×10^6 cells in 15 cm dishes and incubated in Dulbecco's modified Eagle's medium DMEM with 10% FBS at 37°C and 5% of CO_2 . After 24 h, medium was replaced by fresh DMEM with 1% FBS, containing 25 μM of compound 12 or an equivalent volume of DMSO for vehicle control, and cells were incubated for 24 h at 37°C and 5% of CO_2 . After 5 min of stimulation with 10 ng mL^{-1} of epidermal growth factor (EGF), cells were washed with phosphate-buffered saline (PBS) and lysed with ice-cold lysis buffer. Lysates were clarified by 15 min of centrifugation at 16000g and protein concentration was measured by using the bicinchoninic acid method (Thermo Scientific). Before performing the assay, each lysate (50 μL) was separated to analyze the total Ras expression. To analyze active Ras, a Ras-GTP pulldown assay kit (Thermo Scientific) was used by following the manufacturer's instructions. Briefly, lysates were incubated with a glutathione S-transferase fusion of the Ras binding domain (RBD) of Raf1 along with glutathione agarose resin to specifically pull down active Ras. The entire samples obtained after the pulldown assay were boiled for 5 min and loaded onto 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad). Ras proteins were visualized by immunoblotting on nitrocellulose membranes with mouse anti-Ras provided by the manufacturer. Blots were analyzed by densitometry by using ImageJ software (NIH).

Western blot analysis

The expression of different proteins was determined by immunoblot analysis following the conditions previously reported,^[15b,16] as detailed in the Supporting Information.

Thermal shift assay

The assay was carried out by Reaction Biology Corp. by using RasG12V and compound 12 at 25 μM in HEPES buffer (pH 7.5).

Migration, anchorage-independent growth, and invasion assays

Migration was determined by the wound-healing assay, as previously described,^[27] with a Boyden chamber. To evaluate the effect of the compound on the capacity of tumor cells to grow without anchorage, cells were seeded in Matrigel (BD, France). The chemoinvasion assay was performed in a Boyden chamber. Experimental details are included in the Supporting Information.

Ras-dependent colony formation assay

The 50% confluent NIH-3T3 cells in a 10 cm diameter plate were transfected with pSV2-RasV12 plasmid (0.5 μg) plus pSV2-neomycin plasmid (100 ng) with Lipofectamine-Plus reagent (Invitrogen), according to the manufacturer's instruction. After 48 h, cells were treated with trypsin, split to 1/3, and incubated in the presence of complete medium supplemented with geneticin (400 $\mu\text{g mL}^{-1}$; Invitrogen) and different doses of compound until emerging resistant clones appeared (2–3 weeks). The medium was renewed every 3 days. Colonies with a minimum of 50 cells were counted after staining with Fuschin 2%.

MD simulations

Four structures of K-Ras (PDB IDs: 4OBE, 4TQ9, 4TQA, and 3GFT) were obtained from the Protein Data Bank (www.rcsb.org), representing the GDP-bound wild-type K-Ras; GDP-bound K-Ras G12V mutant, GDP-bound K-Ras G13D mutant, and GppNHP-bound K-Ras Q61H mutant, respectively. The apo forms of wild-type K-Ras, G12V, G13D, and Q61H oncogenic mutants were obtained by removing either GDP or the GTP analogue. These apo forms and 20 docking poses of the complex between 12 and Ras (see below) were solvated with explicit simple-point charge (SPC) water in a rhombic dodecahedron box. The distance between the protein and the edge of the box was about 1.5 nm to ensure that the minimum distance between periodic images was satisfied. Na^+ and Cl^- ions were added to neutralize charges, reaching a final concentration of 0.15 M. Model systems were energy minimized and equilibrated with positional restraints on the $\alpha\text{-C}$ atoms by using the GROMACS software v5.0.6,^[7,8] the Amberff99SB-ILDN force field for the protein, and the general Amber force field (GAFF) and HF/6-31G*-derived RESP atomic charges for compound 12. The positional restraints were gradually released by decreasing the force constant on the protein in 4 distinct steps (1000, 500, 10, and 0 $\text{kJ mol}^{-1} \text{nm}^{-2}$) of 10 ns each. After relaxation, 100 ns of MD trajectories were produced. All simulations were run at a constant temperature of 300 K by using separate v-rescale thermostats for the protein and solvent molecules. Lennard-Jones interactions were computed by using a cutoff of 10 Å, and electrostatic interactions were treated by using PME with the same real-space cutoff. A time step of 2 fs was used.

Molecular docking of 12

To obtain an ensemble of protein structures for docking experiments, 20 snapshots collected from the MD trajectories of the apo forms of wild type K-Ras, G12V, G13D, and Q61H mutants (see above). Molecular docking experiments were carried out by using AutoDock Vina.^[19] For each of the 80 protein models obtained in

the MD simulations, 2 overlapping 30 Å³ docking grid boxes centered at the N-terminal effector lobe (residues 1–86) and C-terminal allosteric lobe (87–166) were defined (Figure S4 in the Supporting Information). We employed the default grid spacing (0.375 Å) and performed 20 docking runs on each model (1600 poses), treating the protein as rigid and the ligand as flexible. These docking poses were ranked according to the AutoDock Vina binding-energy score function.

NMR spectroscopy experiments

¹H NMR spectroscopy experiments were acquired with 160 scans on a Bruker AVIII 700 MHz or with 64 scans on a Bruker Avance 600 MHz spectrometer equipped with cryoprobes in both cases. Samples were prepared at a ligand concentration of 250 μM in 20 mM Tris buffer, 150 mM NaCl, 2 mM MgCl₂ at pH 7.4 in D₂O. The compound/protein ratio was 40:1. Protein (RasG12V-GST) was obtained from Jena Bioscience. The same experiment in the absence of the protein was acquired for comparison. Competition experiments with nucleotide were performed by adding GTP to the compound/protein sample to reach a final concentration of 250 μM.

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