Drug Discovery II

Sulindac-Derived Ras Pathway Inhibitors Target the Ras–Raf Interaction and Downstream Effectors in the Ras Pathway**

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In the preceding article[1] we introduced a method to synthesize a library of compounds structurally based on the nonsteroidal antiinflammatory drug (NSAID) sulindac (**1**,

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Table 1). Phenotype-based pathway-selective screening revealed that several members of the library affect the Ras signaling pathway.

In the study described herein, we investigated whether the identified compounds directly affect the Ras protein and its major activities, with the aim of determining the biochemical mechanism responsible for the measured cellular effects.

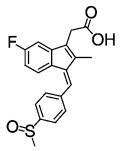
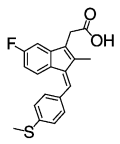
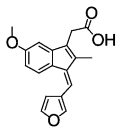
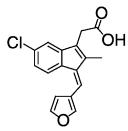
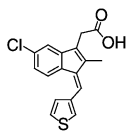
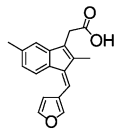
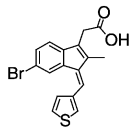
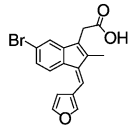
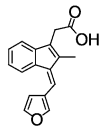
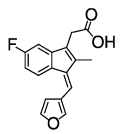
The eight library members with the highest differences between the effective concentration for activity on MDCK-F3 (MDCK = Madine–Darby canine kidney) cells and the effective concentration with MDCK cells were tested to investigate their level of inhibition of the Ras pathway and of the Ras protein in further detail (Table 1). We performed biochemical assays to determine whether these compounds inhibit the interaction of the Ras protein with its major effector Raf. We carried out an NMR analysis of the most active compound and found that this substance does inhibit the Ras–Raf interaction by direct binding to the Ras protein. Next, we showed that four of the new compounds inhibit the activation of the effector protein MAP kinase, which is located downstream of the Ras protein in the signaling pathway.

The oncogenic Ras protein cycles between a bound inactive guanine diphosphate (GDP) and a bound active guanine triphosphate (GTP) state (Figure 1). Active RasGTP can bind and activate the main effector Raf kinase. The activated Raf kinase phosphorylates, and thereby activates, other downstream kinases, of which MAP kinase is the most prominent. This cascade leads to the known cellular effects of the Ras protein, that is, activation of cell proliferation and cell transformation.

To identify potential inhibitors of the Ras–Raf interaction we tested the compounds in a quantitative biochemical assay. This assay is based on the finding that Raf binding lowers the dissociation rate of the RasGTP complex (Figure 1).[2] This so-called guanine nucleotide dissociation (GDI) effect can be quantified spectroscopically by using fluorescently labeled nucleotides. The GDI effect was measured in the presence of the active compounds to look for hints of a possible influence of the compounds on the binding of Raf to RasGTP. Compounds that lower the GDI effect are regarded as inhibitors of the interaction between Raf and RasGTP (Figure 2). Six compounds were found in this assay to inhibit the RasGTP–Raf interaction. Compound **5g** had no effect. Compound **5a** absorbs light at the wavelength applied for fluorescence excitation, which renders its influence on the Ras–Raf interaction undetectable. This compound could therefore not be investigated. The concentrations of the other compounds leading to half maximal inhibition (IC_{50}) ranged between 100 μM and 450 μM, with one exception: compound **5h** inhibited the RasGTP–Raf interaction with an IC_{50} value of 30 μM, which indicates that it has the strongest effect of the tested compounds.

We analyzed the direct interaction of the Ras protein bound to nonhydrolyzable GppNHp (p = phoshpate) with sulindac sulfide (**2**) and **5h** by NMR spectroscopy. ^{15}N HSQC spectra of 50-μM solutions of the protein were recorded in the absence of ligand and with a 300 μM solution of **2** or 290 μM solution of **5h**. HSQC assignments were taken from the study of Ito et al.[3] The binding of **2** was detected by line broadening in the 1D ^{1}H NMR spectrum of a 300 μM solution of the compound in the presence of a 50 μM solution of the protein, compared to the spectrum in the absence of protein (Figure 3A). Few changes in the ^{15}N HSQC spectrum of the

Table 1: Properties of the eight compounds found in the previous cellular screens to have the strongest activity.

No.[a]	Compound	IC_{50} Ras–Raf interaction[b] [μM]	MAPK phosphorylation[c]
1		no effect	no effect
2		210	inhibited
5a		not detectable (absorption by compound)	no effect
5b		180	inhibited
5c		100	inhibited
5d		160	inhibited
5e		220	n.a.[d]
5f		450	n.a.[d]
5g		no effect	n.a.[d]
5h		30	inhibited

[a] The compounds are numbered according to the numbering in the accompanying article.[1] [b] IC_{50} values for inhibition of the RasGTP–Raf interaction. [c] MAPK = mitogen-activated protein kinase. [d] n.a. = not analyzed.

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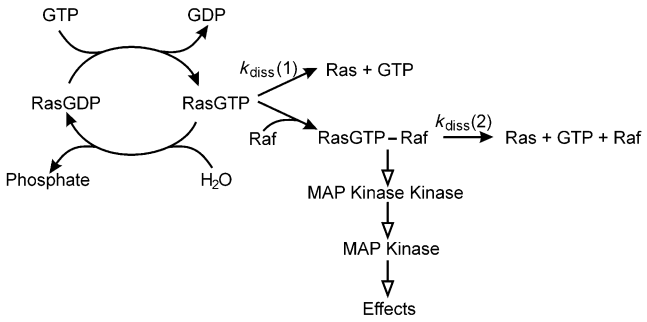

Figure 1. Simplified scheme of the Ras signaling pathway. The Ras protein is activated by the exchange of the bound guanine nucleotide GDP for GTP, and inactivated by the hydrolysis of the bound GTP. Active RasGTP can bind to the effector kinase Raf. The RasGTP–Raf complex activates the downstream effector MAP kinase kinase by phosphorylation (open arrowheads). MAP kinase kinase itself phosphorylates and thereby activates MAP kinase. Phosphorylated MAP kinase can phosphorylate further downstream effectors, which finally leads to the known effects of Ras signaling, such as cell proliferation and transformation. Remarkably, binding of Raf to RasGTP stabilizes the binding of GTP to Ras, which results in a dissociation rate $k_{diss}(2)$ of the RasGTP–Raf complex that is significantly lower than the dissociation rate $k_{diss}(1)$ of RasGTP. This effect is known as the guanine nucleotide dissociation inhibition effect of Raf. We measured the dissociation rates by using fluorescently labeled guanine nucleotides and used the results to identify new inhibitors of the interaction between RasGTP and Raf.

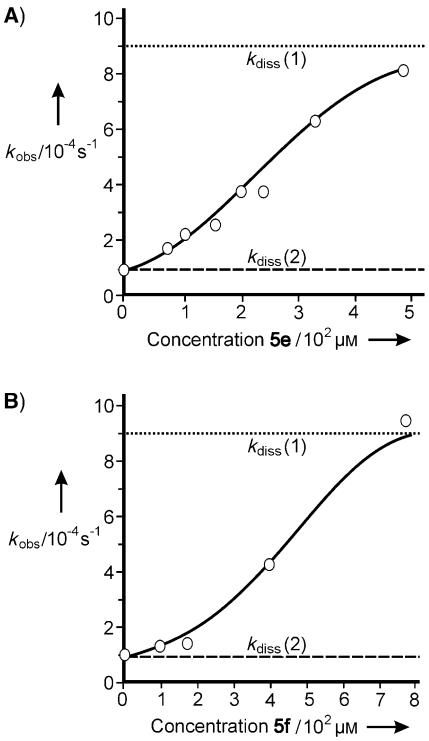

Figure 2. Representative data showing the influence of the new compounds on the RasGTP–Raf interaction. The graphs show the dissociation rate constant of the RasGTP–Raf complex (k_{obs}) as a function of the concentrations of compounds **5e** (A) or **5f** (B), respectively. The nucleotide dissociation rate constant $k_{diss}(1)$ for RasGTP (dotted line) is significantly higher than the dissociation rate constant for RasGTP–Raf ($k_{diss}(2)$, dashed line). The dissociation rate of the RasGTP–Raf complex increases with increasing concentrations of the test compounds. At concentrations higher than 500 μM **5e** or 700 μM **5f**, k_{obs} reaches the value of the dissociation rate constant of RasGTP (dotted line). At these concentrations, only the dissociation rate of RasGTP is measured since the interaction of Raf with RasGTP is inhibited almost completely.

protein could be observed upon addition of ligand (Figure 3B). Small, but significant, shifts were observed for the resonances corresponding to amino acids K16, V29, and Y40. The position of K16, which is located close to the GppNHp nucleotide bound in the active site of Ras, is shown in Figure 3D. The chemical shift of the signal of I163 depends on the concentration of dimethylsulfoxide used (data not shown). Previous structural studies have shown that the Raf-binding site of Ras is close to its GTP-binding site and includes the switch I region (amino acid (aa) 30–37).[4] The switch I and switch II (aa 59–63) regions of Ras do not produce signals in the ^{15}N HSQC spectrum.[3] From our observation by ^{1}H NMR spectroscopy of the binding of **2** and the small changes in the ^{15}N HSQC spectrum for signals of residues close to switch I upon binding, we conclude that the binding occurs almost exclusively in the switch regions of Ras. The binding is thus poor but still observable by HSQC spectroscopy. The same situation occurs with compound **5h** (Figure 3C), which we thus expect to bind to a similar region of the protein to that bound by **2**. The direct binding of the two compounds to the Raf-binding site in the Ras protein further supports the model in which their effects on the Ras signaling pathway are the result of direct interference with the RasGTP–Raf interaction.

Next we investigated whether compounds **5a**–**5d** and **5h** affect the activation of one of the major downstream targets of Ras. We incubated H-Ras-transfected MDCK-F3 cells with the compound to be tested and analyzed the level of MAP kinase phosphorylation. We measured the level of phosphorylated MAP kinase by immunoblot using an antibody specific for phosphorylated MAP kinase. In a parallel control experiment the total amount of MAP kinase was visualized by using an antibody that detects MAP kinase independently of its phosphorylation status. Remarkably, **5b**–**5d** and **5h** decreased the amount of activated phosphorylated MAP kinase (Figure 4). Application of compound **5a** did not lead to a significant decrease in the level of phosphorylated MAP kinase.

In this study the biochemical targets of the newly discovered Ras pathway inhibitors were narrowed down to a few possibilities. In the light of the suggestion that sulindac (**1**) itself influences the Ras pathway by interfering with the interaction between Ras and its downstream effector Raf kinase, the compounds were subjected to an assay that monitors inhibition of the RasGTP–Raf interaction. Six of the eight compounds under investigation inhibited the interaction. In addition, NMR data strongly suggest that this inhibition is a result of direct interaction of the compounds with the Ras protein. While sulindac (**1**) itself showed no inhibitory activity and sulindac sulfide (**2**) showed only very weak activity ($IC_{50} = 210$ μM), the most efficient inhibitor **5h** was significantly more active than the parent compound. Ras interacts with the Raf kinase in a prototypical protein–protein

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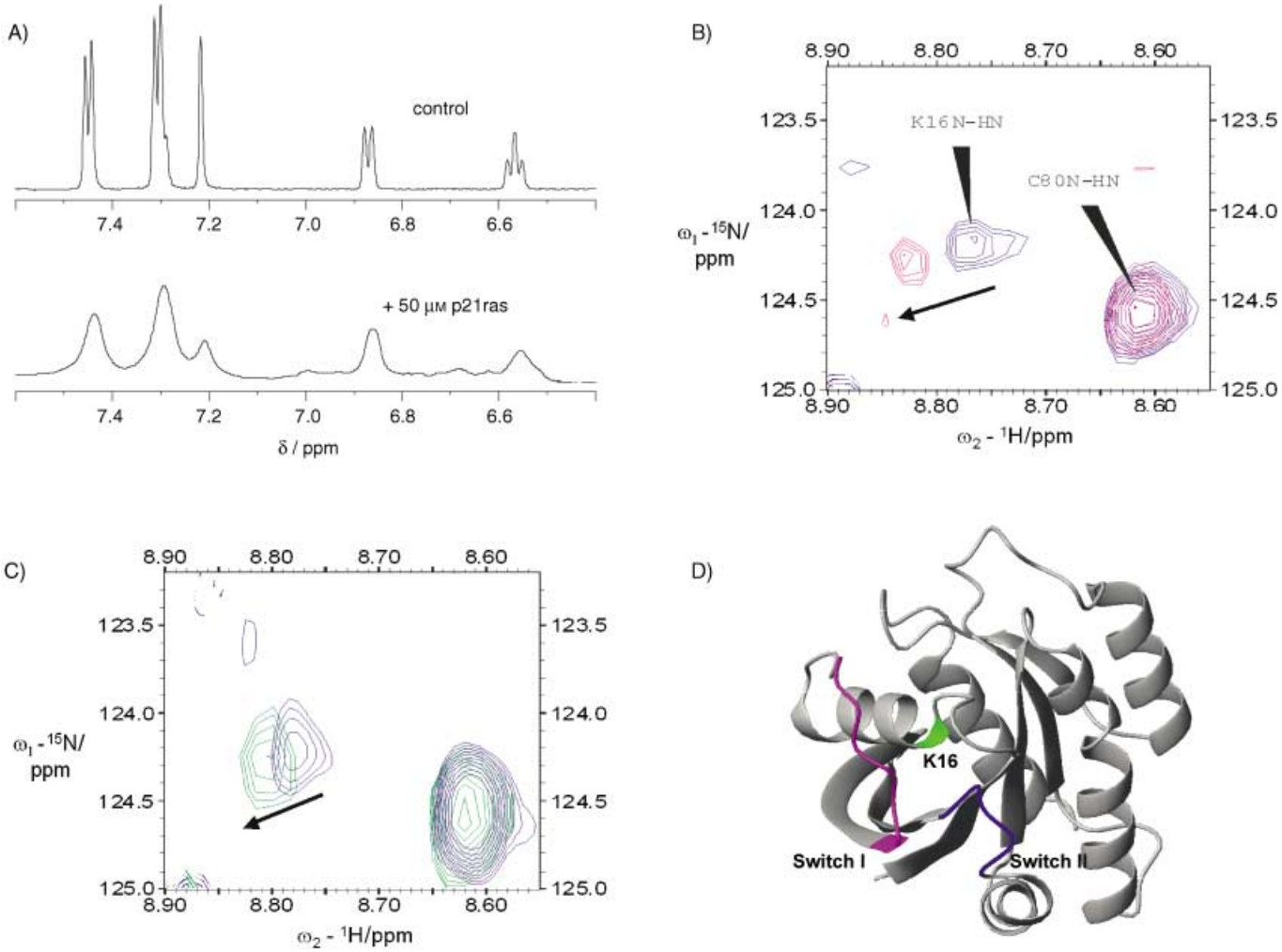

Figure 3. Sulindac sulfide (**2**) and **5h** bind directly to Ras. A) Part of the ^{1}H NMR spectrum of 300 μM **2** in the absence (control) and presence of 50 μM Ras. The significant line broadening in the presence of the protein indicates strong binding. B) Parts of the HSQC spectra of Ras with and without **2**. The reference spectrum of 50 μM Ras without ligand is shown in blue. The spectrum plotted in magenta was recorded in the presence of 300 μM **2**. C) Parts of the HSQC spectra of Ras with and without **5h**. The spectrum plotted in green was recorded in the presence of 290 μM **5h**. D) Spatial relationship of the switch regions I (magenta) and II (blue) in Ras, which are not observable in the HSQC spectra, to the location of K16 (green). The structure was taken from Pai et al.[6]).

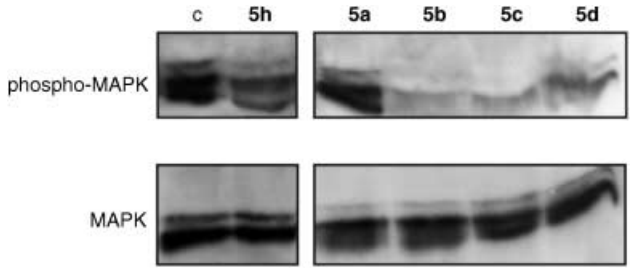

Figure 4. Immunoblot analysis of the levels of phosphorylated MAP kinase (phospho-MAPK) and of total MAPK (bottom) carried out by using an antibody specific for phospho-MAPK and one that binds all MAPK, respectively. The intensities of the bands correlate with the protein levels. The tested compounds do not influence the total level of MAPK. Compounds **5b**, **5c**, **5d**, and **5h** reduce the level of phospho-MAPK. Lysates from MDCK-F3 cells treated with the indicated compound (100 μM) were used for analysis; lysates from untreated MDCK-F3 cells were used as a control (c).

interaction over a very large contact region. Thus, interference with the RasGTP–Raf interaction by using a small molecule is expected to be very difficult. Only very recently was success in this area reported.[5] Our findings may open up a new avenue of research in this area. The results were confirmed in a structural analysis of the interaction between compound **5h** and Ras. NMR data show that **5h** binds to a region of Ras that is important for the interaction with Raf. Finally, we showed that four of the six compounds that inhibit the interaction between Ras and Raf kinase decrease the intracellular level of phosphorylated MAP kinase significantly. Compound **5a** did not decrease MAP kinase phosphorylation. Nevertheless, this compound reverses Ras-induced transformation, which indicates that its cellular target might be a protein located downstream of MAP kinase.

Together with the data detailed in the previous paper, the results of our investigation prove that phenotype-based

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pathway-selective screening followed by detailed biochemical validation provides new small-molecule inhibitors of a tumor-relevant pathway with high fidelity. Only a fairly small compound collection, two cellular screens, and two biochemical experiments were employed in our investigation. This very limited screening effort, however, led to the identification of new inhibitors of the Ras–Raf interaction that are up to 30-fold more active than the parent structure of the compound library, and to a new Ras-pathway inhibitor with a target potentially located downstream of MAP kinase. Our results open up new avenues for research in which signaling pathways are addressed through chemical–biological or medicinal–chemical investigations. The application of similar strategies to other compound classes should reveal new inhibitors of other signal transduction pathways.

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Keywords: bioorganic chemistry · medicinal chemistry · NMR spectroscopy · Ras proteins · signal transduction

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