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Direct Modulation of Small GTPase Activity and Function

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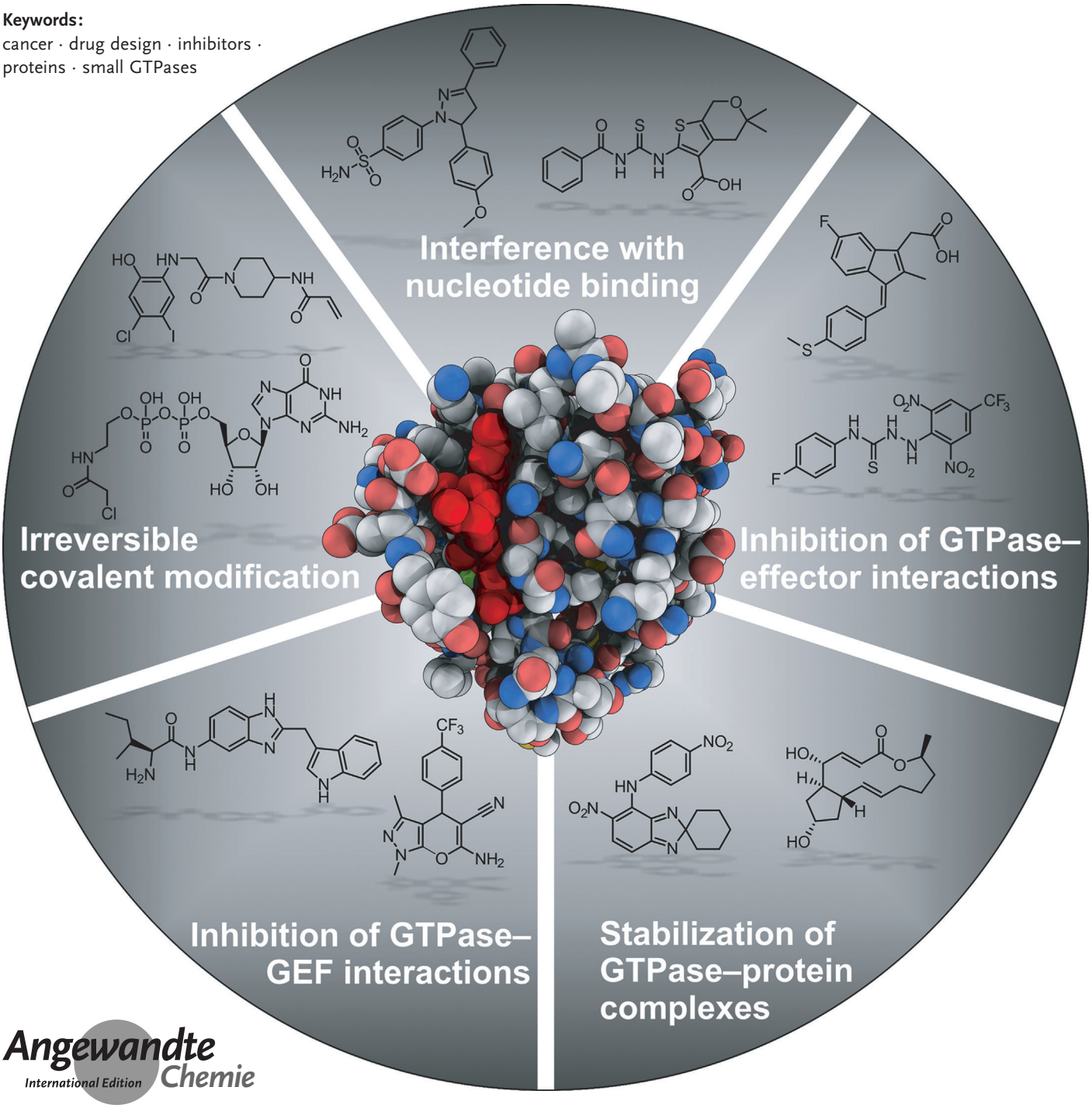
cancer · drug design · inhibitors · proteins · small GTPases



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Small GTPases are a family of GDP-/GTP-binding proteins that serve as biomolecular switches inside cells to control a variety of essential cellular processes. Aberrant function and regulation of small GTPases is associated with a variety of human diseases, thus rendering these proteins highly interesting targets in drug discovery. However, this class of proteins has been considered "undruggable", as intensive decade-long efforts did not yield clinically relevant direct modulators of small GTPases. Recently, the targeting of small GTPases has gained fresh impetus through the discovery of novel transient cavities on the protein surfaces and the application of new targeting strategies. Besides Ras proteins, other small GTPases have attracted increased attention since improved biological insight in combination with novel targeting strategies identified them as promising targets in drug discovery. This Review gives an overview of relevant aspects of the superfamily of small GTPases and summarizes recent progress and perspectives for the direct modulation of these challenging targets.

From the Contents

1. Introduction

GTPases comprise a large group of enzymes which hydrolyze GTP to GDP. Despite this limited enzymatic function, this protein superclass modulates a plethora of essential cellular programs and includes some of the most interesting and challenging targets in drug discovery. GTP hydrolysis results in conformational changes in the G-domain of these enzymes, which makes GTPases molecular switches. This switch function is essential for controlling cellular processes.[1] GTPases can be divided into two major classes: TRAFAC (translation factor related) and SIMIBI (signal recognition particle, MinD and BioD).[1,2] The TRAFAC class contains translation factors, heterotrimeric G-proteins,[3] septins, the dynamin superfamily, and the Ras (Rat sarcoma) superfamily of small GTPases. SIMIBI GTPases involve the signal recognition particle (SRP), its receptor (SR), and some additional families.[1] Although GTPases offer a multitude of promising drug targets, not a single candidate has been approved for modulation of GTPase activity so far.[4]

The members of the prototypical Ras superfamily cycle between an active GTP-bound and inactive GDP-bound state which differ by the conformations of their switch I and switch II regions.[1,5] The activation/deactivation cycle of small GTPases is tightly regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs; Figure 1a).[6,7] GEFs catalyze the exchange of GDP by GTP and thereby activate the small GTPase. GAP binding provides an essential catalytic group to accelerate the slow intrinsic hydrolysis rate of GTPases, which leads to inactivation. In the activated GTP-bound state, small GTPases interact with a variety of effector proteins and promote downstream signaling events. The majority of small GTPases carry a C-terminal hypervariable region (HVR), which is the target of post-translational modifications. Lipidation (usually farnesyl or geranylgeranyl cysteinyl thioethers) of the HVR anchors the small GTPases to membranes and regulates subcellular localization, including distribution in the plasma membrane.[8] To control cellular transport and selective membrane localization, GDIs form soluble complexes with small GTPases which enable intermembrane transport.[6,7]

The human Ras superfamily comprises more than 150 members and consists of the five major Ras, Rho, Rab, Arf, and Ran subfamilies.[9,10] The Ras subfamily includes 36 members in mammals which are key regulators of cell differentiation, proliferation, and survival.[11] The most prominent members of the Ras subfamily are the Ras, Ral, and Rap proteins. The Rho (Ras homologous) subfamily of small GTPases controls actin organization, cell shape and polarity, movement, as well as cell–cell and cell–matrix interactions.[12–14] Thus far, most studies have focused on three of the 22 Rho GTPases identified in mammals, namely, Rho, Rac, and Cdc42. The Rab (Ras-related in brain) and Arf (ADP-ribosylation factor) subfamilies are master regulators of intracellular traffic.[15,16] Rab GTPases represent the

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largest branch of the small GTPase superfamily, with more than 60 members in humans and are involved in intracellular vesicle transport.[17,18] Furthermore, they mediate vesicle formation at the donor membrane as well as vesicle fusion at the acceptor membrane. The 27 members of the Arf GTPase subfamily, with Arf1 being the most studied, also participate in vesicular trafficking and the regulation of endocytosis and secretory pathways. Among the Ras superfamiliy of small GTPases, the Ran (Ras-related nuclear protein) GTPase is unique, as it is the sole subfamily member and is not membrane-bound but soluble.[19,20] Ran is a pivotal regulator of nuclear transport through the nuclear pore complex and the assembly of the mitotic spindle, and is controlled by a spatial gradient of nuclear GEFs and cytosolic GAPs.

As small GTPases participate in a multitude of signaling pathways and control numerous essential cellular functions, their dysregulation is associated with a plethora of human diseases such as various forms of cancer, neurodegenerative diseases, inflammatory disorders, and a plethora of genetic disorders. Excessive and permanent activation of small GTPases was identified as a major driver of many diseases, as it results in enhanced and uncontrolled downstream signaling. Three different mechanisms can lead to inappropriate activation of small GTPases: 1) Enhanced activation of wild-type GTPases as a result of upstream signaling resulting in increased GEF activity, 2) mutations in the small GTPase gene which leads to proteins with impaired hydrolysis ability, and 3) loss of GAP function which prevents GAP-mediated hydrolysis of GTP in complex with the wild-type protein.[21] However, the activation pattern for small GTPases varies between the different subfamilies. The small GTPases of the Rho family are rarely mutated in cancer, thus their overactivation is primarily a result of overexpression or mutation in growth factor receptors or upstream targets, whereas amplified Ras signaling is mainly based on direct mutations in Ras genes.[22]

The most prominent members and founders of the Ras superfamily of small GTPases are the Ras proteins K-Ras (Kirsten-Ras), H-Ras (Harvey-Ras), and N-Ras (Neuroblastoma-Ras) which represent proto-oncogenes and are among the most common drivers of cancer.[23–25] Ras mutations can be found in 20–30% of human tumors and go in hand with poor survival rates.[26–28] The mutations are predominantly found at residues Gly 12, Gly 13, and Gln 61 (Figure 1b), and thwart the intrinsic and GAP-catalyzed hydrolysis of GTP. This results in an accumulation of activated GTP-bound Ras, which is associated with many cancers as it constantly promotes pro-survival and proliferation signaling.[23] However, direct targeting of Ras and other small GTPases has proven extremely difficult and despite extensive efforts in academia and industry has not led to the development of an effective drug for clinical use.[27–31]. According to current knowledge, besides the nucleotide binding pocket, small GTPases rarely harbor cavities suitable for small-molecule binding. In addition, GTPase signaling is mediated by protein–protein interactions (PPIs) that involve extended and shallow surfaces. Consequently, classic targeting approaches have not so far provided selective inhibitors,

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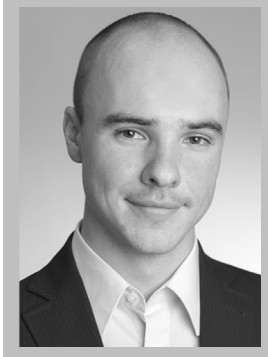

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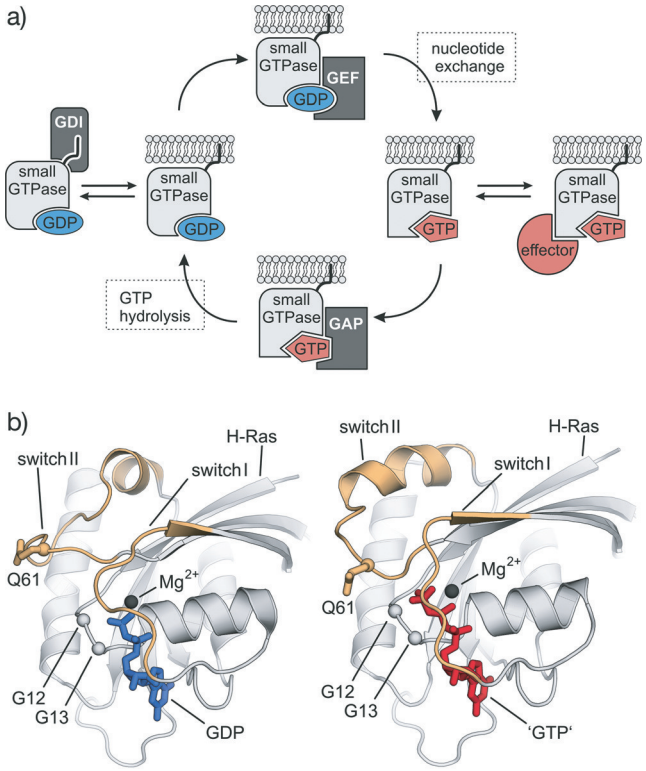

Figure 1. Activation/inactivation cycle of small GTPases: a) GDP-bound small GTPases are activated through GEF-mediated nucleotide exchange. In their GTP-bound form, they interact with effector proteins to trigger downstream signaling events. GAP binding induces GTP hydrolysis and deactivates the small GTPase. GDIs sequester the small GTPases and mediate intermembrane transport through formation of soluble complexes. b) Crystal structures of inactivated (GDP-bound) H-Ras (left) and activated (GTP-bound) H-Ras (right); PDB: 1Q21 and 5P21, respectively. The flexible switch I and switch II regions are shown in orange, the coordinated magnesium in dark gray, and the nucleotides are colored in blue (GDP) and red (GTP). The most common mutation sites found in constitutively activated Ras (G12, G13, and Q61) are highlighted explicitly.

which fostered the notion that small GTPases may in general be "undruggable".

Since Ras is one of the most prominent proto-oncogenes, with its involvement in the development of cancer already identified in 1982,[32] and since subsequent extensive efforts to directly target Ras did not lead to clinically relevant compounds, the Ras protein itself has, in particular, been considered "undruggable". Consequently, major efforts have focused on inhibitors of up- and downstream events as well as compounds that can impair the spatial and temporal organization of Ras. In particular, inhibitors of Ras farnesylation raised high expectations, as they proved efficient in preclinical trials by impairing Ras membrane association.[33] However, their promising preclinical potential could not be translated into clinical efficacy due to compensatory lipidation of Ras by geranylgeranyl transferases.[34] Inhibition of downstream effector pathway members such as the Raf-MEK-ERK kinases is under ongoing exploration, with several compounds approved (such as Sorafenib or Vemurafenib) and in clinical trials.[35] However, these approaches are hampered by the development of drug resistance and complex feedback mechanisms that have not yet been fully revealed.[27,36] Despite these disillusioning outcomes from more than 30 years of research focused on small GTPases, recent advances in targeting Ras and other small GTPases have freshly inspired the field. In this context, the static "on/off" image of small GTPases has begun to change and evolve into a more dynamic picture, with additional conformational states for the Ras proteins having been identified.[37,38] In particular, computer-guided approaches and novel crystal structures of GTPases in previously unknown conformations have led to the identification of small and transient binding pockets, which have been targeted with small molecules.[39]

In this Review we discuss the recent advances in the direct targeting of small GTPases. Given the extensive literature on these proteins, this Review does not aim to be comprehensive. For more detailed discussions, the interested reader should consult additional in-depth reviews about individual Ras subfamilies,[12–18,20,22,27,40,41] their GEF, GAP, and GDI regulation,[6,7,10,42] and their involvement in various diseases.[21,25,29,43–45] This Review will first discuss the potential of compounds that interfere with nucleotide binding, followed by covalent inhibitors that form irreversible adducts with small GTPases (Figure 2). Subsequently, we present inhibitors of small GTPase–GEF interactions and GTPase–effector interactions. Lastly, we describe stabilizers of these PPIs, and conclude by discussing recent advances and offer ideas for future approaches and strategies.

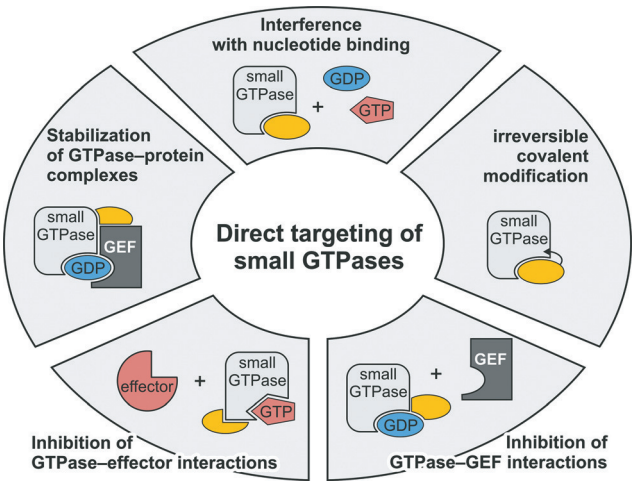

Figure 2. Strategies for targeting small GTPases: This Review discusses five different strategies for directly targeting small GTPases: Interference with nucleotide binding; GTPase inactivation by irreversible covalent modification; inhibition of GTPase–GEF interactions; inhibition of GTPase–effector interactions; and stabilization of GTPase–protein complexes.

2. Interference with Nucleotide Binding

High levels of activated GTP-bound small GTPases promote persistent downstream signaling and have, therefore, been identified as a cause of many human diseases. As the

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activated state of small GTPases is linked to GTP binding, early studies aimed at direct competition with GTP binding. In the case of Ras, modified nucleotides were synthesized with increased affinity compared to GDP, but relatively weak inhibitory potency.[46] The use of nucleotide-based competitive inhibitors is a valid strategy for targeting kinases, as they typically show affinity for ATP in the micromolar range.[47] In contrast to kinases, GTPases show picomolar nucleotide affinities,[48] which renders nucleotide competition in the presence of millimolar intracellular nucleotide pools an extremely challenging task. However, several high-throughput screens (HTSs) identified compounds interfering with GTPase nucleotide binding in vitro.[49–51]

By using a bead-based flow cytometry assay[52] to test 300 000 compounds, CID1067700 (**1**) was identified as a direct competitor for nucleotide binding (Figure 3 a).[49] This compound impairs nucleotide binding by several small GTPases with low nanomolar inhibitory constants (K_i = 12.9–19.7 nM) in vitro. In an identical setup, CID29950007 (ML141, **2**) was identified as a Cdc42-selective noncompetitive allosteric inhibitor (Figure 3 a).[50] In this case, the binding of **2** locks Cdc42 in an inactive conformation, thereby inducing nucleotide release and inhibition of Cdc42-mediated cellular functions such as filopodia formation or cell migration (Figure 3 b). Furthermore, **2** proved useful for combination with colony-stimulation factor (G-CSF) in the mobilization of hematopoietic stem and progenitor cells.[53] Another study identified EHT1864 (**3**) as a Rac1 inhibitor, which effectively reduces Aβ peptide levels in models of Alzheimers diesease in vitro (Figure 3 a).[54] Further investigation of the mode of action led to the postulate that the compound tightly binds Rac1 with low nanomolar affinity (dissociation constant K_d = 40 nM). Compound **3** acts through a noncompetitive mechanism, as observed for compound **2**, which induces nucleotide release upon binding to Rac1.[55]

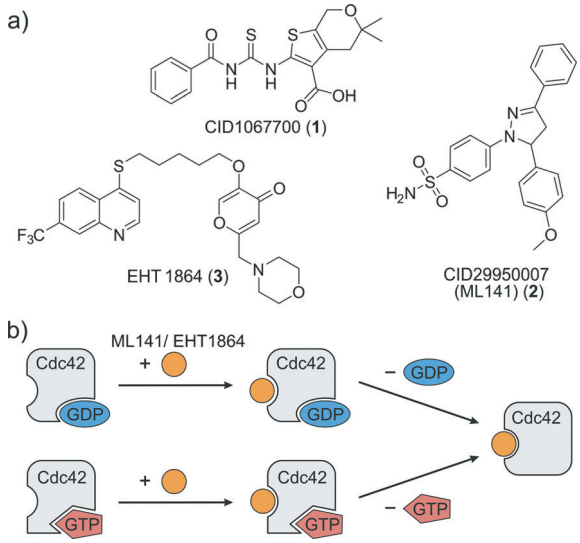

Figure 3. Small molecules interfering with nucleotide binding: a) Chemical structures of representative inhibitors of nucleotide binding. b) Mechanism of action proposed for compounds **2** and **3**.

However, since the efficiency of inhibitors interfering with nucleotide binding is, in general, restrained by the high affinity of small GTPases towards their nucleotides and the intracellular nucleotide concentrations, different strategies to prevent uncontrolled GTPase signaling were explored.

3. Inactivation of Small GTPases by Irreversible Covalent Modification

Several bacterial pathogens modulate GTPase signaling by post-translational enzyme-catalyzed covalent attachment of different small chemical entities. Thereby, they effectively interfere with host signaling pathways to block the host's immune response and enable survival of the bacterium within the hostile environment.[56] In principle, this mechanism validates selective post-translational modification of distinct small GTPases as a general strategy for the effective deactivation of GTPase-mediated signaling pathways. However, despite the fact that covalent inhibitors have a history in drug discovery, there are concerns of potential off-target effects, and only recently has this class of inhibitors attracted renewed increasing interest.[57,58] Two recent examples show that this concept may be applied to small GTPase modulation, even allowing for specific targeting of oncogenic mutants.[59,60]

3.1. Pathogen-Mediated Post-Translational Modifications.

Bacterial pathogens inactivate small GTPases through covalent modification by means of ADP-ribosylating toxins.[56] The C3 exoenzyme from *clostridium botulinum* is a bacterial ADP-ribosyltransferase which covalently attaches an ADP-ribose moiety to Asn 41 of the Rho isoforms RhoA, RhoB, and RhoC to inactivate the cellular functions of these small GTPases.[61,62] ADP-ribosylation at Asn 41 in the switch I region does not inhibit formation of Rho–effector complexes, but prevents nucleotide loading by GEF proteins and release from the GDI complex.[63] Unlike other classical neurotoxins, the bacterial C3 exoenzyme lacks any attributable transport domain and reaches the cytoplasm of target cells by nonspecific uptake.[64] Therefore, chimera comprising of a delivery domain, derived from other toxins, have been constructed and applied.[65] As a consequence of their high substrate specificity, these chimeric toxins are well-established cell-biological tools for simultaneous inactivation of all three Rho isoforms and to prevent the activation of individual Rho isoforms through feedback loops.[66] Rho GTPases play an important role in axonal growth and repair after neuronal injury, and Rho inhibition is a potential strategy to stimulate axonal growth. Inhibition of Rho downstream signaling by C3 causes regrowth and repair of axons in animal models.[67] The BA-210 (cethrin) chimeric C3 exoenzyme is also administered locally to treat spinal cord injury, has shown promising results in phase I/IIa clinical trials,[68] and is planned to enter phase IIb clinical trials under the name VX-210 in late 2015. However, it has been questioned whether the ADP-ribosyltransferase activity is essential for the neurotrophic effects of C3, as an enzyme-deficient peptidic fragment of C3 is also

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neurotrophic.[69] Besides ADP-ribosylation, a variety of other post-translational modifications (glucosylation, adenyllylation, deamidation, etc.) are utilized by pathogens to persistently activate or inactivate the function of various GTPases from different subfamilies.[56] For example, "lethal toxin" from *clostridium sordellii* efficiently inactivates the signaling of Ras family members in intact cells.[70] However, the use of such toxins as biological tools or for medical application has yet to be explored.

3.2. Mutant-Selective Targeting through Small Molecules

In an effort to overcome the limitations associated with nucleotide-competitive binders, the K-Ras-activating mutation G12C was targeted to covalently trap inhibitors through irreversible binding. The G12C mutation is the most common K-Ras mutation in lung cancer, occurring in 7% of these tumors.[26] Based on the nucleotide scaffold, covalent inhibitors were designed which directly target the nucleotide binding site of K-Ras^{G12C}.[59] Supported by molecular-docking studies, a variety of different electrophiles were attached to the β-phosphate group of GDP to target the cysteine residue of the G12C mutation in proximity to the nucleotide binding pocket. Mass spectrometric analysis revealed that the chloroacetyl derivative SML-8-73-1 (**4**) attaches covalently to K-Ras^{G12C} in the presence of millimolar concentrations of nucleotide (Figure 4a). Affinity experiments with the labeled Ras binding domain (RBD) of Raf suggest that inhibitor-bound K-Ras is stabilized in a conformation that resembles the GDP-bound inactive signaling state.[59] This was later confirmed by a crystal structure of compound **4** bound to K-Ras^{G12C}.[71] The inhibitor occupies the nucleotide binding site with an orientation similar to that of GDP and covalently attaches to Cys12, while switch I and switch II of K-Ras assume an open, inactive conformation, which is incompatible with effector binding. A mass spectrometry based profiling experiment in lysates of K-Ras^{G12C} mutant cells confirmed inhibitor selectivity, as only two other GTP-binding proteins, including another small GTPase (Arf-family Arl3), were labeled. However, the experiment involves a nucleotide-removing gel-filtration step, so it does not clearly show whether the inhibitor can compete with cellular nucleotide concentrations.[71] To achieve cell penetration, SML-10-70-1 (**5**), a caged phosphoramidate derivate of **4**, was synthesized, which impairs Akt and ERK phosphorylation in a H358 cell line at a concentration of 100 μM and proliferation in K-Ras^{G12C} mutant H358 and H23 cells with a half-maximum effective concentration (EC_{50}) of 27 μM and 48 μM, respectively. Interestingly, **5** also shows antiproliferative effects in A549 cells, which contain a G12S mutation, thus indicating additional nonselective modes of action.[59]

By using a tethering approach, another set of covalent inhibitors was identified that make use of the G12C mutation as a chemically targetable anchor.[60] A screen of 480 fragments with the potential to form disulfide bonds yielded several compounds which specifically label K-Ras^{G12C}, but do not modify wild-type K-Ras. A cocrystal structure with K-Ras^{G12C}–GDP revealed that the tethered compounds are not

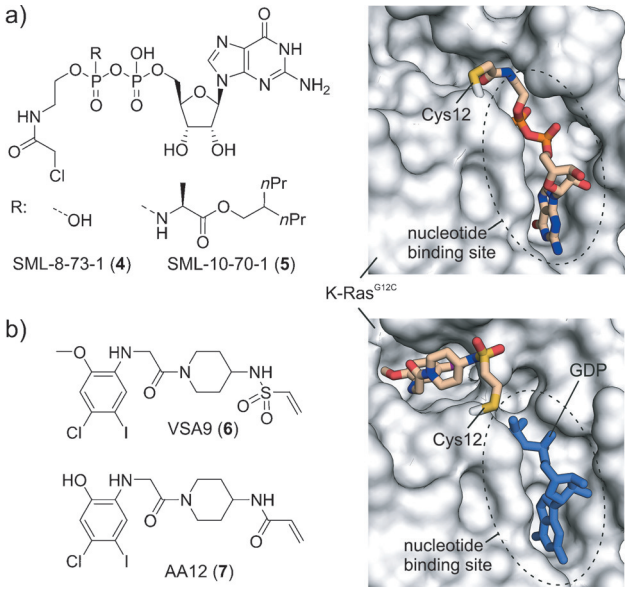

Figure 4. Mutant-selective covalent inhibitors targeting Cys12 of K-Ras^{G12C}: a) The nucleotide-competitive inhibitor **4** and its caged, cell-permeable analogue **5**. Compound **4** attaches to Cys12 to occupy the nucleotide binding site (PDB: 4NMM). b) The vinyl sulfonamide based inhibitor **6** and acrylamide-based inhibitor **7**. The binding of **6** to K-Ras^{G12C}–GDP results in significant rearrangement of the switch II region to form a new subpocket in proximity to the nucleotide binding pocket, as observed by X-ray crystallography (PDB: 4LYJ).

located at the nucleotide binding site, but occupy a novel pocket (termed S-IIP) that extends from Cys12 opposite to the nucleotide binding site mostly composed of residues of the switch II region. Several derivatives with acrylamide and vinyl sulfonamide electrophilic groups were synthesized and subsequently optimized. These compounds react with mutant K-Ras^{G12C}, but not wild-type K-Ras and cause reduced levels of activated K-Ras–GTP. Vinyl sulfonamide VSA9 (**6**) and acrylamide AA12 (**7**) allosterically control nucleotide affinity and decrease the relative GTP-affinity of K-Ras compared to GDP (by ca. 4-fold). Moreover, they completely abolish SOS (Son of Sevenless) catalyzed nucleotide exchange to prevent GTP loading of K-Ras. Treatment of K-Ras^{G12C}–mutant cells results in reduced Ras–effector binding, as shown by co-immunoprecipitation. Notably, the inhibitors promote apoptosis and reduce the viability of different K-Ras^{G12C}–mutant cell lines with varying potency, while cells that lack this mutation are insensitive to compound treatment. Consistent with the in vitro labeling efficiency, compound **7** displays a half-maximum effective concentration of $EC_{50} = 0.32$ μM in K-Ras^{G12C}-mutant H1792 cells.

4. Inhibition of GTPase-GEF Interactions

As small GTPases show very high affinity towards their nucleotides, GEF proteins are required to accelerate the exchange reaction to provide efficient activation in cells.[6,7] Nucleotide exchange is a complex multistep reaction, in which the GEF remodels the nucleotide binding site of the small GTPase to trigger nucleotide release. Initially, the

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inactivated GDP-bound small GTPase forms a low-affinity complex with its GEF. After nucleotide release, a high-affinity nucleotide-free GTPase–GEF complex is formed which dissociates upon binding of a new nucleotide. As the binding affinity of small GTPases for GDP and GTP is similar, activation occurs as a result of a roughly 10-fold higher cellular concentration of GTP compared to GDP. Despite the fact that the exact mechanism of GEF activation is not conserved throughout the Ras superfamily, an elementary mode of action can be defined. In a first step, GEFs deform the phosphate binding site (switch I and P-loop), which results in reduced nucleotide affinity. They then interact with switch II to stabilize the nucleotide-free form of the small GTPase. For a more detailed discussion and illustration of the mechanism, the reader should consult additional reviews.[6,7] However, small GTPases of the Ras family are activated by GEFs containing a CDC25 homology domain and a Ras exchanger motif (REM). The RasGEF Son of Sevenless (SOS) uses a α-helix to open the nucleotide binding site of Ras and allow nucleotide release.[42] Unlike the RasGEF RasGRF-1 (Ras guanine nucleotide release factor 1), SOS needs to be activated by allosteric Ras–GTP binding to induce nucleotide exchange.[72] In contrast to the Ras subfamily, the Rho subfamily of small GTPases is regulated by a multitude of GEFs (more than 75 GEFs) and GAPs (more than 60 GAPs).[22] However, despite this large number of GEF proteins, only two activating domains for RhoGEFs have been identified. Most GEFs of the Rho family such as TrioN, Tiam1, or LARG share a Dbl-homology domain (more than 69 GEFs),[73] while a minor group of Rho GEFs activates Rho GTPases through a Dock homology domain (11 members).[74] Arf family GTPases are activated by ArfGEFs bearing a Sec7 domain, and RCC1, the only identified RanGEF, catalyzes Ran–GTP loading. RabGEFs contain either a Vsp9 domain or a DENN domain or bind to MSS4, which is presumed to act as a chaperone with weak nucleotide exchange activity.[75] However, as the activation of small GTPases depends on GEF-mediated nucleotide exchange, the GTPase–GEF interaction has emerged as highly interesting and a valid target in small GTPase drug discovery.

4.1. Ras GTPases

The first compounds reported to impair nucleotide exchange on Ras were designed to compete for nucleotide binding (for example SCH53239 (**8**), Figure 5a). Mass spectrometry identified the compounds in ternary complexes with Ras and GDP, and NMR analysis placed them in an area close to the nucleotide binding site.[76] To improve their water solubility and potency, derivatives were designed which can inhibit RasGRF-1-catalyzed nucleotide exchange in vitro (half-maximum inhibitory concentration (IC_{50}) = 35–80 μM).[77] Reduced cell growth in Ras-dependent cell lines as well as decreased downstream signaling were observed at high micromolar concentrations, but the exact mode of action still remains elusive and off-target effects have to be considered in certain cases. For another compound of the SCH compound family (SCH51344 (**9**), Figure 5a) which was reported to modulate the Ras signaling pathway through a novel mechanism,[78] the human mutT homologue hMTH1 was recently identified as the actual target.[79]

Based on the Ras-binding epitopes of its GEFs, rationally designed orthosteric peptidic inhibitors were developed to impair Ras nucleotide exchange.[80–82] The RasGEF SOS forms a tight complex with nucleotide-free Ras after insertion of a helical hairpin motif between the Ras switch I and II regions to open the nucleotide binding pocket and trigger GDP release (Figure 6a).[6,7] This α-helix (αH) was used as

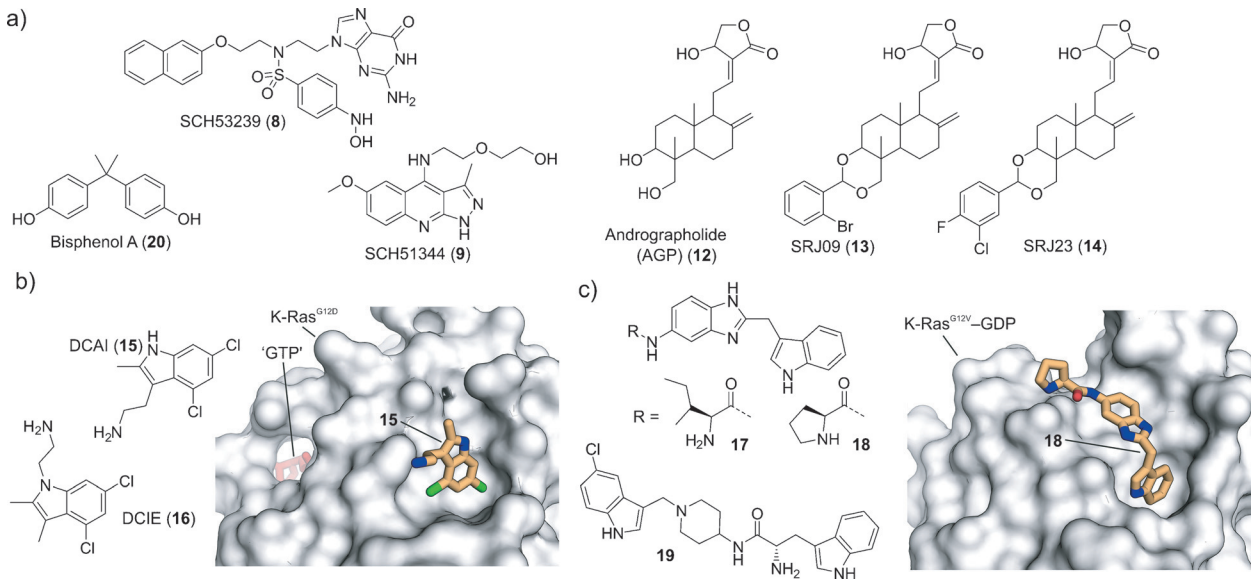

Figure 5. Small-molecule inhibitors of the Ras–GEF interaction: a) Chemical structures of the Ras–GEF inhibitors. b) Crystal structure of **15** bound to K-Ras^{G12D}–"GTP" (PDB: 4DST). c) Chemical structures of fragments **17**–**19** and crystal structure of **18** in complex with K-Ras^{G12V}–GDP (PDB: 4EPY).

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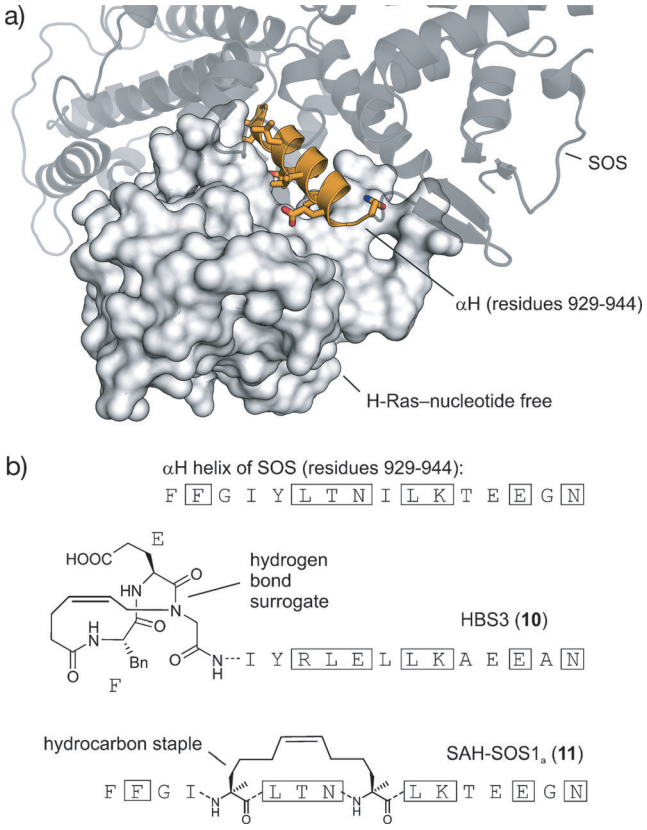

Figure 6. Structural basis for inhibition of the Ras–SOS interaction: a) Crystal structure of nucleotide-free Ras (gray) in complex with its GEF SOS (dark gray; PDB: 1NVW). The helix αH is highlighted (orange) and the interacting residues are shown explicitly. b) Schematic representation of the α-helical, stabilized peptides **10** and **11**, as well as the corresponding sequences. Both peptides are derived from αH. The interacting residues are labeled with boxes.

a starting point to design peptide-based inhibitors that prevent Ras activation.[80,81] Stabilized α-helices have proven highly successful in inhibiting protein–protein interactions (PPIs), with hydrogen-bond surrogates (HBS) and stapled peptides resembling the most advanced approaches within that field.[83,84] HBS3 (**10**) is a derivative of αH with enhanced solubility and was designed as an inhibitor of the Ras–SOS interaction (Figure 6b). It shows moderate affinity for GDP-bound ($K_d = 158$ μM) as well as nucleotide-free (nf) H-Ras ($K_d = 28$ μM).[80] Although the affinity of HBS3 for Ras–GDP is approximately tenfold lower than that of the parent protein SOS itself, HBS3 impairs SOS-mediated nucleotide exchange in vitro ($IC_{50} = 25$ μM) and reduces ERK activation in serum-starved HeLa cells. However, a recently reported stapling approach of the same SOS αH helix seems to have a much more drastic effect than HBS stabilization. It yields a stapled peptide SAH-SOS1$_a$ (**11**) with low nanomolar affinity towards nucleotide-bound wild-type and mutant K-Ras ($EC_{50} = 60$–140 nM; Figure 6b).[81] Surprisingly, introduction of the hydrocarbon peptide staple into the αH helix results in a dramatically increased affinity, even compared to the full catalytic domain of SOS (K_D(H-Ras–GDP–SOS$_{cat.}$) ≈ 14.5 μM),[85] and although K-Ras undergoes conformational changes within the switch regions upon nucleotide exchange, compound **11** counterintuitively binds GDP- and GTP-bound K-Ras with similar affinity. Although GEF-mediated nucleotide exchange was not investigated, **11** impairs the viability of K-Ras-dependent cancer cell lines ($IC_{50} = 5$–15 μM) and reduces K-Ras downstream signaling in Panc 10.05 cells.

According to computational studies and molecular dynamics (MD) simulations, the Ras protein surface is less rigid than previously thought and can form several transient pockets which might be capable of binding small molecules.[39,86,87] Docking experiments identified two small-molecule binders which reduce the phosphorylation of downstream ERK as well as Ras–GTP levels in U251 glioblastoma cells ($IC_{50} = 10$–30 μM), but further investigations will be needed to verify their proposed mode of action.[86] By using previously identified transient pockets on the surface of Ras, GDP-bound K-Ras was proposed as the target of the anticancer agent Andrographolide (AGP, **12**) and its benzylidene derivatives (Figure 5a).[88] Ensemble molecular docking placed **12** at the Ras–GEF interface, where it impairs GEF-catalyzed Ras activation. Consequently, the derivatives of **12**, SRJ09 (**13**), and SRJ23 (**14**) reduce wild-type Ras-GTP levels and ERK phosphorylation in cells. Decreased levels of mutant K-Ras^{G12V}–GTP were reported after long-term treatment (3 days) with compounds **13** and **14**. The slow intrinsic GTPase activity of mutant Ras^{G12V} together with blocking of GEF activation was proposed as the cause of this effect, although additional modes of action could not be ruled out.[88]

Besides these computational approaches, fragment-based ligand discovery[89] has led to the identification of previously unknown surface cavities and novel ligand scaffolds.[90–92] A fragment library of 3300 compounds was screened for binding to a 1:1 mixture of K-Ras bound to GDP and a nonhydrolyzable GTP analogue (henceforth termed "GTP") using an NMR-based saturation transfer difference assay.[90] The 240 primary hits were further validated by two-dimensional NMR spectroscopy to yield 25 confirmed hits which can be mapped to a distinct binding site of GDP-bound K-Ras. High-resolution cocrystal structures with K-Ras-"GTP" identified a previously not observed hydrophobic pocket between switch I and II as a conserved interaction area for all hits. This novel pocket opens up after fragment binding and overlaps with the SOS binding site. Two of the hit molecules, DCAI (**15**) and DCIE (**16**), can impair SOS-catalyzed nucleotide exchange and nucleotide release in vitro (Figure 5b). Binding of **15** leads to a reorientation of two side chains, which enlarges the binding pocket and simultaneously prevents the formation of two essential salt bridges that stabilize the Ras–SOS complex. Despite its low affinity to K-Ras ($K_d = 1.1$ mM), **15** inhibits the nucleotide exchange and release reaction with moderate potency ($IC_{50} = 342$ μM and 155 μM, respectively). Furthermore, in live-cell experiments, **15** prevents membrane recruitment of the fluorescence-labeled Ras-binding domain (RBD) of c-Raf kinase (Raf-RBD) with a surprisingly low EC_{50} value of 15.8 μM. This finding suggests that another distinct mechanism may be operating in cells in addition to the orthosteric inhibition of SOS-mediated Ras activation.

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In a different study, an NMR-based screen of 11000 fragments identified 140 hits which directly bind to GDP-bound K-Ras^{G12D} with millimolar affinity ($K_d = 1.3$–2.0 mM).[91] As these compounds bind with similar affinity to wild-type K-Ras and H-Ras they most likely occupy a binding site conserved among different Ras proteins. High-resolution cocrystal structures with K-Ras–GDP revealed binding to the same hydrophobic pocket between switch I and II as previously identified for **15**.[90] A small library of the initial indole-based scaffold yielded compounds **17** and **18** as sufficient for inhibition of SOS-mediated nucleotide exchange, with an affinity towards K-Ras–GDP in the high micromolar range ($K_d = 190$ μM and 340 μM, respectively; Figure 5c). However, the potential of this indole scaffold to serve as a starting point for fragment growing appears to be limited, as compound **19** was also reported to be an activator of SOS-catalyzed nucleotide exchange ($EC_{50} = 14$ μM).[93] Compound **19** binds to a hydrophobic pocket formed by the CDC25 domain of SOS and increases cellular Ras–GTP levels in a concentration-dependent manner, whereas no effect on the Ras-specific GEF RasGRF-1 was observed. Further investigation of **19** will be required, as it shows a biphasic response and decreases AKT and ERK activation downstream of Ras through an unknown mode of action, thereby underlining the complexity of the Ras signaling network.[93] In a further fragment-based approach from a library of less than 100 fragments, bisphenol A (**20**) was identified as a binder of K-Ras and Rheb (Ras homologue enriched in brain) with low to moderate potency ($K_d = 600$ μM (K-Ras) and 1830 μM (Rheb), respectively; Figure 5a).[92] Although compound **20** impairs SOS-mediated nucleotide exchange and molecular docking places it in the same hydrophobic binding pocket occupied by **15** and compound **17**, the strongly hydrophobic character and the relative low binding affinity indicate a possible unspecific mode of interaction.

4.2. Ral GTPases

Within the last decade, the Ras-like GTPases RalA and RalB have emerged as important drivers of multiple human cancers.[41,94,95] Despite their high sequence identity of 82%, RalA and RalB execute distinct functions in tumor growth and metastasis.[96] Whereas RalA is important for tumor initiation, RalB activity is more crucial for tumor metastasis. The RalGEFs RalGDS, Rgl, and Rgf are direct effectors of activated Ras–GTP,[97] which results in continuous activation of RalA and RalB in Ras-dependent tumors. Similar to other small GTPases, no clinically relevant inhibitor of Ral GTPases has been described. Nevertheless, Ral is considered to be a valuable target in drug discovery. Based on the idea of preventing Ral activation by inhibiting the interaction with its GEFs, the RalA–GDP structure was used as a starting point to identify small-molecule binders. A structure-based virtual screening approach by individual docking of 500000 compounds to a defined binding site adjacent to switch II identified 88 initial hits.[98] Measuring the amount of activated RalA–GTP after compound treatment identified two compounds, RBC6 (**21**) and RBC8 (**22**), which were capable of

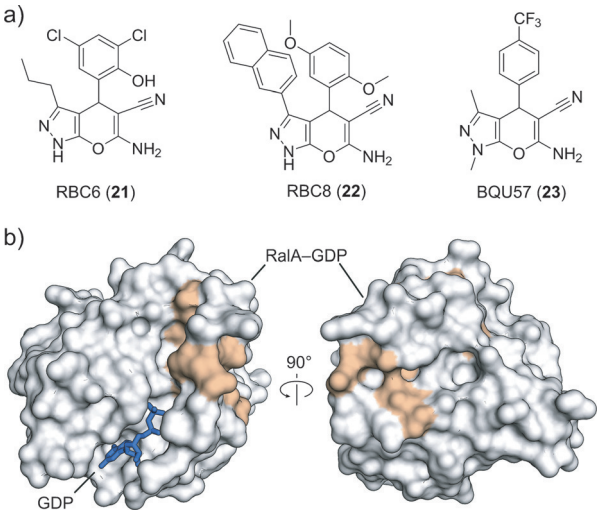

Figure 7. Ral GTPase inhibitors: a) Chemical structure of the Ral–GEF inhibitors **21**, **22**, and **23**. b) Crystal structure of RalA–GDP (PDB 1U90). Orange: residues which show a chemical shift difference $> +2$ s.d. after incubation with 100 mM **23**. The experiment was carried out using RalB–GDP and the implied residues mapped onto RalA–GDP.

effectively reducing RalA–GTP levels in cells (Figure 7a). As both compounds share the same scaffold, several derivatives were synthesized, with BQU57 (**23**) being the most potent Ral binder (Figure 7a). NMR analysis of **23** and RalB–GDP confirmed the hypothesized binding site. Isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) experiments revealed low micromloar binding affinity ($K_d = -7.7$ μM and 4.7 μM, respectively), whereas no binding to RalB–"GTP" was detected. Compound **23** inhibits colony formation of human lung cancer cell lines with an IC_{50} value of 1.3–2.0 μM and is effective in mouse xenograft models of human lung cancer. Tumor immunoblots exhibit a significant decrease in activated RalA and RalB levels without any effect on activated Ras or RohA.

4.3. Rho-Family GTPases

The Rho-family GTPases, notably Rho, Rac, and Cdc42 are regulators of the actin cytoskeleton and several signaling pathways.[12,13] Rac and Cdc42 share a relatively high sequence identity (ca. 70%) as well as a common binding motif of around 15 residues—the CRIB (Cdc42/Rac-interactive binding) motif.[99] In contrast, the sequence similarity between RhoA with Rac and Cdc42 is only about 45%. Nevertheless, selective modulation of all three Rho-family GTPases is achieved with a variety of regulating proteins. Rho-family GTPases are involved in the immune response and in inflammation, and contribute to various steps of cancer progression.[22,44] These properties render Rho-GTPases and their signaling pathways interesting targets in drug discovery. However, the identification of effective Rho-family GTPase inhibitors has proven very challenging, with no direct inhibitors identified for clinical trials, thus far.[44] Nevertheless, screening a library of 570 pharmacologically active

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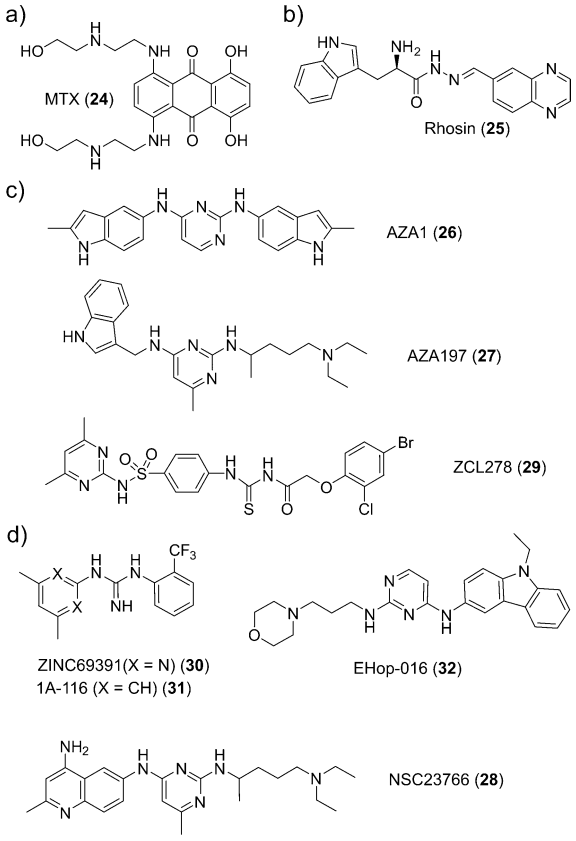

Figure 8. Inhibitors of the Rho subfamily: a) Pan inhibitor for RhoA, Cdc42, and Rac1. b) The Rho-specific inhibitor Rhosin which blocks the Rho–GEF interaction. c) Inhibitors of the Cdc42–GEF interaction. d) Rac-selective inhibitors of the Rac–GEF interaction.

compounds using AlphaScreen technology identified the topoisomerase II targeting drug mitoxantrone (MTX, **24**) as a pan inhibitor of Rho-GTPases (Figure 8a).[100] Compound **24** impairs GTP loading of RhoA/Rac1/Cdc42 in vitro, F-actin reorganization, and cell migration in a wound-healing assay. However, as each member of the Rho-GTPase family exerts defined functions by regulating distinct signaling pathways, selective inhibitors instead of pan inhibitors are desired.

4.3.1. *RhoA*

Different computer-aided docking approaches were explored to develop a RhoA-selective inhibitor.[101–104] Structure-based virtual screening identified several small molecules as potential RhoA inhibitors.[101] After binding was verified by SPR measurements, 41 analogues were synthesized and their biological activity evaluated. Two compounds showed low micromolar inhibition of RhoA activity (IC_{50} = 1.2–1.7 μM) and impaired phenylephrine (PE) induced thoracic aorta artery ring contraction. Further optimization enhanced the solubility of the initial hits and increased their ability to inhibit PE-induced contraction in thoracic aorta artery rings.[102] However, the exact mode of action of these compounds still remains elusive and demands further investigation. Another virtual screening approach led to the identification of the Rho-specific inhibitor Rhosin (**25**, Figure 8b).[103,104] The virtual screen was designed to yield small molecules that bind to RhoA near to Trp58. The analysis of the crystal structure of RhoA with its GEF LARG previously identified the surface around Trp58 as a suitable binding site for small molecules to interfere with GEF binding.[105] Trp58 resembles a ridge between two shallow pockets within the GEF binding area of RhoA. The small molecule **25**, which contains two aromatic fragments tethered by a properly spaced linker, binds to RhoA with submicromolar affinity (K_d = 350 nM) and interferes with the RhoA–GEF interaction.[103] Compound **25** shows no effect on Rac1 and Cdc42 activation by their GEFs, and its binding to RhoA is sensitive to point mutations in the proposed binding region. As the closely related isoforms RhoB and RhoC share the recognition motif around Trp58 with RhoA, their GEF-mediated activation is equally affected by **25**. In a cellular analysis, **25** blocks RhoA-specific downstream signaling and the formation of actin stress fiber without any impact on the downstream signals of Cdc42 and Rac1. Furthermore, **25** induces a dose-dependent inhibition of MCF7 cell growth, impairs invasion by these breast cancer cells, and promotes neurite outgrowth from neuronal cells.

4.3.2. *Cdc42*

Similar to the other Rho GTPases Rho and Rac, Cdc42 controls actin organization and cell shape. Nevertheless, a thorough understanding of Cdc42 activity and function is still missing, and consequently only a few small-molecule modulators of Cdc42 have been reported.[106–108] AZA1 (**26**) inhibits the activation of Cdc42 by its GEFs, and also inhibits Rac1 activation (Figure 8c).[106] The exact mode of action of **26** is unclear. However, it reduces lamellipodia and filopodia formation, migration of prostate cancer cells, downstream signaling through p21-activated kinase (PAK1), AKT, and ERK, and is effective in human prostate cancer xenograft models. A close analogue AZA197 (**27**), which belongs to the same chemotype as **26** and the Rac1 inhibitor NSC23766 (**28**), stands out by its selective inhibition of Cdc42 (Figure 8c).[107] Compound **27** blocks nucleotide exchange of Cdc42 in colon cancer cells without any effect on RhoA or Rac1. It reduces colon cancer cell migration and invasion, downregulates PAK1 signaling, diminishes tumor growth, and prolongs animal survival in human colon cancer xenograft mouse models.

Virtual screening of 197000 compounds identified ZCL278 (**29**) as a selective low micromolar inhibitor of Cdc42 (K_d = 11.4 μM; Figure 8c). It binds to a surface groove close to Phe56, which is essential for the binding of GEFs to Cdc42.[108] Upon exposure to cells, **29** inhibits microspike formation and decreases cellular levels of activated Cdc42–GTP. Compound **29** impedes wound healing without affecting cell viability, as shown for human prostate cancer cells. As Cdc42 deactivation is associated with a significantly reduced number of neurites and disrupted filopodia function,[109] treatment of primary cortical neurons with **29** suppressed neuronal branching. However, the specific Cdc42 inhibitors **29** and **27** might provide further insights into Cdc42-

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controlled functions and Cdc42-associated human diseases such as cancer and neurological disorders.

4.3.3. Rac

Structural analysis of the Rac1 interaction with its GEFs revealed that an area in the vicinity of Trp56 is essential for GEF binding.[110] These studies pinpointed Trp56 as the critical determinant for GEF binding (similar to Trp58 in RhoA and Phe56 in Cdc42), because the $Rac1^{W56F}$ mutant cannot interact with certain Rac GEFs. However, $Cdc42^{F56W}$ gains sensitivity towards these RacGEFs, thus underlining the high structural similarity between Rac1 and Cdc42. Virtual screening of more than 140000 small molecules for binding to the shallow pocket around Trp56 on Rac1 identified **28** as an inhibitor of the Rac–GEF interaction (Figure 8d).[111] Compound **28** selectively inhibits the interaction of Rac1 with its GEFs Tiam1 and TrioN with medium micromolar affinity ($IC_{50} \approx 50$ μM) in vitro. In cell-based assays, it has no effect on the interaction between RhoA and Cdc42 with their GEFs. The application of **28** to prostate cancer cells inhibits lamellipodia formation, cell proliferation, and tumor cell invasion in a dose-dependent manner. Recent studies report therapeutic effects on osteoarthritis (OA) in mice after lowering of the Rac1–GTP levels using **28**.[112] Hyaluronic acid/chitosan microspheres incorporating **28** were injected weekly into OA joints to provide more constant levels of **28**.

Inspired by the initial success of **28**, an improvement of the affinity and efficiency was attempted.[113–115] Based on another virtual screen inspired by the crystal structure of **28**,[116] compounds with a different chemotype were identified which showed IC_{50} values between 12 and 58 μM.[113] Closely related analogues of **28**, such as **26** and **27**, showed different selectivity. They were either active on Rac1 and Cdc42 (**26**) or Cdc42-selective (**27**).[106,107] Studies of the structure–activity relationship of **28** led to a further slightly improved affinity and efficiency.[114] However, another docking-based virtual screen proved to be more effective and identified ZINC69391 (**30**) and its optimized analogue 1A-116 (**31**), which reduces cellular Rac1–GTP levels with low micromolar affinity ($IC_{50} = 4.0$ μM) (Figure 8d).[115] Compound **31** inhibits Rac-mediated tumor cell proliferation in cell-based assays and metastatic activity in vivo by reducing the formation of metastatic lung colonies in mice by 60%. The most potent Rac inhibitor is the small molecule EHop-016 (**32**), which shows inhibition of Rac activity in cells at low micromolar levels ($IC_{50} = 1.1$ μM; Figure 8d).[117] Compound **32** has no effect on RhoA, but slightly reduces Cdc42 activation at concentrations above 10 μM. At a low micromolar concentration (2 μM), **32** significantly reduces Rac-mediated formation of lamellipodia and cell migration without interfering with Cdc42-derived filopodia extension. Compound **32** significantly reduces tumor growth, metastasis, and angiogenesis in breast cancer mouse models.[118] Compared to **28**, Rac1 inhibition can be achieved with a 10–50-fold lower concentration of **32**.[117]

5. Inhibition of GTPase–Effector Interactions

After GTP loading, small GTPases interact with numerous effector proteins to trigger downstream signaling events. Inhibition of these PPIs is a promising approach to modulate GTPase signaling, especially in cases in which mutations render the protein constitutively active. A paramount example is constitutively active Ras, where different mutations prevent GTP hydrolysis and lead to an enrichment of activated species and permanent signal propagation. Once the small GTPase is in its activated, GTP-bound state, reversal is slow. Thus, inhibitors that prevent GTP loading have to be highly efficient to have a significant impact on downstream signaling. The prominent role of constitutively active Ras in the development of human cancer has motivated various efforts to find small molecules that inhibit the formation of GTPase–effector complexes. The medicinal value of this approach has been validated by intracellular expression of antibody fragments directed against the effector binding switch I region of Ras–GTP.[119] Cells expressing these fragments display blocked effector signaling downstream of Ras, which is sufficient to prevent K-Ras-mediated tumor initiation and growth in a transgenic mouse model of human lung cancer.[120]

5.1. Orthosteric Inhibitors

5.1.1. Small-Molecule Ras Inhibitors

The lack of tractable surface cavities and the highly dynamic nature of the effector binding regions are significant challenges for the application of small-molecule-based inhib-

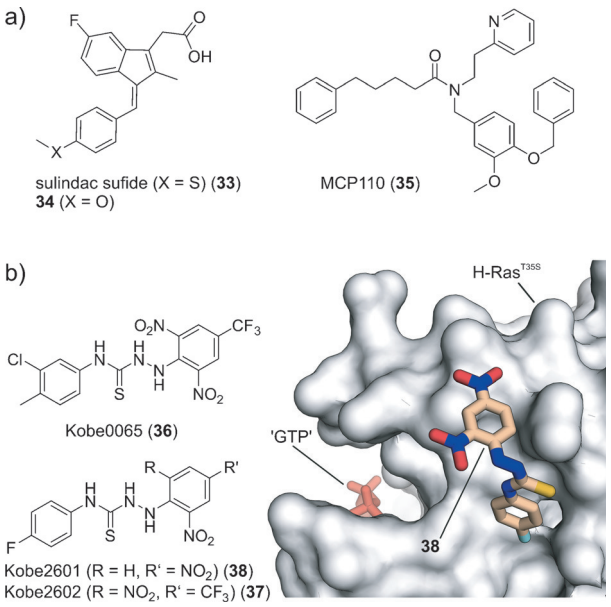

Figure 9. Small-molecule orthosteric inhibitors of Ras–effector interactions: a) Chemical structures of representative orthosteric Ras inhibitors. b) Structural basis for the orthosteric inhibition by Kobe compounds. The NMR structure of $H\text{-}Ras^{T35S}$–"GTP" shows that the binding site of **38** overlaps with effector-binding sites and should result in steric hindrance (PDB: 2LWI).

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itors. However, a compound that inhibits the formation of the Ras–Raf complex is sulindac sulfide (**33**; Figure 9a), a metabolite of the nonsteroidal anti-inflammatory drug sulindac.[121] A sulindac-inspired compound library yielded inhibitors with 20-fold higher potency than **33** ($IC_{50} = 10\ \mu M$ for the best molecule **34**). ^{15}N HSQC NMR shift experiments showed a direct interaction, with Ras in proximity to the switch I region overlapping with effector binding interfaces.[122] Another series of orthosteric Ras–effector inhibitors originated from a yeast two-hybrid screen[123] and was subsequently optimized.[124] MCP compounds disrupt the Ras–Raf interaction in vitro and in cells, reduce ERK phosphorylation, and show antiproliferate activity in cells expressing constitutively active Ras.[125] Following intraperitoneal and intravenous administration, MCP110 (**35**) sensitizes tumor cells and shows synergistic effects with other anticancer agents in mouse xenograft models of human cancer (Figure 9a).[126] Unfortunately, these early examples of GTPase–effector inhibitors lack rigorous biophysical characterization, thus hampering a stringent structure-based optimization process. Moreover, the question remains whether most of their activity has to be attributed to considerable off-target effects, as molecules such as sulindac are promiscuous.[127]

Recent progress in the discovery of orthosteric small-molecule inhibitors of Ras–effector interactions was based on the virtual screening of a 41000 compound library for binding to the structure of M-Ras^{P40D}-"GTP",[128] a close homologue of Ras. 97 candidates were identified and characterized in vitro.[129] Kobe0065 (**36**) efficiently inhibits the association of M-Ras^{P40D}–GTP and H-Ras–GTP with the RBD of c-Raf ($K_i = 46\ \mu M$; Figure 9b). A structure-based similarity search of 160000 compounds yielded 273 hits, of which Kobe2602 (**37**) could be confirmed in vitro ($K_i = 149\ \mu M$). As the residues forming the binding pocket are well-conserved among different Ras family members, both compounds were tested for binding to other small GTPases (Figure 9b). According to NMR experiments, both compounds bind to the Ras-family members M-Ras, Rap2A, and RalA, but not to the Rho-family members RhoA and Cdc42. Both **36** and **37** interfere with different Ras–effector interactions in cells to impair downstream signaling pathways in a dose-dependent manner. They decrease the amount of Ras-bound c-Raf-1 and reduce the phosphorylation of MEK and ERK downstream of Raf as well as RalA activation and phosphorylation of Akt, downstream of RalGDS and PI3K, respectively.

Although the compounds do not block binding to the catalytically active site of SOS, they inhibit the binding of Ras–GTP to the distal site of SOS, thereby impairing the allosteric acceleration of its GEF activity. This is in agreement with NMR-binding experiments of Kobe2601 (**38**), a water-soluble low-affinity analogue, and H-Ras^{T35S}–"GTP" (Figure 9b). NOE signals indicate binding of **38** to hydrophobic surface pockets in proximity to switch I and II, respectively, which is predicted to overlap with different Ras–effector binding interfaces and to cause steric hindrance. Moreover, at low micromolar concentrations, the compounds inhibit colony formation and anchorage-dependent proliferation in various cell lines carrying activated *ras* oncogenes and are active in a xenograft model of human cancer cells in nude mice.

5.1.2. *Peptide-Based Ras Inhibitors*

To overcome the lack of distinct surface pockets and to account for the shallow, extended interaction surfaces, several peptide-based inhibitors were developed to target GTPase–effector complexes. Short linear peptide sequences derived from Raf served as orthosteric inhibitors of Ras–effector interactions, but they are only moderately potent.[130] Rigidified peptides have been suggested to better address the highly flexible effector binding regions and to offer more druglike properties. Two combinatorial libraries of cyclized peptides were tested for their potential to bind to K-Ras and to impair the K-Ras–c-Raf interaction.[131,132] Screening of approximately 1.5 million compounds originating from a combinatorial library of bicyclic peptides (3–5 variable residues per ring) yielded four molecules that bind K-Ras–GTP with affinities in the low micromolar range ($K_d = 0.5\ \mu M$–$6.8\ \mu M$). The peptides display a preference for the GTP-bound state, inhibit the Ras–Raf interaction in vitro (best IC_{50}: $1.4\ \mu M$), but show only poor antiproliferative activity, most probably because of poor cell penetration.[132]

Another library of six million cyclic peptides containing a variable stretch of 4–6 residues and a FK506-binding protein 12 (FKBP) binding moiety was generated.[131] By analogy to rapamycin, the FKBP-binding moiety was included to allow formation of a binary peptide–FKBP complex with increased interaction surface with the target protein.[133] Screening of about 3 million compounds yielded several Ras-binding peptide–FKBP complexes. Interestingly, compound **39** disrupts the interaction of K-Ras^{G12V} and Raf–RBD in vitro with IC_{50} values of $0.7\ \mu M$, even in the absence of FKBP. According to fluorescence polarization (FP) and surface plasmon resonance (SPR) measurements, **39** binds to K-Ras with a K_d value of $0.83\ \mu M$, but does not attenuate MEK or ERK phosphorylation in cells, possibly because of insufficient cell penetration (Figure 10a). In a second-generation bead-based library of monocyclic peptides, the potential cell-penetrating motif of **39** was retained and the remaining residues were replaced with a combinatorial peptide sequence of up to five amino acids.[134] Screening of this 1.6 million library identified two peptides that bind K-Ras^{G12V} with submicromolar affinity and inhibit the Ras–Raf interaction in vitro. Subsequent iterative sequence optimization yielded highly potent Ras inhibitors (best $IC_{50} = 14\ nM$) with various cell permeabilities. The cell-permeable peptide Cyclorasin 9A5 (**40**) preferably binds to Ras^{G12V} in its GTP-bound form and inhibits the Ras–Raf interaction ($IC_{50} = 0.12\ \mu M$) by targeting the effector-binding region of Ras, as suggested by ^{15}N HSQC NMR experiments. The treatment of different cancer cells with **40** blocks EGF-stimulated Akt, MEK, and ERK phosphorylation in a dose-dependent manner, which indicates inhibitory effects on different signaling pathways downstream of Ras. A 3 h treatment of N-Ras^{Q61K}-mutant H1299 cells with compound **40** at $10\ \mu M$ resulted in a 2.3-fold increase of caspase-3 activity as well as increased apoptosis, as observed by flow cytometry analysis and induced cell rounding and reduced cell size.

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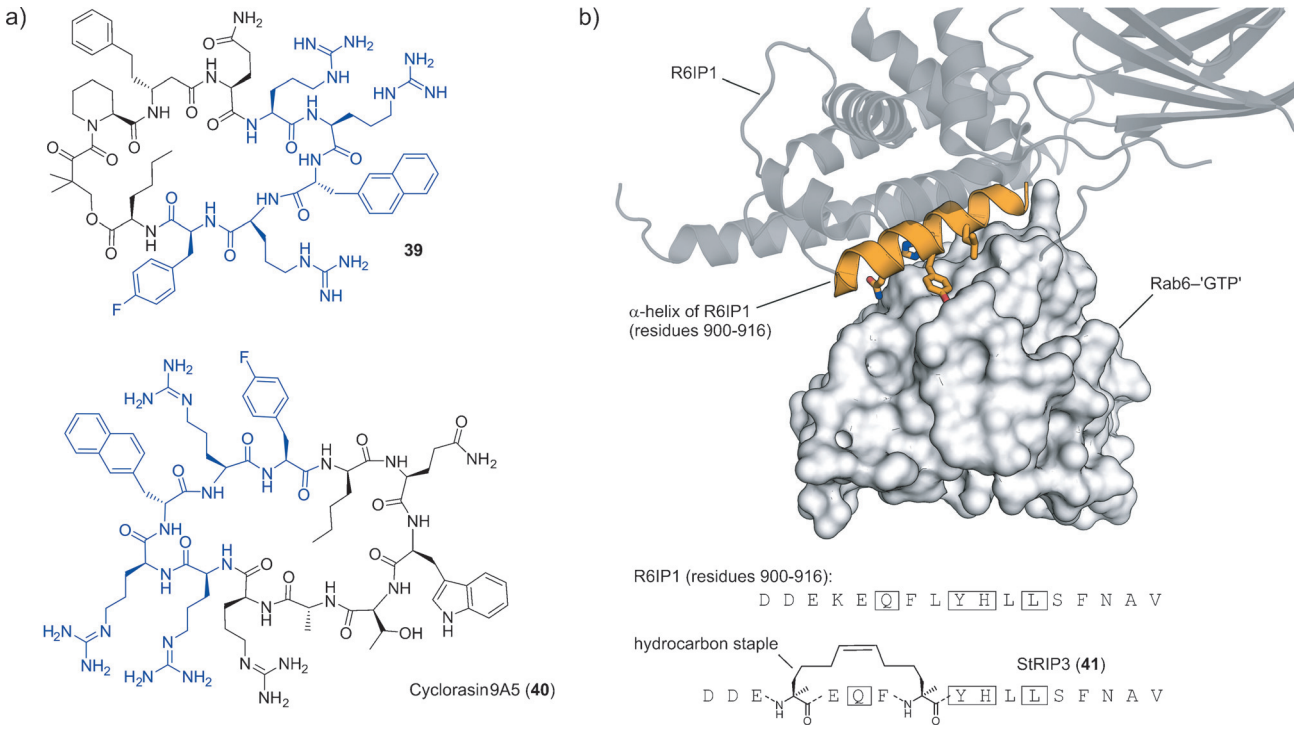

Figure 10. Peptide-based orthosteric inhibitors of GTPase–effector interactions: a) Chemical structure of macrocyclic orthosteric Ras inhibitors. Potential cell-penetrating motifs are highlighted in blue. b) Crystal structure of Rab6–"GTP" (gray) and its effector Rab6-interacting protein 1 (R6IP1, dark gray; PDB: 3CWZ). The α-helix used for hydrocarbon peptide stapling is marked in orange and the interacting residues are shown explicitly (top). Sequences of the hydrocarbon stapled peptide **41** and the wild-type sequence with interacting residues highlighted in boxes (bottom).

5.1.3. ***Peptide-Based Rab Inhibitors***

In an effort to generate inhibitors of Rab–PPIs, hydrocarbon-stapled peptides[135] were designed based on α-helical binding motifs of natural Rab interaction partners.[136] Peptide stapling[84] can be used to stabilize the α-helical conformation of a given peptide by introduction of an all-hydrocarbon macrocyclic bridge to interconnect two turns of a helix. In a first step, complexes of Rab proteins with various interaction partners were analyzed with respect to their interaction surface to identify suitable α-helical binding domains. The top-rated peptide fragments were tested with a representative set of six different Rab proteins in all nucleotide binding states to yield four peptides which bind nucleotide-free Rab proteins in the low micromolar range. Incorporation of hydrocarbon staples into these peptide sequences produced a library of stapled peptides which bind a representative set of Rab proteins in the nucleotide-free form with varying selectivity and affinity down to the submicromolar range. Notably, the stapled peptide StRIP3 (**41**) selectively binds to Rab8a in its activated, GTP-bound form with $K_d = 22\ \mu M$ and inhibits the interaction of Rab8a and its effector OCRL1 in vitro (Figure 10b). Despite moderate potency, these results validate the applicability of stapled peptides for targeting GTP-bound small GTPases and raise the question whether other strategies for the stabilization of interaction domains such as different helix stabilizers or β-sheet mimetics might be suitable for the modulation of Ras[137] and other constitutively active GTPases.

5.2. Allosteric Inhibitors

A different approach to inhibit the formation of Ras–effector complexes takes advantage of the dynamic structural properties of GTP-bound Ras proteins. ^{31}P NMR spectroscopy revealed that GTP-bound Ras exists in a dynamic equilibrium of two conformational states which are almost equally populated in the wild-type Ras protein and interconvert on the millisecond time scale.[138] The conformational state 2 corresponds closely to the conformation found in Ras–effector complexes and displays high affinity for effector binding, whereas state 1 represents a conformation with strongly reduced affinities to effectors by more than two orders of magnitude.[38,139] State 1 is generated upon GTP-binding during the nucleotide exchange reaction, but transition to state 2 is required to render Ras functional.[140] In conformational state 1, Ras reveals unique surface pockets, which can be exploited for allosteric stabilization to shift the equilibrium towards the low affinity conformation and impair downstream signaling. Both Zn^{2+}– and Cu^{2+}–cyclen complexes bind H-Ras–"GTP" in its state 1 conformation with millimolar affinity (Figure 11).[141,142] ^{31}P NMR shift analysis suggests two distinct binding sites, one with the metal ion coordinating the γ-phosphate of the bound nucleotide, and the other located close to the C-terminus and loop 7. However, binding site 2 seems to have a low impact on the stabilization of the state 1 conformation. Mono- and dinuclear Zn^{2+}–cyclen peptide conjugates were synthesized to enhance the affinity of the complexes, but resulted in Ras aggrega-

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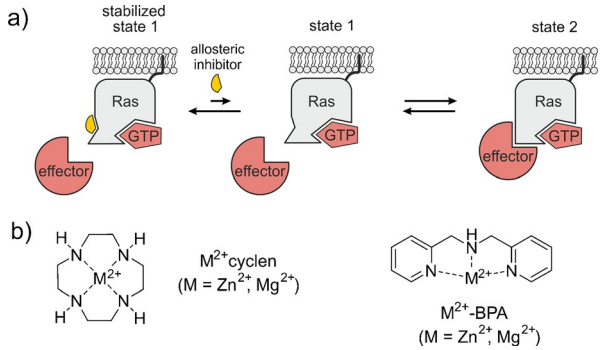

Figure 11. Allosteric inhibitors of Ras–effector interactions: a) The switch I region of GTP-bound Ras proteins occupies two distinct conformational states, of which only state 2 allows effector binding. State 1 may be stabilized by binding of molecules to an allosteric site to prevent effector binding. b) Chemical structures of allosteric inhibitors of Ras–effector interactions.

tion.[143] In a similar study, bis(2-picolyl)amine (BPA) complexes of Zn^{2+} and Cu^{2+} bind Ras in millimolar concentrations at two distinct binding sites (Figure 11).[144] Site 2 is located close to the C-terminus and does not stabilize state 1. In contrast, binding site 1 is located at the active center for the M^{2+}–BPAs, no longer in proximity to the nucleotide binding pocket. Interestingly, BPA complexes are at least five times more potent than the cyclen complexes in interfering with oncogenic mutated Ras^{G12V}. So far, these organometallic complexes provide only a proof-of-concept for the design of allosteric Ras signaling inhibitors because of their low affinities and poor druglike properties. Nevertheless, the design concept of allosteric conformational stabilizers could in principle be expanded to other small GTPases, as these conformational states vary in effector binding affinity and are also present in other GTPases, each with a defined state 1/state 2 population ratio.[140]

6. Stabilization of GTPase–Protein Complexes

An alternative approach to modulate GTPase activity involves the stabilization of complexes of small GTPases and their interaction partners. Molecules that bind at the interface of such complexes can potentially trap distinct functional or nonproductive conformations. Stabilizers of PPIs are to date underrepresented in chemical biology and drug discovery, although several highly potent examples, such as Cyclosporin A, FK506, or Rapamycin, underscore the potential of this strategy.[145,146] The natural product Brefeldin A (BFA, **42**) is a well-known stabilizer of a GTPase–PPI (Figure 12a), but this class of compounds has only recently been reconsidered for other families of small GTPases. In principle, this approach may help to circumvent some of the major challenges in the direct targeting of small GTPases. In the stabilization of a protein–protein complex, large macromolecular interaction partners do not have to be mimicked by small chemical entities, and the formed small GTPase–protein complex might provide novel tractable cavities not present on the particular small GTPase.

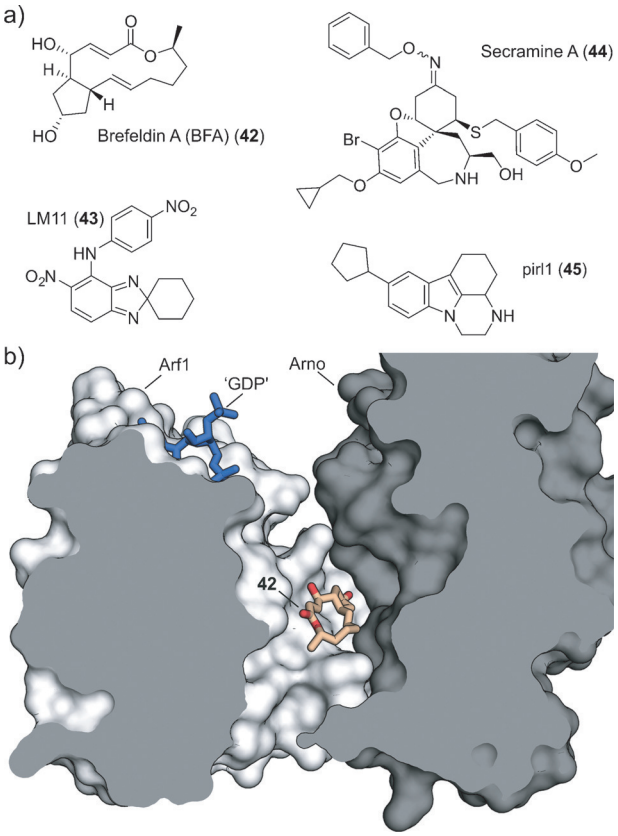

Figure 12. Stabilization of GTPase–protein interactions: a) Chemical structures of different stabiliziers of GTPase–protein interactions. b) Cutaway view of the crystal structure (PDB: 1R8Q) of **42** (orange) bound to the interface of a complex of Arf1-'GDP' (gray) and the catalytic Sec7 domain of ARNO (dark gray). Compound **42** occupies an interfacial cavity to stabilize an early stage conformation of the nucleotide exchange reaction.

6.1. Arf–ARNO

The prototypical stabilizer of GTPase–protein interactions is the fungal metabolite **42**, which inhibits Arf-regulated cellular trafficking and membrane transformation.[147] Compound **42** is a 13-membered lactone ring that binds to a hydrophobic cavity at the interface of the complex of Arf–GDP and the catalytic Sec7 domain of its cognate GEFs, such as ARNO (Figure 12b).[148] Only the ternary complex is targeted and no binding to isolated Arf, the Sec7 domain, or the nucleotide-free complex is observed.[149] By stabilizing an early transient intermediate of the nucleotide exchange reaction, **42** acts as an uncompetitive inhibitor. It traps Arf in an abortive complex which cannot proceed to the subsequent nucleotide dissociation step, thereby blocking Arf activation and downstream signaling.[150]

Inspired by **42**, an interfacial inhibitor targeting a flexible pocket near the Arf1–GDP–$ARNO^{E156K}$ interface and remote from the BFA binding site was identified in a virtual screen of the Chembridge database.[151] 17 hit compounds were tested for inhibition of Arf1 and the Sec7 domain of ARNO, which carried four BFA-sensitizing mutations ($ARNO^{4M}$). The most potent compound LM11 (**43**) reduces the apparent exchange rate constant by a factor of 3.6 (Figure 12a). Unlike **42**, **43** acts

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by a noncompetitive mechanism. It binds to both the Arf1–GDP and the Arf1–GDP–ARNO complex and it inhibits the spontaneous as well as the ARNO-mediated nucleotide-exchange reaction. In this context, **43** induces formation of an abortive complex that is distinct from the **42**-bound conformation. Despite its moderate inhibitory potency, **43** displays cellular activity and blocks ARNO-mediated migration of MDCK cells in a dose-dependent fashion.[151]

6.2. Cdc42–RhoGDI

The natural product-like small molecule Secramine A (**44**) is also believed to be a PPI stabilizer (Figure 12a). The compound was identified in a phenotypic screen from a library of approximately 2500 galanthamine-inspired small molecules[152] as a potent inhibitor of transport from the Golgi apparatus to the plasma membrane and actin polymerization.[153] Compound **44** is a Cdc42-specific inhibitor that prevents Cdc42–effector interactions, without affecting nucleotide exchange or binding. It interferes with Cdc42 shuttling and membrane association by a mechanism dependent on the cognate guanine dissociation inhibitor RhoGDI. Although definitive structural data are still missing, **44** is believed to stabilize the Cdc42–RhoGDI interaction to trap the small GTPase in an inactive complex and decrease its availability for downstream signaling.[153] Compound **44** has shown different cellular activities, such as disruption of Golgi polarization during cell migration after wounding,[153] inhibition of cell spreading,[154] and blocking of the secretion of collagen I from vascular smooth muscle cells.[155] The structurally unrelated small molecule pirl1 (**45**) and some of its derivatives may also inhibit Cdc42 downstream signaling by stabilization of the inactive Cdc42–RhoGDI complex (Figure 12a).[156] Although some experiments suggest that the compounds mode of action involves stabilization of the Cdc42–RhoGDI complex, additional data are still required to support this theory.

6.3. Ras–SOS

Interfacial pockets in the complex of Ras and its GEF SOS have recently been reported through the discovery of small molecules that bind the Ras–SOS complex and enhance SOS-mediated nucleotide exchange.[93] Although this mode of action is not necessarily beneficial for oncology applications, it still indicates the potential for targeting of the Ras–SOS complex and the compounds might serve as a starting point for the design of PPI stabilizers.

In the pursuit to develop small-molecule stabilizers for the Ras–SOS complex, an X-ray fragment screening of a 1160 member library was conducted by soaking these fragments into protein crystals of H-Ras in complex with the catalytic domain of SOS (SOS_{cat}).[157] Binding fragments were confirmed by TROSY-HSQC NMR titrations with the stable isotope-labeled H-Ras–SOS complex and displayed dissociation constants in the low millimolar range. A pocket identified on SOS matches a binding site previously described

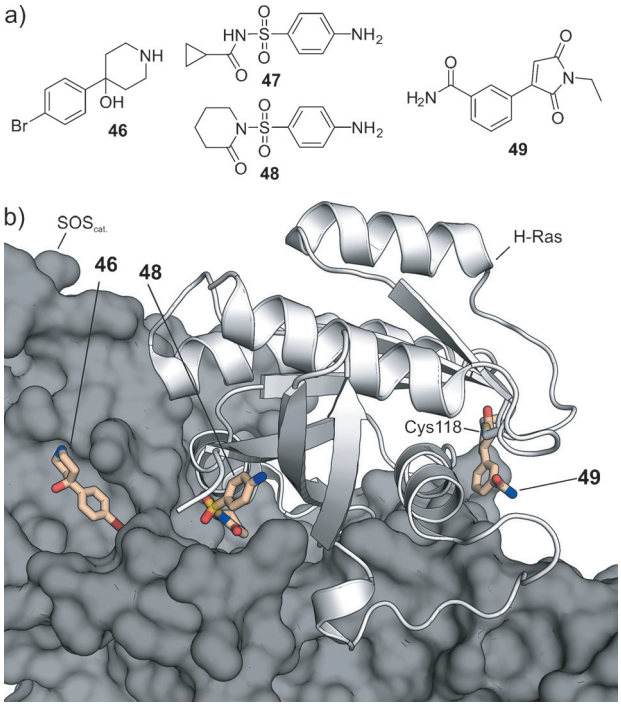

Figure 13. Small molecules targeting the Ras–SOS complex: a) Chemical structures of small-molecule fragments. b) Aligned crystal structures of fragments (orange) binding to different binding sites of the H-Ras–SOS complex. Compound **46** binds to a remote pocket (site A) on SOS (dark gray; PDB: 4URV), while **47** and **48** target an interfacial cavity (site B) of the H-Ras–SOS complex (PDB: 4URZ). SOS binding results in reorganization of the Ras (gray) nucleotide binding pocket and exposes the Cys118 residue, which allows covalent binding of **49** (site C; PDB: 4US2).

for **19**, and some fragments such as compound **46** show corresponding binding modes (Figure 13a,b).[93] The binding of one or two fragments to this relatively flexible cavity occurs with limited movement of protein side chains compared to the H-Ras–SOS_{cat} structure. In contrast to the earlier study, the fragments do not show any increase in the rate of SOS-mediated nucleotide exchange.[157] Another fragment binding site comprises a largely solvent-inaccessible pocket located at the interface of H-Ras and $SOS_{cat.}$ which corresponds to a site described in studies of uncomplexed Ras proteins.[90,91] Although small-molecule binding to this pocket prevented SOS binding and SOS-mediated nucleotide exchange in the previous studies, binding of the smaller fragment **47** to the H-Ras–$SOS_{cat.}$ interface does not lead to steric hindrance and interference with SOS binding (Figure 13a,b). For the synthesis of PPI stabilizers, variation of the sulfonamide substituents of **47** resulted in fragment **48**. Notably, the binding of **48** expands the binding pocket at the H-Ras–$SOS_{cat.}$ interface. However, several rounds of structure optimization did not result in compounds that could significantly stabilize the protein complex or show functional activity.[157] Therefore, a set of covalent inhibitors was designed, targeting Cys118 of H-Ras in proximity to the GDP binding site, which becomes solvent accessible in the Ras–SOS complex as a result of reorganization of the nucleotide-binding pocket upon SOS binding. A library of 400 fragments with potentially cysteine-

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reactive functional groups was screened by mass spectrometry for covalent binding. Among the identified hits, an *N*-ethylmaleimide fragment showed favorable reactivity and selectivity for Cys 118 over other cysteine and histidine side chains. Subsequent growing of the fragment into an adjacent lipophilic groove as well as probing of interactions with Glu 942 of the SOS yielded compound **49**, which covalently attaches to Cys 118, as shown by X-ray crystallography (Figure 13 a,b).[157] Preincubation of the compounds with the K-Ras–GDP–SOS complex results in them preventing K-Ras activation in vitro, whereas preincubation with K-Ras–GDP or SOS results in only modest inhibition. Moreover, **49** shows functional activity with both wild-type K-Ras and the oncology-relevant, mutated forms K-Ras^{G12V} and K-Ras^{G12C}. However, the inhibitory effects of the fragments are abolished in the presence of 1 mM DTT, thus indicating the need for compounds of higher affinity, but lower electrophilic reactivity.

7. Conclusions

Small GTPases are regulators of many pivotal cellular functions and processes. Dysregulation of these cellular switches as a result of direct mutations, over-activation, or loss of function is associated with a variety of human diseases. For many diseases, the accumulation of activated (GTP-bound) GTPases has been identified as a pathogenic driver and extensive efforts have not yet yielded any drug candidates. Ras targeting was solely achieved indirectly by using farnesyl transferase inhibitors (FTIs), which proved effective in preclinical trials and animal models. However, FTIs ultimately failed in clinical trials because of a lack of efficacy in humans. The high expectations for FTIs led to stagnation in Ras-focused research as the possible impact of FTIs could have rendered additional Ras inhibitors redundant. After FTIs failed in clinical trials, Ras-focused research was revived. These efforts led, for example, to the inhibition of the GDI-like solubilizing factor PDEδ, which sustains Ras intermembrane transport.[158] This approach might allow a more-selective modulation of the spatial and temporal localization of Ras. Meanwhile, a better understanding of small GTPases of the Ras superfamily in general has identified more small GTPases as interesting targets for drug development. In combination with advanced targeting strategies, this resulted in the various novel modulators of small GTPase activity summarized and discussed in this Review. Most of the highlighted work was conducted within the last decade and the increasing number of publications on small GTPase targeting in the last few years underlines the reawakened interest in the field. The recently pursued development of direct small GTPase modulators can be subdivided into five major categories (Sections 7.1–7.5; see also Figure 2).

7.1. Interference with Nucleotide Binding

Thus far, the nucleotide binding pocket of small GTPases resembles the only identified cavity suitable for high-affinity binding of traditional small-molecule entities. In comparison to kinase inhibitors that target the ATP-binding site,[47,159] competition with the binding of GDP or GTP to small GTPases is much more challenging. Kinases typically show affinities for ATP in the micromolar range[47] and their nucleotide turnover is magnitudes of order higher than the turnover in small GTPases. Small GTPases, on the other hand, exhibit picomolar binding affinities toward GDP and GTP and show very slow off-rates, which render the development of effective nucleotide-competitive inhibitors a highly challenging endeavor.[45] Most of these nucleotide-competitive inhibitors were identified in high-throughput screens at nonphysiological nucleotide concentrations, which limits the validity and use of such hits for the development of GTPase inhibitors. The two Rho family inhibitors **2** and **3** which trigger nucleotide release and form an inactive inhibitor–GTPase complex after binding to the nucleotide-loaded GTPase act through an alternative mode of action. This approach needs further investigation, but high nucleotide concentrations will probably affect the postulated mechanism (Figure 3) as they will shift the equilibrium towards the nucleotide-bound states. However, the introduction of an electrophilic reactive group which covalently attaches to the small GTPase upon binding might turn out to be beneficial for this mode of action. Covalent attachment, as observed for **4**, could help to overcome the drawbacks associated with compounds interfering with nucleotide binding.

7.2. Inactivation of Small GTPases by Irreversible Covalent Modification

Bacterial toxins such as the C3-exoenzyme modulate the activity of small GTPases of the Ras superfamily by covalent post-translational modification such as APD ribosylation.[61,62] The effective silencing of distinct signaling pathways of small GTPases allows escape of the bacteria from the host's immune system. The effects verify that small GTPases can be interesting targets for drug discovery and further studies will reveal if cell-permeable chimera of these exoenzymes can be beneficial for the treatment of human diseases.

Notably, selective targeting of mutated Ras^{G12C} has been achieved for the first time by means of small-molecule covalent inhibitors.[59,60] The inhibitors exploit the nucleophilic thiol of Cys 12, which is not present in the wild-type protein, for irreversible attachment. As the oncogenic mutations of the Ras subfamily affect single amino acids, discrimination between mutated and wild-type Ras GTPases is difficult to achieve. Using the oncogenic mutation itself is an elegant approach to simultaneously differentiate and inactivate the carcinogenic Ras^{G12C} mutation. However, the currently available compounds can only be considered as proof of principle and require further optimization. In particular, the nucleotide-competitive SML compounds are not druglike and compete with nucleotide binding which raises the question if nucleotide exchange rates are sufficient to provide the required inhibitory activity. Compounds **6** and **7** are of special interest, as they do not compete with nucleotide binding and occupy the previously unknown binding pocket

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S-IIP.[60] These results might promote the development of further covalent inhibitors for small GTPases. However, the low accessibility/absence of suitable nucleophilic residues as anchor points for electrophilic groups on the surface of small GTPases hampers the broad implementation of this approach. The application of covalent inhibitors additionally raises the question of toxicity and off-target effects, but similar to small GTPase targeting, the field of covalent drugs has experienced a renaissance as safety issues are becoming better understood.[57,58] A careful optimization of the reactivity and binding of the covalent inhibitors leads to improved pharmacokinetic profiles and minimizes side effects.

7.3. Inhibition of GTPase–GEF Interactions

Interfering with small GTPase–GEF interactions to reduce levels of activated GTPases resembles the most common approach to target members of the Ras superfamily. Several inhibitors for the different subfamilies have been identified, but the question whether the GTPase–GEF interaction is a valid target for the treatment of cancer is controversial.[27,29,31,45] Since most direct mutations of small GTPases impair their ability to hydrolyze GTP even in the presence of GAPs, the non-GEF-mediated intrinsic nucleotide exchange might be sufficient for activation. However, the blocking of GTP loading through the inhibition of GTPase–GEF interactions can be considered a promising approach if hyperactivation of the GTPase results from increased GEF activity arising from upstream mutations or mutation in the GEF itself—provided that the GTPase is still capable of conducting intrinsic or GAP-mediated GTP hydrolysis. This holds true for the Rho subfamily and Ral GTPases, as they are rarely mutated.[22,95] Ras GTPases are mutated in 20–30% of all human cancers, which renders them less vulnerable to inhibition of the Ras–GEF interaction. In the case of the Rho subfamily GTPases, in particular, inhibition of the GTPase–GEF interaction could provide a valuable strategy to identify druglike inhibitors with preclinical perspectives. The identified binding areas around the essential GEF-recognizing residues Trp58, Trp56, and Phe56 for Rho, Rac, and Cdc42, respectively, resemble a good starting point. Further studies will have to prove if these cavities can be filled with sufficient chemical matter for high affinity and selective targeting. The Rho subfamily GTPases are activated by more than 75 different GEFs, and potential inhibitors will have to compete with numerous native binding partners. The Ral inhibitor **23** is the first of its kind, and fuels the hope for a better understanding of this Ras downstream pathway.

7.4. Inhibition of GTPase–Effector Interactions

High-affinity binders for activated (GTP-bound) small GTPases which block the interaction with effector proteins would be the most universal approach for targeting the Ras superfamily of small GTPases. Irrespective of the origin of GTPase over-activation (direct mutation, upstream mutation, or GAP loss of function), blocking interactions between small GTPases and effectors could impair signal propagation. However, a competition with effector proteins which show affinities in the medium to low nanomolar range, and the absence of well-defined surface pockets for compound binding, is an incredibly challenging task. Thus far, small-molecule targeting of small GTPase–effector interactions has only met with limited success. Identified small molecules of the Kobe family are a promising starting point, but further studies have to prove whether the identified pocket on Ras can be expanded.[129] In this context, initial success has been achieved by screening libraries of mono- and bicyclic peptides which exhibit sub-micromolar affinity and interfere with the Ras–Raf interaction in vitro (Figure 10).[131,132,134] The first generation of these inhibitors suffered from inefficient cell penetration, but permeability was improved in a very recent second generation of cyclorasins.[134] Their performance in in vivo studies needs to be investigated for assessment of the full potential of these promising inhibitors of Ras–effector interactions. As a consequence of their larger interaction surfaces, macrocycles and stabilized structured peptides are highly interesting compound classes for PPI inhibition,[160] and might provide sufficient binding affinity and selectivity for small GTPase targeting. The identification of the stapled peptide StRIP3 (**41**) as an inhibitor of Rab8–effector interactions[136] also indicates that such modified macromolecules can act as effective modulators of activated small GTPases. Further studies will have to prove if the presented examples are only exceptions or if these compound classes bear the potential for universal targeting of small GTPases.

The concept of allosteric inhibitors which stabilize the small GTPase in a GTP-bound state with a significantly reduced affinity for effector binding represents an intriguing strategy. In its inactive state 1, Ras displays additional transient surface pockets which allow specific targeting of this conformation. Nevertheless, the metal complexes identified as state 1 stabilizers lack binding affinity and are not druglike.[142,144] Alternative chemotypes will be needed to assess the full potential of this approach.

7.5. Stabilization of GTPase–Protein Complexes

The opposite approach to inhibiting small GTPase PPIs, that is, a stabilization of these PPIs, represents another appealing strategy to impair small GTPase activity. The small GTPase cannot function properly when trapped in a protein complex, which can lead to reduced overall activity. The benefit of stabilizing a PPI is based on the fact that the compounds do not have to compete with large high-affinity binding partners of the small GTPases.[146,161] PPI stabilizers target the complex upon its formation, which allows the targeting of potential novel binding pockets that can arise at the interface of the PPI. Despite highly effective PPI stabilizers such as Cyclosporin A or Rapamycin, the field of PPI stabilizers is still in its infancy.[146,161] However, the potential of this class of molecules for small GTPases is exemplified by the natural product Brefeldin A (**42**) which inhibits the activation of Arf by stabilizing the interaction with its GEF domain Sec7.[148] Besides its moderate in vitro

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affinity, **42** provides considerable cellular activity as it acts through a noncompetitive mode of action by trapping Arf in an inactivated complex with its GEFs. Exemplified by the recent study of AstraZeneca that targeted the Ras–SOS interaction,[157] PPI stabilization resembles a highly interesting approach for targeting small GTPases.

7.6. Concluding Remarks

The direct targeting of small GTPases in general, and Ras targeting in particular, represents a very demanding and challenging problem involving sophisticated and complex feedback mechanisms and regulations which are still not fully understood.[27] A deeper understanding of these processes is required to target Ras and other small GTPases in a disease-relevant manner. Additionally, a more thorough discrimination between the various forms of over-activated Ras tumors can allow the better development of compounds. Although GEF inhibition is the most common approach for targeting small GTPases, it might only turn out be beneficial for a subgroup of tumors which do not show direct mutations within the small GTPase itself. Small-molecule covalent inhibitors bear the potential to effectively discriminate between wild-type and mutated GTPases. This approach is the first to target mutated Ras (Ras^{G12C}) selectively without any effect on the wild-type form and might be advanced into clinical candidates. Mutant-selective inhibitors for EGFR have already reached clinical trials,[162] and even if this does not solve the problem entirely it may be beneficial for certain subpopulations.

The targeting of Ras and other small GTPases will most likely be a long-term process, and novel, alternative, and maybe unorthodox strategies which might or might not turn out to be suitable targeting approaches are in high demand. This category includes screening for Ras-destabilizing molecules which reduce functional Ras protein levels in cells,[163] or engineering a E3 ubiquitin ligase, which targets K-Ras for ubiquitination and degradation.[164] These ideas might not solve the problem entirely, but they can lead the way to new technologies and fresh ideas.

Following the setback from strategies targeting lipidation and post-translational modification of small GTPases such as the FTIs, the field is experiencing a renaissance with the application of various strategies for PPI inhibition, such as structure-guided and fragment-based approaches, stabilized peptide secondary structures, or macrocycles. Various approaches already launched in the 1990s and abandoned in light of the FTI trials might be worthy of reinvestigation. Today's corporate compound libraries are very different from the libraries in the early 1990s, and rescreening for inhibitors or stabilizers of small GTPase PPIs might turn out to be useful. In particular, PPI stabilization can be useful for targeting "undruggable" proteins such as small GTPases, since protein complexes might offer additional pockets for compound binding. Furthermore, inactivation of the target protein occurs in a noncompetitive manner, which means that the inhibitor does not have to compete with high affinity and optimized binding partners. Screening for PPI stabilizers is an underexplored approach for PPI modulation. Inspired by the success of the identified PPI stabilizers thus far, one might recapitulate such efforts.

In general, the GTPase field and, in particular, Ras-focused research has expanded greatly within the last decade and numerous novel insights have been gained that encourage fresh approaches. As this protein class has remained recalcitrant to small-molecule modulation, a single agent is probably unlikely to solve the problem. Encouraged by the recent developments in targeting small GTPases of the Ras superfamily, small GTPase-driven diseases might finally succumb step by step to the various targeting approaches. This diverse and complex field comprises many challenges with different levels of difficulty and even if they can not all be solved, we can be optimistic that the current strategies have the potential to generate promising results and reach beyond the seemingly impenetrable surface of this arduous endeavor.

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