RhoGEFs as Therapeutic Targets

Anne Blangy

Chapter N

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1.1 Introduction.

1.1.1 *The relevance Rho GTPase exchange factors as therapeutic targets.*

Rho GTPase signaling pathways are major regulators of eukaryotic cell dynamics, which control normal and pathological processes (Cook et al., 2014). They participate in cell migration, morphology, polarity and differentiation during embryonic development(Duquette and Lamarche-Vane, 2014; Fort and Théveneau, 2014). They are also involved in the pathological mechanisms of a variety of diseases, including hypertension(Shimokawa et al., 2016), cancer(Lin and Zheng, 2015), and neurodegenerative diseases(Stankiewicz and Linseman, 2014).

Mammals have 20 Rho GTPases(Boureux et al., 2007) and 82 Rho GTPase exchange factors (RhoGEFs) that distribute between two families: the Dbl-related and the Dock-related RhoGEFs. The Dbl family counts 71 members (Jaiswal et al., 2013)(Cook et al., 2014) and there are 11 proteins in the Dock family(Gadea and Blangy, 2014). They activate RhoGTPase via their catalytic domain called the DH domain for the Dbl family or the DHR2 domain for the Dock family. The RhoGEFs are multidomain proteins: their catalytic domain is accompanied by various functional domains that can mediate the association of the GEF with membrane receptors or lipids for instance, or that provide the GEF with other enzymatic activities, such as kinase, phosphatase, or even GEF or GAP function towards other Ras-like GTPases.

Rho GTPase signaling pathways can be targeted at various levels: the GTPases themselves but also GEFs, GAPs and downstream effectors.

Drugs have been developed against downstream effectors of RhoGTPAses, such as Y-27632, which inhibits the kinase Rock(Uehata et al., 1997) or IPA3 that targets the Pak kinases(Deacon et al., 2008). Rock inhibitor Fasudil is used in the clinics to modulate pulmonary hypertension and Ripasudil for the treatment of glaucoma(Defert and Boland, 2017). Several inhibitors of Rho GTPases exist naturally in bacteria, such as Clostridium botulinum C3 exoenzyme that targets RhoA, RhoB and RhoC(Sekine et al., 1989). Others were developed for scientific applications, such as Rac inhibitors EHT1864, NSC23766 and its derivative EHop-016 (Gao et al., 2004; Montalvo-Ortiz et al., 2012; Shutes et al., 2007) and Cdc42 inhibitor ML141(Surviladze et al., 2010) and RhoA inhibitor Rhosin (Shang et al., 2012). But the ubiquitous expression of most Rho GTPases and their implication in fundamental cellular processes do not make Rho GTPase inhibitors suitable for therapeutic applications. In fact, the knock down of many Rho GTPases is deleterious, for instance RhoA-null(Pedersen and Brakebusch, 2012), Rac1-null(Sugihara et al., 1998) and Cdc42-null(Chen et al., 2000) mice die early during embryonic development.

RhoGEFs activate their target Rho GTPases in response to different signals, usually transmitted from the extracellular medium by membrane receptors. This results in local and temporal regulation of the activation of Rho GTPases. The majority of RhoGEFs have a restricted tissue and/or subcellular distribution and they are specific for one Rho GTPase (Cook et al., 2014; Gadea and Blangy, 2014). In pathological contxt, a number of RhoGEFs were found overexpressed, including Dbl, Vav1/2/3, Ect2, Tiam1/2, P-Rex1/2 in cancer, or bearing activating mutations, such as LARG, BCR (Lin and Zheng, 2015) in cancer, Vav1 in multiple sclerosis (Jagodic et al., 2009), Dock2 in Alzheimer's disease (Cimino et al., 2013) and Dock3 in muscular dystrophy (Alexander et al., 2014). Conversely, there are only sporadic examples of mutation or overexpression described for RhoGTPase, unlike Ras GTPases that are often found mutated in cancers. Thus, RhoGEFs are attractive targets to optimize efficacy and specificity of Rho GTPase signaling inhibition. Several RhoGEFs already qualify as relevant therapeutic targets and different types of inhibitors have been developed through a variety of strategies that are described in this chapter (Table 1).

Table 1: Inhibitors of RhoGTPase exchange factors

Target GEF	Inhibitor	Validation	Pathologies	References
		Peptide inhibitor		
Trio (D2), Tgat,	TRIPα, $TRIP^{E32G}$	Cell-free assay, Cellular expression	T-Cell Leukemia	(Bouquier et al., 2009a; Schmidt et al., 2002)
Dock2	DCpep-4-NH2	Cell-free assay, Cell culture	Immune disorders, Alzheimer's disease	(Sakamoto et al., 2017)
		RNA inhibitor		
Tiam1	K91	Cell-free assay	Various cancers	(Niebel et al., 2013)
		Chemical inhibitor		
Trio (D1)	ITX3	Cell-free assay, Cell culture	Glioblastoma, Breast cancer	(Blangy et al., 2006; Bouquier et al., 2009b)
Dock5	C21 [(a)]	Cell-free assay, Cell culture, Mouse models of pathologies	Osteolytic diseases, osteoporosis, bone metastases, inflammatory diseases	(Vives et al., 2011, 2015)
Dock2	CPYPP	Cell-free assay, Cell culture, Mouse (T-Cell homing)	Immune related disorders, Alzheimer's disease	(Nishikimi et al., 2012)
LARG	Y16	Cell-free assay, Cell culture	Accute myeloid Leykemia	(Shang et al., 2013)
Lbc	A13	Cell-free assay, Cell culture	Various cancers	(Diviani et al., 2016)

[(a)] The inhibitor of Dock5 (CAS 54129-15-6) should not be mistaken for the inhibitor of the protein arginine methyltransferase PRMT1 (CAS 1229236-78) and for the non-peptide selective AT2 receptor agonist M24 (CAS 477775-14-7), which were also named C21.

1.1.2 *Targeting RhoGEF activity.*

The activation of a GTPase by an exchange factor is a complex enzymatic reaction. The precise molecular mechanism driving the catalysis of nucleotide release by the GTPase is different between Dbl- (Rossman et al., 2002) and Dock-related RhoGEFs (Yang et al., 2009). But overall, the sequence of interactions between the GTPase and the

GEF is similar. The first step is the formation of a complex between the GEF and the inactive GDP-bound GTPase. In this low affinity complex, the exchange factor provokes conformational modifications in the GTPase causing the release of the guanine nucleotide. This results in a stable complex between the nucleotide-free GTPase and the GEF. GTP destabilizes this complex and then binds to the empty nucleotide pocket of the GTPase, provoking the release of the active GTP-bound GTPase from the GEF.

To inhibit the exchange reaction, the strategy is to target the catalytic DH or DHR2 domain of the GEF. Aiming more specifically at the interface between RhoGEF and the GTPase during the nucleotide exchange reaction can render the inhibition even more specific. This approach is made feasible with the increasing number of RhoGTPase-GEF complexes available in databases (http://www.rcsb.org/pdb). The approach used so far to obtain RhoGEF inhibitors has been to prevent the interaction between the RhoGEF and the GTPase. This is the strategy used in Nature by enteric bacterial pathogens that produce type III effector EspH, a small 20 kDa protein that interact with various GEFs for RhoA, preventing their binding to the RhoGTPase (Dong et al., 2010). Another solution, which could be considered for the future design of RhoGEF inhibitord, is to stabilize an intermediate step of the exchange reaction, thereby freezing the GEF-GTPase complex and compromising the activation of the GTPase. This is again a strategy efficiently developed in Nature by fungi; they produce the macrocyclic lactone Brefeldin A, inhibit the activation of ARF-family GTPases by locking the complex between the GTPase and the GEF (Peyroche et al., 1999).

1.2 Different Types of RhoGEF Inhibitors.

1.2.1 *Peptides and nucleic acids.*

1.2.1.1 *Peptidic inhibitors*

Historically, the first inhibitor of a RhoGEF is TRIPα (Trio Inhibitory Peptide α), which targets the exchange factor Trio. Trio is an unusual exchange factor in that is exhibits two exchange domains(Debant et al.,

1996): Trio-D1 can activate the GTPases Rac1(Debant et al., 1996) and RhoG(Blangy et al., 2000) and Trio-D2 activates the GTPase RhoA(Debant et al., 1996). The *Trio* gene is expressed as several splice variants including Tgat, an oncogenic form of Trio identified in Adult T-Cell Leukemia (ATL) patients. Tgat only has the Trio-D2 exchange domain, responsible for its oncogenic activity(Yoshizuka et al., 2004).

The strategy used to find inhibitors of Trio-D2 was to identify peptides able to bind the GEF using the yeast 2-hybrid system. The screening was performed on a library of $2x10^6$ plasmids expressing random 20-aminoacid peptides (peptide aptamers), which conformation is constrained by fusion to the bacterial protein Thioredoxin A(Colas et al., 1996). The expression of a peptide binding to Trio-D2 translates into the ability of yeast to grow in appropriate selective medium(Schmidt et al., 2002). As the screening is performed in a living organism, a toxic aptamers result eliminated, as it prevent yeast growth. This system allows easy testing of aptamer selectivity for the GEF of interest, by monitoring its ability to bind other GEFs. This way, TRIPα was selected as able to bind to Trio-D2 but not to other GEFs for RhoA, such as Dbl, p115-RhoGEF and PDZ-RhoGEF(Schmidt et al., 2002). Mutagenesis can be performed in the aptamer sequence to identify important residues and increase the interaction potential. Thereby, mutations were selected in TRIPα that increase its capacity of binding the GEF. Still, binding does not mean inhibition and the effect of the peptide on the exchange reaction must be tested. This can be done either in a cell-free exchange assay, where the GTPase, the GEF and the peptide aptamer are combined as purified proteins, or in a cellular system in which the activation of the GTPase in response to the expression of the GEF, with and without the aptamer, is monitored by pull-down assays that detect the active GTP-bound GTPase. In both systems, TRIPα proved an efficient inhibitor of Trio-D2(Blangy et al., 2006; Schmidt et al., 2002) and more potent inhibitors where derived by mutagenesis of TRIPα. When expressed in NIH-3T3 cell transformed with Tgat, TRIPα derivative $TRIP^{E32G}$ was able to diminish the activation of RhoA and Tgat oncogenic potential; $TRIP^{E32G}$ expression also reduced the growth of Tgat-transformed cells after their subcutaneous engraftment in nude mice (Bouquier et al., 2009a).

Very recently, a small peptide was developed to inhibit Dock2 (Sakamoto et al., 2017), an exchange factor for Rac GTPases. Dock2 controls lymphocyte activation and migration and it is considered an interesting target in the context of immune-related disorders (Gadea and Blangy, 2014) and also in Alzheimer's disease (Cimino et al., 2013). The identification of peptides able to interact with Dock2 was done by phage display. Random peptides displayed by the T7 phage were selected for their ability to bind to the DHR2 exchange domain of Dock2 immobilized on beads and then to be displaced by Rac1. Several rounds of amplification selected a phage expressing the 17-aminoacid peptide DCpep-4-NH2 (LNRCVAKYHGYPWCRRR). DCpep-4-NH2 inhibited the ability of Dock2 to bind and activate Rac1 in a cell free assay. Conversely, it did not affect the interaction of Rac1 with Dock1, a GEF closely related Dock2. DCpep-4-NH2, fused or not to a cell penetrating peptide, and added to the culture medium hindered sphingosine-1-phosphate-induced lymphocytic cell migration, a process known to rely on Dock2 (Sakamoto et al., 2017).

1.2.1.2 *RNA inhibitors.*

Small nucleic acid molecules (nucleic acid aptamers) are able to bind proteins with high affinity and specificity. Methods to develop such molecules as targets of various types of proteins using the SELEX (systematic evolution of ligands by exponential enrichment) have been expanding for the last three decades (Mallikaratchy, 2017). The first RNA aptamer targeting the activity of an exchange factor was M69, which is active towards Cytohesins, a family of GEF for ARF-type small GTPases (Mayer et al., 2001). Some twelve years later, the first RNA aptamer inhibitor of a RhoGEF was engineered (Niebel et al., 2013): K91 is a inhibitor of the Rac GEF Tiam1, T-lymphoma invasive and metastasis inducing protein 1, which involved in cancer (Boissier and Huynh-Do, 2014). The approach was to select for RNAs able to bind *in vitro* to the purified exchange domain of Tiam1 among a library of $4x10^{14}$ random 50-nucleotide aptamer RNAs constrained within a constant 40 nucleotide RNA sequence. Sixteen rounds of selection followed by PCR amplification led to the identification of 33 RNA

aptamers with high binding affinity for Tiam1 exchange domain (Niebel et al., 2013). Similar to peptide aptamers, the ability of the RNA aptamer to inhibit the exchange reaction must be confirmed as the selection procedure is only based on its ability to bind the target GEF. RNA aptamer K91 was found to inhibit the activation of Rac1 by Tiam1 *in vitro*, but its ability to inhibit Tiam1 in cellular systems remains to be confirmed (Niebel et al., 2013).

RNA and peptide aptamers proved efficient at inhibiting RhoGEF activity and they are easily amenable to optimization by mutagenesis. They can be expressed in cell to monitor their biological effects and test their toxicity. Still they suffer an important limitation regarding therapeutic usage. In fact, RhoGEFs are intracellular targets and the difficult challenge to vectorize RNAs and peptides into a cell within a living organism remains a big limitation to their utilization as therapeutic agents.

1.2.2 *Chemical compounds.*

Thus far, small chemical compounds represent the vast majority of therapeutic agents. Several molecules were developed to target RhoGEFs through various approaches (Table 1).

1.2.2.1 *Functional approach: the yeast exchange assay.*

The first inhibitor of a RhoGEF was designed to target Trio-D1 (Blangy et al., 2006), one of the two DH domain of Trio that is specific for Rac1(Debant et al., 1996) and RhoG(Blangy et al., 2000). This inhibitor was identified taking advantage of the yeast exchange assay, a method developed in live yeast to monitor the activation of a RhoGTPase by an exchange factor (De Toledo et al., 2000). In this reporter assay, the expression of Trio-D1 induced the activation of RhoG resulting in its binding to its effector kinectin. This was monitored by the expression of ß-galactosidase and by yeast becoming auxotrophic for histidine (Blangy et al., 2006). In this experimental set up, a chemical library of 2,640 compounds was screened for species able to inhibit yeast growth in histidine-deprived medium, as indicative of the inhibition of RhoG activation by Trio-D1. In parallel, the same library was screened in

growth medium supplemented with histidine, to eliminate any molecule that would inhibit yeast growth, indicative of cytotoxicity. Thereby, a series of compounds were identified as the first chemical inhibitors of a RhoGEF. Among these, three molecules displayed selectivity: they efficiently inhibited Trio-D1 but not Arhgef17, a GEF for RhoA, nor cytohesin-2, a GEF for the small GTPase Arf1 (Blangy et al., 2006). Toxicity assays on mammalian cells restricted usable TRIO-D1 inhibitors to compound ITX1 (Inhibitor of Trio eXchange 1: 2-(5-chloro-2-ethoxybenzylidiene) [1,3] thiazolo [3,2-a] benzimidazol-3(2H)-one) (Bouquier et al., 2009b). Analogs of ITX1 were tested and molecule ITX3 (CAS 347323-96-0) proved efficient at inhibiting Trio-D1 in cell free assays and also a variety of Trio-dependent cellular functions, ranging from myogenic differentiation (Bouquier et al., 2009b) to leukocyte transendothelial migration (van Rijssel et al., 2012) and endothelial barrier formation (Timmerman et al., 2015).

The Yeast Exchange Assay was also used to identify the first chemical inhibitor of a RhoGEF from the Dock family. C21 (N-(3,5-dichlorophenyl) benzenesulfonamide, CAS 54129-15-6) was characterized as an inhibitor of Dock5, an exchange factor for Rac (Vives et al., 2011). C21 can inhibit Dock5 in cell free assays, in culture cells and *in vivo* in the mouse (Vives et al., 2011, 2015) (see paragraph 1.3 of this chapter).

1.2.2.2 *Cell-free protein-protein interaction.*

Another strategy to find inhibitors of exchange factors is to look for molecules that can disrupt the interaction between the GEF and the GTPase. A library of 9,392 chemical compounds was screened for molecules able to prevent the binding of Rac1 to the immobilized DHR2 exchange domain of Dock2. This cell-free approach picked up CPYPP (CAS 310460-39-0), which inhibits the activation of Rac1 by Dock2 in cell free assays as well as in HEK293T cells. CPYPP also prevents lymphocyte migration and leukocyte activation in culture, two processes relying on Rac activation by Dock2. In the mouse, intra-peritoneal injection of CPYPP (250 mg/kg), just one hour before the adoptive transfer of spleen cells, was found to prevent T-cell homing to the lymph

node and to the spleen (Nishikimi et al., 2012). This was the first example of a RhoGEF inhibitor proven to be active in a whole organism.

1.2.2.3 *Rational design.*

Rather than performing a physically screen, the rational design is based on computational modeling. From the crystal or modeled structure of the target, virtual molecules are screened or designed according to their capacity to dock onto the target, which is likely to interfere with the pathway of interest. Rational design proved successful for the development of inhibitors of RhoGTPases including Rac1 (NSC23766 (Gao et al., 2004)) and RhoA (Rhosin (Shang et al., 2012)). Several crystal structures of GEF-RhoGTPase complexes were solved in the recent years, which allow following the same strategy to develop molecules that target the RhoGEF instead of the GTPase. In fact, the RhoGTPase-RhoGEF co-crystal unravels the exact binding regions between the two proteins and highlights the interactions between individual aminoacids of each protein that are necessary to form the complex and/or important for the nucleotide exchange reaction (Snyder et al., 2002; Yang et al., 2009). Thereby, it is possible to highlight small pockets in the RhoGEF where the binding of a small chemical compound is likely to interfere with the formation of the RhoGTPase-RhoGEF complex.

LARG, p115RhoGEF and PDZ-RhoGEF are GEFs for RhoA; they are RGS-RhoGEFs meaning that they are regulated by heterotrimeric G-proteins (Chikumi et al., 2004). These GEFs are relevant therapeutic targets in the context of various pathologies including cancer (Reuther et al., 2001), lung hypertension (Guilluy et al., 2010) and obesity (Chang et al., 2015). The crystal structure available for LARG exchange domain in complex with RhoA (PDB 1X86) was used to identify interaction sites between the GEF and the GTPase. This highlighted a concave region in LARG, between residues Asn975 and Arg986, into which RhoA sends a protrusion. The structure of this small domain was used for the screening *in silico* of 4 million compounds in the ZINC library of virtual compounds, to identify molecules able to dock into the groove. The 49 best hits were synthetized and further validated for their capacity to

affect the interaction between RhoA and LARG. This rational drug design strategy identified Y16 (CAS 429653-73-6) as able to prevent the interaction between LARG and RhoA (Shang et al., 2013). Y16 possesses a good selectivity as it affects the binding of RhoA to LARG, p115RhoGEF and PDZ-RhoGEF but not to the closely related RhoA GEFs Dbl and Lbc. Y16 has no effect on the binding of Rac1 to Tiam1 and of Cdc42 to its GEF Intersectin-1. In culture cells, Y16 blocks stress fiber formation in response to lysophosphatidic acid, a process driven by RhoA activation downstream of heterotrimeric G-proteins. Interestingly, Y16 and the RhoA inhibitor Rhosin display a synergistic effect to block the proliferation and invasion of breast cancer cells (Shang et al., 2013).

Y16 does not bind to Lbc, another RGS-RhoGEFs closely related to LARG. A model for the RhoA-Lbc complex was built by structural analogy after the RhoA-LARG complex and the aminoacids involved in the interaction between the RhoA and Lbc were deduced (Diviani et al., 2016). A virtual screening on the ZINC database highlighted 30 compounds likely to interfere with the formation of the RhoA-Lbc complex. They were synthetized and tested for their ability to block the interaction between RhoA and Lbc in a cellular system, which selected molecule A13 (4-[(4Z)-3-methyl-5-oxo-4-[[5-[3(trifluoromethyl) phenyl] furan-2-yl] methylidene] pyrazol-1-yl]benzoate). A13 affected the activation of RhoA induced by Lbc expression in 293T cells. A13 successfully inhibited the cellular effect driven by RhoA activation by Lbc, in particular NIH-3T3 cell transformation. Interestingly, A13 and Y16 display distinct selectivity for RhoA GEFs. A13 was found to block the binding of RhoA to Larg and PDZ-RhoGEF but not to p115RhoGEF. A13 also interfered with other RhoA GEFs: p114RhoGEF, p190RhoGEF and GEF-H1, but not p63RhoGEF and Net1 (Diviani et al., 2016).

Hopefully, *in vivo* assays will soon confirm the potentiality of Y16 and A13 RhoGEF inhibitors in the context of cancer.

1.3 An Example of Preclinical Application of a RhoGEF Inhibitor: Dock5 and Osteolytic Diseases.

1.3.1 *Dock5 is necessary for bone resorption by osteoclasts.*

Osteoclasts are essential for the maintenance of the skeleton. They degrade old or damaged bone, and osteoblasts replace it with new bone. The balanced activity of osteoclasts and osteoblasts throughout life is essential to maintain the health of the skeleton and its adaptation to loading constraints. But a variety of physiological and pathological situations exacerbate osteoclast activity, causing an excess of bone resorption over formation. This leads to progressive bone loss, osteoporosis and bone frailty. This occurs upon sexual hormone decay, for instance after menopause (Frenkel et al., 2010), in inflammatory diseases such as rheumatoid arthritis (Redlich and Smolen, 2012) and in bone metastasis, in particular of breast cancer (Weilbaecher et al., 2011). Increased osteoclast activity is also a iatrogenic effect of various medical treatments including corticosteroids (Canalis et al., 2007) and cancer chemotherapy (Drake, 2013). In these situations, medications to inhibit osteoclast activity are often associated with the front line treatment of the disease, to prevent osteoporosis and reduce the risk of fractures, pain and disability.

Dock5 was identified as an activator of Rac1 essential for bone resorption by osteoclasts in culture and *in vivo* in the mouse (Vives et al., 2011). The activation of Rac1 by Dock5 participates in the organization of osteoclast adhesion structures into a belt of podosomes to form the architecture of the bone resorption apparatus (Touaitahuata et al., 2014a). In the absence of Dock5, osteoclasts adhere on the bone but they fail to degrade it. In the mouse, the genetic deletion of Dock5 expectedly results in increased bone mass, while animals grow and behave normally and they remain fertile (Touaitahuata et al., 2014b; Vives et al., 2011). Therefore, Dock5 appears an attractive target in the context of osteolytic diseases to control the excess of bone resorption by osteoclasts.

1.3.2 *An inhibitor of Dock5 can prevent pathological bone loss.*

Small molecule hindering the activation of Rac1 by Dock5 where identified using the Yeast Exchange Assay (Blangy et al., 2006) and validated further using biochemistry and cell culture assays (Vives et al., 2011, 2015). In particular the molecule C21 prevented Rac1 activation by Dock5 in cell-free and reporter cell assays. In osteoclast in culture, C21 reduced the activity of Rac1, disorganized the belt of podosomes and hindered their capacity to resorb the bone. *In vivo* in the mouse, daily injections of C21 up to 25 mg/kg during one month did not provoke any measurable side effects on the behavior, the blood cell counts and the weight of the mice; it caused no toxic effect to the liver and the kidneys. C21 was tested for its ability to protect against pathological bone loss in mouse models of human osteolytic diseases: sexual hormone deficiency, inflammation and bone metastases. In these three disease models, the systemic administration of C21 during one month efficiently protected the animals against pathological bone loss (Vives et al., 2015). Therefore, targeting Rac1 activation by Dock5 appears as a beneficial and feasible strategy in the context of osteolytic diseases.

This study is a proof of concept that RhoGEF can indeed constitute relevant therapeutic targets and that they are amenable for long-term inhibition in the context of a whole organism.

1.4 References

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