

Small inhibitors of ADP-ribosylation factor activation and function in mammalian cells

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Abstract

Small GTP-binding proteins of the ADP-ribosylation factor (Arf) family control various cell functional responses including protein transport and recycling between different cellular compartments, phagocytosis, proliferation, cytoskeletal remodelling, and migration. The activity of Arfs is tightly regulated. GTPase-activating proteins (GAPs) inactivate Arfs by stimulating GTP hydrolysis, and guanine nucleotide exchange factors (GEFs) stimulate the conversion of inactive GDP-bound Arf to the active GTP-bound conformation. There is increasing evidence that Arf small GTPases contribute to cancer growth and invasion. Increased expression of Arf6 and of Arf-GEFs, or deregulation Arf-GAP functions have been correlated with enhanced invasive capacity of tumor cells and metastasis. The spatiotemporal specificity of Arf activation is dictated by their GEFs that integrate various signals in stimulated cells. Brefeldin A (BFA), which inactivates a subset of Arf-GEFs, has been very useful for assessing the function of Golgi-localized Arfs. However, specific inhibitors to investigate the in-

dividual function of BFA-sensitive and insensitive Arf-GEFs are lacking. In recent years, specific screens have been developed, and new inhibitors with improved selectivity and potency to study cell functional responses regulated by BFA-sensitive and BFA-insensitive Arf pathways have been identified. These inhibitors have been instrumental for our understanding of the spatiotemporal activation of Arf proteins in cells and demonstrate the feasibility of developing small molecules interfering with Arf activation to prevent tumor invasion and metastasis.

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Key words: Golgi; Vesicular transport; Migration; Adhesion; Actin

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ADP-RIBOSYLATION FACTORS

Introduction

ADP-ribosylation factors (Arf) are small monomeric GDP/GTP-binding proteins (20 kDa) that belong to the Ras superfamily, which is divided into five subfamilies: Ras, Rho, Rab, Ran and Arf^[1]. Like other small GTPases Arfs are regulated through a cycle of GTP binding and GTP hydrolysis, which activates and inactivates them, respectively^[2]. The Arf subfamily comprises six isoforms (numbered from 1 to 6) grouped into three classes based on their amino acid sequence homology. Arf class I is

composed of Arf1, 2, and 3, and class II of Arf4 and 5, while Arf6 is the unique member of class III^[3]. Arfs are not found in prokaryotes, but are expressed in yeast (Arf2-3) and in complex eukaryotic organisms such as mammals (Arf1-6). The homology between yeast and mammalian Arfs is over 74%^[4] and mammalian Arfs are able to functionally substitute for Arf depletion in yeast.

Arf functions

Class I / II Arfs: Class I and II Arfs exhibit several functional homologies since they are generally associated with the Golgi apparatus and regulate the secretory machinery in mammal cells^[5]. Arf class I (Arf1, 2 and 3) and class II (Arf4 and 5) are functionally redundant since silencing them separately has no measurable impact on Golgi morphology in mammalian cells^[6]. Active GTP-bound class I / II Arfs translocate reversibly to Golgi membrane compartments while inactive Arfs are cytosolic^[5]. Once associated with the membranes, these Arfs recruit several coat and cargo proteins such as COPI (coatamer), clathrin adaptor protein 1 (AP-1), or Golgi-localized γ -ear-containing Arf binding proteins (GGA), that are essential for vesicular biogenesis and anterograde ER-Golgi and retrograde Golgi-ER traffic^[5,7]. Sequential activation and inactivation of Arfs is required for regulation of ER-to-Golgi and Golgi-to-ER membrane traffic. Thus, Arf1-GTP in the Golgi membrane is required for vesicle budding, while Arf1 inactivation or dissociation from the membrane is necessary for vesicle detachment from the Golgi and coat disassembly^[8]. Class I / II Arfs also regulate several signalling pathways, in particular those issued from lipid metabolism, by stimulating the production of phosphatidic acid (PA) and of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase D (PLD) and PI4-/PI(4)P5-kinases [PI(4)P5K], respectively^[9,10].

Class III Arfs: There are fewer structural and functional homologies between Arf6 and other Arfs compared to those between class I and II Arfs. Contrary to other Arfs, Arf6-GTP is rarely found in the Golgi, and translocates from the cytosol to the plasma or endosomal membranes in cells^[7]. Arf6 contributes to membrane trafficking essentially *via* the regulation of lipid metabolism. PLD and PI(4)P5K are amongst the major effectors of Arf6^[11]. Stimulation of PLD1^[12] and possibly PLD2^[13] by Arf6 produces PA, and that of PI(4)P5K generates PIP2^[14-16]. Arf6-dependent synthesis of PIP2 can regulate proteins involved in exocytosis and endocytosis^[15,16], and the Arf6-PLD signalling axis is involved in regulated secretion^[17]. Internalization of several G protein-coupled receptors (GPCRs) from the cell surface to endosomal compartments^[18] and remodelling of the actin cytoskeleton^[7] are also regulated by Arf6.

guanine nucleotide exchange factors (GEFs) while the hydrolysis of bound GTP is highly accelerated by GTPase-activating proteins (GAPs)^[19]. The active form of Arf is characterized by a structural conformation that deploys a N-terminally myristoylated arm that facilitates membrane binding^[7].

Guanine nucleotide-exchange factors for Arfs

Arf-GEFs determine the amount and the spatiotemporal distribution of active Arfs by interacting with GDP-bound Arfs^[2]. Despite low sequence homology Arf-GEFs share one common central region of 200 amino acids similar to that of yeast Sec7p protein and known as the Sec7 domain. Arf-GEFs interact with Arfs through their Sec7 domain, which is necessary and sufficient for GEF activity^[20]. The Sec7 domain is regulated by other domains and contributes to substrate specificity of various Arf-GEFs^[20].

The crystallographic structure of the isolated or Arf1-bound Sec7 domain has been resolved^[21-23]. A hydrophobic groove composing the catalytic core of the Sec7 domain is delineated by two conserved regions interacting with the switch I and switch II domains of Arfs. The Sec7 domain contains an invariant glutamate residue necessary for GEF activity. Interaction between GDP-bound Arf and the GEF brings the invariant glutamate to a position that catalyzes the expulsion of the bound GDP from the GTP-binding site by charge repulsion. Then Arf binds GTP and the Arf-GTP complex dissociates from the GEF^[23]. The Sec7 domain is targeted by Brefeldin A (BFA), a fungal metabolite secreted by *Eupenicillium brefeldianum*, and designated by early studies as an inhibitor of secretion in mammalian cells^[24]. The Arf-GEFs are divided into two major classes based on their molecular weight, sequence homology, and sensitivity to BFA.

High-molecular weight GEFs: Also called large Arf-GEFs due to their molecular weight (> 100 kDa), these proteins are sensitive to BFA^[25,26]. In addition to the common Sec7 domain, these Arf-GEFs share other functional domains such as Dimerization and Cyclophilin-Binding, Homology Upstream of Sec7 and Homology Downstream of Sec7^[27,28]. The large Arf-GEFs found in mammals include the Golgi-specific Brefeldin A-resistance factor (GBF)^[29], and the Brefeldin A-inhibited GEF (BIG) families^[30]. Gea1p/Gea2p expressed by *S. cerevisiae*^[31] and GNOM expressed by *Arabidopsis thaliana*^[32] are GBF orthologs, while the Sec7p protein is the only BIG ortholog characterized in yeast^[28,33]. In mammalian cells, large Arf-GEFs preferentially localize to the Golgi where they activate Arf class I and II. The rapid and reversible BFA-induced dislocation of the Golgi is due to inhibition of Arf-GEF functions^[33]. Over-expression of GBF^[34] or of BIG^[35] can overcome BFA-mediated Golgi disassembly.

Low-molecular weight GEFs: The best-characterized small Arf-GEFs are proteins of the EFA6, Brefeldin A-Resistant Arf-GEFs (BRAG), and cytohesin families. These proteins are insensitive to BFA. They are gener-

REGULATION OF ADP-RIBOSYLATION FACTORS ACTIVITY

The exchange of GDP for GTP on Arfs is catalyzed by

ally cytosolic and are recruited to the plasma membrane in activated cells^[33]. Their central Sec7 domain is flanked by other functional domains that contribute directly or indirectly to the regulation of their activity. The Pleckstrin Homology (PH) domain in the C-terminus interacts with membrane PIP2/PIP3 and can be in part responsible for small Arf-GEF translocation to membranes^[36]. However, the PH domain of the BRAG family members seems to be insensitive to membrane PIP2/PIP3^[37]. The coiled-coil (CC) domain in the C-terminus of EFA6 or in the N-terminus of cytohesin family Arf-GEFs allows the formation of homodimers or of heterodimers with other CC domain-containing proteins. The BRAG family contains an additional PDZ domain and IQ motifs^[27,28].

Small Arf-GEFs exhibit some preferences for Arf family members due to substantial specificities in the Sec7 domain^[38]. EFA6 catalyzes the GDP/GTP exchange exclusively on Arf6 *in vitro* and *in vivo*^[39]. The other small Arf-GEFs can activate all the Arfs *in vitro* or *in vivo* depending on the cellular context^[28,33].

Due to their distinct cellular localization, the roles of small Arf-GEFs are different from those of the large Arf-GEFs. EFA6D is ubiquitously expressed in mammals, EFA6A is restricted to the brain and the intestinal epithelium, and EFA6B to spleen, thymus, pancreas, and the placenta^[40]. The EFA6 family members regulate actin remodelling^[41,42] and polarization of the intestinal epithelium by maintaining the integrity of tight junctions^[43]. The expression of BRAG2 is ubiquitous and that of other BRAG family members is more restricted to the nervous system (BRAG1/3)^[44,45]. In non-neuronal cells, BRAG2 is localized in endosomes where it preferentially activates Arf6^[37,46,47]. BRAG2 inhibition results in a defect in recycling to the cell surface of proteins such as integrins^[46], which interferes with cell adhesion, transferrin receptors, and E-cadherin^[47].

Cytohesins (1-4) are found in vertebrates^[28], and their orthologs are expressed in flies^[48] and in amoebae^[49]. Though *in vitro* cytohesins are more potent on Arf class I^[19], *in vivo* they preferentially catalyze the GDP/GTP exchange on Arf6^[28,50]. The expression of cytohesin-2/3 is ubiquitous in mammalian cells, and that of cytohesin-1 is more confined to leukocytes^[51]. Cytohesin-1 regulates integrin functions in T cells^[50], dendritic cells^[52], monocytes^[53,54], and neutrophils^[55-57], which affect their capacity to adhere and migrate. In non immune cells cytohesins control the recycling to the cell surface of integrins^[58], and GPCRs^[59,60]. Cytohesins interact with the insulin receptor complex and regulate insulin-mediated signalling in mammals^[36,61-63] and flies^[48].

INHIBITORS OF ADP-RIBOSYLATION FACTOR ACTIVATION BY Sec7 PROTEINS

Inhibitors of the high-molecular weight Arf-GEFs

BFA: BFA causes Golgi collapse through inhibition

of anterograde/retrograde transport between the ER and the Golgi and induces tubulation of recycling endosomes, which mix with the *trans*-Golgi network^[64,65]. BFA-induced effect is caused by inhibition of multiple Golgi-associated GEFs that act on Arfs^[66,67], mainly Arf class I and II. Arf1 recruits the coat protein COPI to the *cis*-Golgi enabling ER-to-Golgi vesicle transport^[68]. Activation of Arf1 and possibly of Arf5 by GBF1, a *cis*-Golgi GEF, regulates the association of COPI with vesicular tubular clusters^[29]. In contrast BIG1 and BIG2 localize to the *trans*-Golgi where Arf1 initiates the assembly of AP-1/clathrin coat protein complexes^[69]. BFA acts to stabilize an abortive complex between the GEFs and Arf in the GDP-bound form^[70]. The exchange activity of cytohesin-2 and of cytohesin-1 is not inhibited by BFA^[20,71]. Swapping the Sec7 domain of cytohesin-2 with Gea proteins confers resistance to BFA^[70]. The determinant of BFA sensitivity was analyzed by comparing the Sec7 domain of the yeast BFA-sensitive GEFs, Gea1p/Gea2p and Sec7p^[70,72], and of bovine p200, also known as GBF1 in humans, with that of BFA-resistant GEFs^[73,74]. The sec7 residues Y695 of Gea1p, D965 and M975 of Sec7p were conserved in all BFA-sensitive GEFs whereas the amino acids corresponding to F190, S198 and P208 in the Sec7 domain of cytohesin-2 were found in BFA-insensitive GEFs. Double substitutions F190Y/A191S or S198D/P208M (S199D/P209M in cytohesin-1) are sufficient for conferring BFA sensitivity to the cytohesin-2 Sec7 domain^[70,72]. These Sec7 residues that are critical for binding and stabilizing BFA in an abortive complex with Arf1 lie in a core region forming a direct interaction with Arf1^[22,75,76]. Note that not all Golgi Arf-GEFs can make a stable abortive complex with their preferred Arf substrates. No abortive complex formation between GBF1 localized to the ERGIC and the *cis*-Golgi and Arf class II that functions in ER-to-Golgi traffic was detected^[77].

LM11: Virtual screening was initiated to discover small inhibitors that fit in a pocket at the Arf1/cytohesin-2 Sec7 domain interface identified in the crystal structure of the Arf1-GDP/cytohesin-2 complex^[78]. The authors characterized a compound, LM11, that targets the Arf1-GDP/cytohesin-2 complex and acts as a non-competitive inhibitor. Structure-activity analysis indicated that LM11 in the Arf1-GDP/cytohesin-2 complex interacts with K38 in the switch 1 region of Arf1. Mutation of K38 renders Arf1 insensitive to inhibition by LM11 while Arf1^{K38} activation by a BFA-sensitive Sec7 domain remains sensitive to BFA^[79]. Residues R152 and N201 of the cytohesin-2 sec7 domain are conserved in several Arf-GEFs and contribute to LM11 sensitivity. LM11 does not distinguish between BFA-insensitive and sensitive Arf-GEFs. Activation of Arf1 and Arf5 by the Sec7 domain of BIG1 was LM11-sensitive *in vitro*. Similarly to BFA, LM11 interferes with Arf1 but not Arf6 activation *in vitro*^[23,80]. LM11 inhibits Arf1 in cells. Treatment of MDCK cells with LM11 causes dispersion of *cis*-Golgi markers, an effect that is reminiscent of GBF1 inhibition

by BFA^[68,69], and cytohesin-2-dependent migration^[78].

Golgicide A: Golgicide A (GCA) was identified using a high-throughput screen for small inhibitors that protect cells from Shiga toxin toxicity^[81]. GCA disperses *medial*- and *cis*-Golgi with rapid cytoplasmic redistribution of the coat protein COPI^[82]. The effect of GCA differs from that of BFA in that the coat protein AP-1 and GGA3 remain associated with the *trans*-Golgi network (TGN) until the Golgi collapses. Dispersion of the coat protein COPI but not of the TGN-associated coat protein complexes suggests that GCA may target GBF1^[68]. The morphological effects of GCA on *medial*- and *cis*-Golgi were reminiscent of those caused by dominant-inactive GBF1^[83] and silencing of GBF1^[84]. Canine GBF1 is naturally resistant to BFA due to leucine substitution for methionine 832 within the Arf-binding site of GBF1^[34,70]. M832L substitution in BFA-sensitive GBF1 renders the GEF resistant to both BFA and GCA^[82], suggesting that the binding site of GCA overlaps with that of BFA within the GBF1-Arf1 interfacial region. Over-expression of GBF1-M832L in GCA-sensitive cells protected them from the morphological and functional effects of the compound on the Golgi and on protein secretion, respectively. As expected, treatment with GCA caused a decrease in Arf1 activation and over-expression of the GBF1-mutant in cells reversed GCA effects on Arf1-GTP levels^[82].

Replication of Hepatitis C virus (HCV) and of enteroviruses depends on GBF1^[85,86]. Treatment with GCA of cells that support productive infection of viral particles reduces HCV replication and interferes with the biogenesis of virions in an Arf1-dependent manner^[85]. Inactivation or silencing of Arf1 redistributes viral proteins of the replication complex from ER-like vesicular membranes to the rims of lipid droplets^[87]. GCA reduces the RNA levels of protein 3A of enterovirus poliovirus and coxsackievirus B3^[88-90], a protein of the replication complex interacting directly with GBF1. The effect of GCA on viral RNA replication was countered by over-expressing GBF1 or the GCA-resistant GBF1-M832L, further supporting the dogma that GCA targets GBF1^[86]. This study suggests that viral proteins of the replication cycle hijack GBF1 and reduce GBF1-induced Arf activation to support the biogenesis of viral particles. GCA is more selective than BFA to dissect molecular mechanisms of the HCV and enterovirus replication cycles^[85,87].

Exo1: Exo1 was identified using a phenotypic screen based on the export of the vesicular stomatitis glycoprotein fused to GFP (VSVG-GFP) from the ER to the Golgi^[91]. Exo1 reproduces the effects of BFA such as the tubulation and collapse of the Golgi^[64]. Though Exo1 induces a rapid release of Arf1 from the Golgi and disappearance of COPI-coated vesicles, its effects differ from those of BFA in that redistribution of the TGN-associated coat proteins is delayed and the endocytic pathways are not affected^[92]. Unlike BFA and GCA^[70,82],

Exo1 does not interfere with the nucleotide exchange activity of various Arf-GEFs on Arf1. Whether Exo1 has a stimulatory effect on Arf-GAPs by interfering with the assembly/stability of the COPI coat with the Arf1-GTP-GAP complex required for GTP hydrolysis or through another mechanism remains to be clarified^[77,92].

Exo2: Exo2 was discovered using the imaging screen described by Yarrow *et al.*^[91] to search for inhibitors of the secretory pathway. In BSC1 and Vero cells Exo2 has no significant effect on the integrity of the TGN or on retrograde trafficking of cholera toxin from the plasma membrane to the ER through the TGN^[93]. The effect of Exo2 is cell type specific since it disperses the TGN in HeLa cells and retards trafficking of Shiga toxin to the ER^[94]. Unlike BFA Exo2 does not induce tubulation of early endosomes and mixing with the TGN but disrupts the COPI-dependent anterograde transport of VSVG-GFP from the ER to the Golgi^[77,92,93]. Though the data suggest that Exo2 interferes with the functions of Arfs or Arf-GEFs localized to the ER-Golgi intermediate compartment or the TGN, the targets of Exo2 remain unknown. Exo2 reduces Arf1 activation by the Sec7 domain of BIG1 but the effect is weak compared to BFA^[95]. Whether Arf activation by GBF1 is sensitive to Exo2 remains to be established.

LG186: Based on the 3D model structure of Sec7 domains in complex with BFA^[22,23], Exo2 was used as a prototype molecule to design an inhibitor of GBF1^[95]. The drug was engineered to fill a pocket overlapping the BFA binding site at the interface between the Sec7 domain of GBF1 and Arf1-GDP^[22,75]. Though LG186 slightly inhibited the exchange activity of the Sec7 domain of BIG1 compared to BFA *in vitro*, demonstration that LG186 is an inhibitor of GBF1 was not convincing^[95]. Nevertheless, cells treatment with LG186 reduces Arf activation, induces dissociation of the COPI vesicle coat from the Golgi and accumulation of GBF1 on the ER-Golgi intermediate membrane compartment as described for BFA^[77] and for GCA^[82]. An interesting property of LG186 is the inhibition of canine GBF1 activity which is insensitive to inhibition by BFA and GCA^[34,82] due to a methionine substitution in its Sec7 domain. Characterization of LG186 as a selective GBF1 inhibitor awaits further confirmation.

Inhibitors of the low-molecular weight Arf-GEFs

RNA Aptamers M69 and K61: RNA aptamers are nucleic acid species that have the capacity to bind specifically to target proteins with high affinity and to interfere with their functions *in vivo* and *in vitro*. RNA aptamers are identified using a technical procedure named SELEX (systematic evolution of ligands by exponential enrichment) which consist of repeated rounds of *in vitro* selection by screening the affinities of large RNA libraries for a specific target^[96]. Famulok and colleagues used recombinant cytohesin-1^[97] and cytohesin-2^[98] as baits to identify bind-

ing partners from libraries containing 10^{14} and 10^{15} RNA aptamers, respectively.

The M69 RNA aptamer was reported to interact with full-length cytohesin-1 and to target the Sec7 domain but not the PH or the C-terminal domains of cytohesin-1^[97]. *In vitro*, M69 inhibited GDP/GTP exchange on Arf1 catalyzed by full-length cytohesin-1 and cytohesin-2 or their Sec7 domains, but not that stimulated by the large Arf-GEF Gea2, thereby demonstrating specificity for the cytohesin family^[97]. A vaccinia virus-based intramer expression system was used to allow expression of the M69 RNA aptamer in mammalian cells. Over-expression of M69 in Jurkat cells inhibited adhesion to the integrin ligand ICAM-1 and impacted cell morphology by affecting actin cytoskeleton remodelling^[97]. These *in vivo* effects of M69 are reminiscent of those described for catalytically inactive cytohesin-1 on Jurkat cell adhesion^[99] and spreading^[100].

Another RNA aptamer, K61, discriminating cytohesin-2 from cytohesin-1, was also identified by the group of Dr Famulok^[98]. K61 RNA aptamer binds more tightly to CC-Sec7 ($K_d = 400$ nmol/L) than to Sec7 alone ($K_d = 800$ nmol/L) or to Sec7-PH ($K_d = 1000$ nmol/L) domains of cytohesin-1, but is capable of inhibiting nucleotide exchange on Arf1 catalyzed by cytohesin-2 *in vitro*. When used to transfect HeLa cells, both the K61 RNA aptamer and cytohesin-2 siRNA, but not the cytohesin-1 siRNA, markedly decreased serum-induced ERK phosphorylation and serum-mediated transcriptional activation^[98]. Of note, targeting the CC domain of cytohesin-2 with aptamer K61 has the same inhibitory effect as that of the GEF-deficient E156K mutant of cytohesin-2 on serum-mediated transcriptional activation. Though an indirect effect of aptamer K61 on Arf activation due to miss-localisation of cytohesin-2 through inhibition of protein interaction with cellular partners other than Arfs cannot be excluded, another study showed a role for cytohesin-2 and Arf6 activation in GPCR-mediated signalling through the ERK/MAP kinase pathway^[101].

Sec7 inhibitor H3: Inhibitory RNA aptamers were subsequently used to screen libraries for small molecules that could displace the binding of fluorescent M69 RNA aptamer from the cytohesin-1 Sec7 domain and act as inhibitors of cytohesins^[63]. One of the cell permeable inhibitors, named Sec7 inhibitor H3 (secinH3), was found to bind to and to inhibit the GEF activity of cytohesin-1, -2, and -3, and steppe, an insect ortholog for cytohesins^[48,63]. The affinity of secinH3 for other Arf-GEFs such as EFA6 was very low and their nucleotide exchange activity was not inhibited by this compound^[63]. Cell treatment with a high concentration of secinH3 does not disrupt the Golgi, contrary to a low dose of BFA, which precludes a role for cytohesins in maintaining Golgi morphology^[63]. Feeding mice with secinH3 resulted in hepatic insulin resistance^[63] and insulin-induced signalling is disrupted in flies fed with secinH3^[48]. Treatment of HepG2 hepatocytes with secinH3 abrogates the association of

cytohesins and Arf6 with the insulin-receptor complex^[63]. More recently, secinH3 was reported to inhibit glucose-stimulated insulin secretion in cultivated rat and human β pancreatic cells through inhibition of the cytohesin-2/Arf6 signalling axis and downstream activation of Rac1 and Cdc42^[102].

The valproic acid-induced neurite outgrowth in N1E-115 neuroblastoma cells, which involves cytohesin-2 and downstream activation of Arf6, was reversed by treatment with secinH3^[103]. Similarly secinH3 interferes with preadipocyte 3T3-L1 cell migration through inhibition of the cytohesin-2/Arf6 signalling axis^[104]. SecinH3 was also reported to inhibit the growth of human lung cancer cells through inhibition of cytohesin-2-mediated autophosphorylation of ERB receptors, but independently of cytohesin-2-induced Arf activation^[105].

SecinH3 inhibits several human neutrophil functions such as degranulation and superoxide anion production *via* the regulation of the cytohesin-1-Arf6-PLD signalling axis^[57]. Cytohesin-1 was first characterized as a regulator of β -2 integrin-dependent adhesion through direct interaction with LFA-1^[99]. As anticipated, secinH3 inhibited activation of the β -2 integrin LFA-1 in neutrophils^[55]. Adhesion of Jurkat T cells to the specific β -2 integrin substrate ICAM-1^[106] and ICAM-1-dependent adhesion of neutrophils to endothelial cells^[55] were inhibited by secinH3. Though secinH3 inhibits the function of LFA-1, we reported that secinH3 activates Mac-1, another neutrophil β -2 integrin, and stimulates Mac-1-dependent responses such as migration, adhesion to fibrinogen and phagocytosis^[56]. SecinH3 was also reported to regulate the internalization of β -1 integrins in mammalian cells^[58].

Using secinH3 as a template, virtual screening of a library of small chemicals led to the discovery of more potent inhibitors of cytohesin-1 such as secin16, secin69, secin132^[106] and secinB7^[107]. Secin16 and secinB7 were up to 10-fold and 26-fold more potent in inhibiting nucleotide exchange on Arf1 catalyzed by cytohesin when compared to secinH3, respectively. SecinH3, as well as lower concentrations of secin16 or secinB7, markedly reduced the proliferation of PC9 cells^[107].

TARGETING ADP-RIBOSYLATION

FACTORS FOR ANTICANCER AND ANTI-INFLAMMATORY THERAPIES

Recent evidence indicates that the dynamics of β 2 integrin activation and of other neutrophil functional responses are controlled by Arf6 GDP-GTP cycling, which is regulated by the Arf6-GEF cytohesin-1^[55-57], and ARAP3^[108], a dual GAP for Arf6 and RhoA. Therefore, the use of small inhibitors of cytohesin-1 causing down regulation of Arf6 may be a therapeutic option in autoimmune diseases to prevent tissue damage and inflammation associated with excessive neutrophil responses.

Arf6 is highly expressed in some breast carcino-

Table 1 Targets and effects of the ADP-ribosylation factor inhibitors

Inhibitor	Arf regulators	Arf	Phenotype/function	Cell type
Brefeldin A	GBF1	Arf1	Tubulation and collapse of Golgi apparatus	Mammalian cells
	Gea1p/2p	Arf5		
	Sec7p	Arf1p	Inhibition of protein secretion	Yeast
LM11	Cytohesin-2	Arf1	Dispersion of Golgi markers	MDCK cells
	BIG1	Arf5	Inhibition of migration	
Golgicide A	GBF1	Arf1	Protection from Shiga toxin toxicity	Mammalian cells
			Dispersion of cis- and medial-Golgi	
			Inhibition of protein secretion	
			Inhibition of viral replication: (hepatitis C virus; enteroviruses; poliovirus; coxsackievirus)	
EXO1	ArfGAPs	Arf1	Tubulation, collapse of Golgi	Mammalian cells
EXO2	BIG1	Arf1	Dispersion of the trans-Golgi network	HeLa cells
			Interfere with shiga toxin trafficking to the endoplasmic reticulum	
LG186	BIG1	Arf1	Dissociation of COP I vesicles from the Golgi	HeLa cells
	GBF1			
M69 aptamer	Cytohesin-1	Arf1	Actin remodelling	Jurkat T cells
	Cytohesin-2		Inhibition of adhesion	
K61 aptamer	Cytohesin-1	Arf1	Inhibition of serum-induced ERK MAP Kinase activation	HeLa cells
	Cytohesin-2			
SecinH3	Cytohesin-1	Arf1	Insulin resistance	HepG2 cells; rat and
	Cytohesin-2	Arf6		human β pancreatic cells
	Cytohesin-3		Inhibition of superoxide anion production	Neutrophils
			Inhibition of degranulation	
			Activation of Mac-1-dependent adhesion, phagocytosis and migration	
			Inhibition of LFA-1 dependent adhesion	
			Inhibition of migration	3T3-L1 cells
			Inhibition of neuritis outgrowth	N1E-115 neuroblastoma cells
			Regulation of β -1 integrin internalization and of β -1 integrin-dependent adhesion	HeLa cells
Steppke			Insulin resistance	Fly cells

Arf: ADP-ribosylation factor; GBF: Golgi-specific Brefeldin A-resistance factor; BIG: Brefeldin A-inhibited GEF.

mas^[109], glioma cell lines^[110], as well as in primary lung cancers^[111]. Several studies have correlated the levels of Arf6 protein expression with increased cell motility^[110] and the invasive phenotype^[109,110,112]. The invasive capacity and the metastatic behavior of cancer cells are under the control of Arf6^[113]. Silencing of Arf6 or expression of dominant negative mutants reduces cancer cell motility and invasion^[109,110,112,113]. In addition to enhanced expression of Arf6, aberrant functions of regulators of Arfs (including subsets of Arf-GEFs and Arf-GAPs) have also been associated with the invasive and metastatic phenotype of cancer cells^[114]. The expression of GEP100/BRAG2 (an Arf-GEF for Arf6) is increased in invasive breast carcinoma compared to non-invasive breast tumors^[115]. The recruitment of GEP100 to EGF receptors in breast cancer cells activates Arf6 and stimulates cell invasion^[115]. As previously reported for Arf6 in breast carcinomas^[109], silencing of GEP100 reduces the invasive and metastatic phenotypes of these breast cancer cells^[115]. Elevated expression of EFA6, another Arf6-GEF, recently reported in low and high-grade glioma tissues, has also been linked to cell invasiveness^[116]. Although there is no available inhibitor of Arf6 activation by GEP100 or EFA6A, the proof of concept that small-inhibitors of Arf-GEFs could be used for cancer therapeutics is now emerging^[114]. Depending on the cellular context the selective inhibition of Arf6-GEFs may block cell motility^[78],

invasion and metastasis whereas targeting Arf1-GEFs would induce inhibition of cancer cell growth^[117].

CONCLUSION

The availability of inhibitors of Arfs with selectivity for individual BFA-sensitive Arf-GEFs localized to the Golgi were instrumental to understanding the spatiotemporal activation and function of Golgi-associated Arfs in anterograde (ER-to-Golgi) and retrograde (Golgi-to-ER) transport mechanisms in cells. Inhibitors that target the BFA-insensitive pathways have been developed (Table 1). However these inhibitors are specific for the cytohesin family of Arf-GEFs and cannot be used to discriminate between the Arf pathways regulated by cytohesin-1, cytohesin-2 or cytohesin-3. Though there is no inhibitor for the BRAG family of Arf-GEFs, several proofs of principle indicate that Arf-GEFs are druggable targets *in vivo* and that the design of compounds targeting the Arf/Arf-GEF complexes could yield inhibitors with potential for development into anti-cancer and anti-inflammatory drugs.

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