Acta Pharmaceutica Sinica B 2023;13(10):4089–4104

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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REVIEW

Arf6 as a therapeutic target: Structure, mechanism, and inhibitors

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Received 23 February 2023; received in revised form 28 April 2023; accepted 2 June 2023

KEY WORDS

Arf6;
Mechanism;
Structure;
Inhibitors;
Drug resistance

Abstract ADP-ribosylation factor 6 (Arf6), a small G-protein of the Ras superfamily, plays pivotal roles in multiple cellular events, including exocytosis, endocytosis, actin remodeling, plasma membrane reorganization and vesicular transport. Arf6 regulates the progression of cancer through the activation of cell motility and invasion. Aberrant Arf6 activation is a potential therapeutic target. This review aims to understand the comprehensive function of Arf6 for future cancer therapy. The Arf6 GEFs, protein structure, and roles in cancer have been summarized. Comprehending the mechanism underlying Arf6-mediated cancer cell growth and survival is essential. The structural features of Arf6 and its efforts are discussed and may be contributed to the discovery of future novel protein-protein interaction inhibitors. In addition, Arf6 inhibitors and mechanism of action are listed in the table. This review further emphasizes the crucial roles in drug resistance and attempts to offer an outlook of Arf6 in cancer therapy.

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Peer review under the responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2023.06.008

1. Introduction

The ADP-ribosylation factors (Arfs), the members of the Ras superfamily, are guanine nucleotide binding proteins that regulate the cytoskeleton and membrane traffic. The Arf family includes six isoforms in mammalian cells, which can be divided into three classes according to its homology[1]. The classes I (Arf1, Arf2 and Arf3) (humans lack Arf2) and II (Arf4 and Arf5) localize at the endoplasmic reticulum[2]. The sites of Arf6 (class III) are the plasma membrane and endosomal, which are distinguished from other members. Arf6 is involved in the cytoskeletal organization of cell surface actin. It plays important roles in exocytosis, endocytosis, plasma membrane reorganization, etc.[3].

Like other small GTPases, Arf6 exists in two forms: guanosine triphosphate (GTP)-Arf6 and guanosine diphosphate (GDP)-Arf6, respectively[4]. The former is its active form, and the latter reacts with enzymatic catalysis to produce the former for its action. The cycling of Arf6 between binding to GDP and binding to GTP is tightly mediated by regulators. Mechanically, both GAPs (the Arf6 GTPase activating proteins) and GEFs (the Arf6 guanine nucleotide exchange factors) can replace GDP and allow GTP to bind to active Arf6[5]. In the resting state of cells, the existing form is GDP-Arf6. When cells are stimulated by agonists, GDP is exchanged for GTP through Arf6 GEFs. GEFs are classified into 3 families: cytohesin, EFA6 and BRAG family[1]. Then, GTP-bound active form of Arf6 interacts with its effectors, and is converted to inactive form by Arf6 GAPs. GAPs are classified into 4 families: GIT, SMAP, ARAP and ACAP family[5,6]. Arf6 affects disease in many ways, including regulating cell division, polarity, adhesion, etc.[3,7]. In the study of cancer cell invasion and metastasis, aberrant Arf6 activation is found.

Arf6 is considered as a potential target for cancer therapeutics. It exists a meaningful direction to understand the mechanism and inhibitors of Arf6 activation during past years. This review focuses on the structure and biological functions of Arf6 and its effectors, summarizes the recent development of the relative inhibitors of Arf6 activation in cancer treatment and speculates on the future of developing Arf6 inhibitors based on drug sensitization.

2. Activation machinery of Arf6 through GEFs

The activation of Arf6 is regulated by GEFs. The structures of GEFs (approximately 200 amino acids) contain a catalytic Sec7 domain, accelerating the dissociation of GDP from the Arf6 and switching Arf6 to an activated state[4]. Domain structures of GEFs are summarized and illustrated in Fig. 1.

2.1. *Cytohesins*

Arf6 GEFs, as upstream partners of Arf6, can regulate the GDP/GTP exchange of Arf6[1]. There are three kinds of Arf6 GEFs in higher organisms, one of which is cytohesin family. The cytohesin family consists of four members, cytohesin 1−4, being identified in vertebrates[8]. Cytohesins are composed of a central Sec7 domain, a N-terminal coiled-coil domain (CC), and a C-terminal PH domain[4]. Cytohesin 1, cytohesin 2 and cytohesin 3 are detected ubiquitously in mice, but cytohesin 4 is expressed in leukocytes[8]. Cytohesin 1−3 promote GDP/GTP exchange on Arf6[9,10]. Cytohesin 2, referred to as ARNO, acts as GEF to Arf1, Arf3 and Arf6. ARNO is composed of heptad repeats, a PH domain, a Sec7 domain, and a polybasic motif[11]. The PH domain exerts significant effects on recruiting Arf6 to the cell membrane. Furthermore, the Sec7 domain is essential for the transportation of intra-Golgi[11]. Arf6 regulates actin polymerization by recruiting ARNO to the membrane and EspG blocks the effect by antagonizing ARNO recruitment[12]. The mechanism of ARNO involvement in endocytosis differs from EFA6. The peripheral ARNO regulates endocytosis through Arf6-mediated clathrin assembly[13]. In addition, ARNO and Arf6 are also involved in regulating the endocytic degradative pathway[14]. In this process, the a2-isoform of proton-pumping vacuolar ATPase (V-ATPase) interacts with ARNO, and the c-subunit interacts with Arf6. V-ATPase/ARNO/Arf6 regulates the endocytic degradative pathway. A subsequent study finds that aldolase is one of ARNO effectors[15]. The aldolase forms a complex with ARNO/Arf6 and the V-ATPase. The complex remodels the actin cytoskeleton and the trafficking. However, ARNO/Arf6 signaling cascade can be upstream of Cdc42 and Rac1 activation steps which is necessary for glucose-stimulated insulin secretion[16]. Arf6-mediated Rac1 activation is essential for glioma cell invasion that requires IQ-domain GTPase-activating protein 1[17]. ARNO/Arf6 can connect with BRAG2 to improve the phosphorylation of vascular endothelial growth factor receptor 2, which participates in diabetic retinopathy[18]. Moreover, RLIP76, a novel R-Ras effector, recruits ARNO to activate Arf6 and contributes to cell spreading and migration[19]. ARNO/Arf6 can be involved in some disease-related responses, including tumors, through regulating Arf6 and even other factors such as PI3K and PIP3. The detailed pathways are summarized in the previous review[20]. Here, the activation of Arf6 is described (Fig. 2). The auto-inhibition state of cytohesins exists in the solution. PH domain is inhibited by the C-terminal helix/polybasic domains and linkers between Sec7 and PH domain positioned by the catalytic Sec7 domain, which block access to the binding site for Arf[21]. It is recruited to the membrane, which is encouraged by a specific and direct interaction between Arf6-GTP and PH domain. The auto-inhibition is regulated by PH domain and alleviated by Arf6 binding[22]. The PH domain interacts specifically with $PI(4,5)P_2$ or $PI(3,4,5)P_3$[23].

2.2. *EFA6*

EFA6 family proteins are the most Arf6-specific GEFs. EFA6 protein family's common domains consist of a pleckstrin homology (PH) domain, a catalytic Sec7 domain and a C-terminus (Ct), including a coiled-coil domain and two proline-rich regions[24]. The PH domain controls the localization of plasma membrane. The Ct domain connects with the plasma membrane, and EFA6 proteins are recruited. EFA6 can transform Arf6-GDP into Arf6-GTP to active Arf6. Activation of Arf6 with EFA6 suppresses axon regeneration, reduces neurite growth and regulates integrin transport[25]. Likewise, EFA6 recruits endophilin to the endocytic zones of the plasma membrane, where EFA6 cooperates with endophilin to activate Arf6 and then regulate endocytosis[26]. In addition, CD13 can recruit EFA6 and IQ motif-containing GAP 1 to the cell membrane to activate Arf6, which is critical in the process of β1 integrin endosomal trafficking and cell-ECM interactions[27]. EFA6 family contains EFA6A to D members. The four members possess similar amino acid sequences. In the key Sec7 domain, EFA6D has high consistency with EFA6C (70%), EFA6A (66%), and EFA6B (60%)[28]. Each isoform has diverse physiological functions in different tissues[29,30]. Among them, EFA6A regulates the formation of actin bundles, and is an essential regulator of ciliogenesis, in which

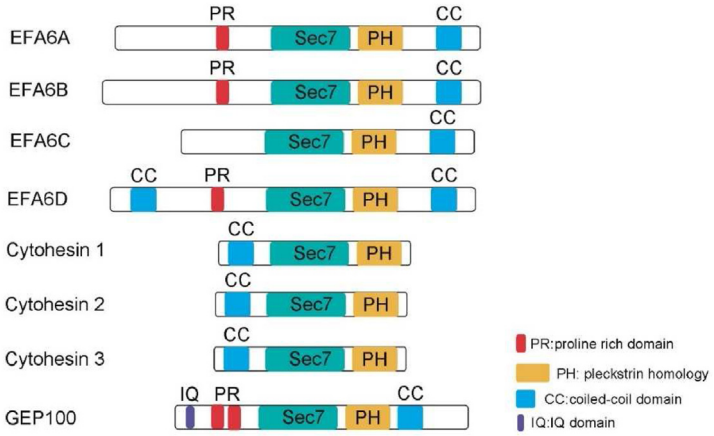


Figure 1 Structures of Arf6 GEFs.

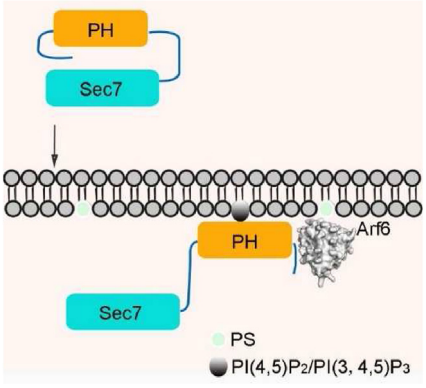


Figure 2 Model of activation of Arf6 by cytohesins. The PH domain of cytohesins specifically binds with PI(4,5)P_2 or PI(3,4,5)P_3 and Arf6 at the plasma membrane.

EFA6A plays a role together with several small G proteins, including Arf6, Arl13B and Rab8[31]. EFA6/Arf6 can induce the reorganization of the actin cytoskeleton, but Arf6 also directly transport Rho GTPases to actin polymerization[32]. EFA6B is associated with tumor cell metastasis as well as poor prognosis in breast cancer[33]. EFA6D can activate Arf6 to induce the reorganization of the actin cytoskeleton, and it has been identified as a biomarker for ovarian cancer[34]. In conclusion, EFA6 can activate Arf6 to regulate fundamental cellular processes. Most importantly, the EFA6−Arf6 pathway is mainly involved in integrin transport, endocytosis and actin cytoskeleton. The mode of Arf6 activation by EFA6 is summarized. An increase of PIP_2 induces the recruitment of EFA6 subfamily members to the plasma membrane. The PH domain controls its localization[35]. The Sec7 domain binds with Arf6, and stimulates the exchange of GDP/GTP (Fig. 3A). Negative feedback loop exists in EFA6 to regulate Arf6. Although the autoinhibition of EFA6 is devoid, it can sustain the level of Arf6-GTP *via* its negative feedback loop. ARNO is recruited and exerts its GEF activity by utilizing Arf6-GTP pool (Fig. 3B, Top). ARNO generating Arf6-GTP and Arf1-GTP maintains its own activities and shutdowns the GEF activity of EFA6[36]. The activation of Arfs is mainly performed by ARNO at that stage (Fig. 3B, Bottom).

2.3. *BRAGs*

The BRAG family contains three genes, BRAG1, BRAG2 and BRAG3. BRAG2, also referred to as GEP100, is detected in a variety of other tissues[37]. Other two BRAGs are enriched in the postsynaptic densities[38]. GEP100 possesses the substrate specificity to Arf6, but the specificity of BRAG1 and 3 is still unclear. In addition to several domains reported in other ARF6 GEFs, GEP100 includes an IQ-like domain[4]. The role of IQ domain regulating GEP100 GEF activity is unknown. GEP100 partially co-localizes with Arf6 at the endosome and cell periphery. Then, Arf6 is activated to adjust endosomal and membrane functions[39,40]. GEP100 is involved in cell adhesion, invasion and actin cytoskeleton, which is independent of Arf6 activation[40]. However, GEP100 exerts certain functions, such as regulating apoptosis, which does not depend on Arf activation[41]. The PH domain GEP100 possesses unique features. The expanded PH domain can be regarded as the linker of C terminus of PH domain and N terminus of Sec7 domain[42]. In the PH domain, a conserved lysine recognizing PI(4,5)P_2 is replaced by Glu639, which impairs PI(4,5)P_2 binding, but does not prevent membrane binding[42]. The research also finds that GEP100 is not auto-inhibition by detecting the activity of the short isoform GEP100 in solution. In BRAGs, the Sec7 domain binds with Arf6, and the negative or positive feedback loop is not found (Fig. 4A). The comparison of GEP100 and cytohesin 3 PH domain shows that kinked C-terminal helix exists in cytohesin 3, and the helix in GEP100 is straight, which may be adverse effects on forming the autoinhibitory structure (Fig. 4B)[42].

2.4. *The comparison of 3 families of ARF6 GEFs*

The GEFs of ARF6, EFA6, cytohesin and BRAG families possess divergent N-terminal domain, but the C-terminal domain comprises a PH and Sec7 domain. However, the different regulatory functions are found in their C terminus. The PH and Sec7 domain are the state of autoinhibition by obstructing its Arf-binding sites in cytohesins[43]. It exists a positive feedback loop involving the activation of Arf6 and Arf1[22]. EFA6 families are not autoinhibited by the PH domain and the nucleotide exchange depends on phosphoinositides[36]. EFA6 performs certain

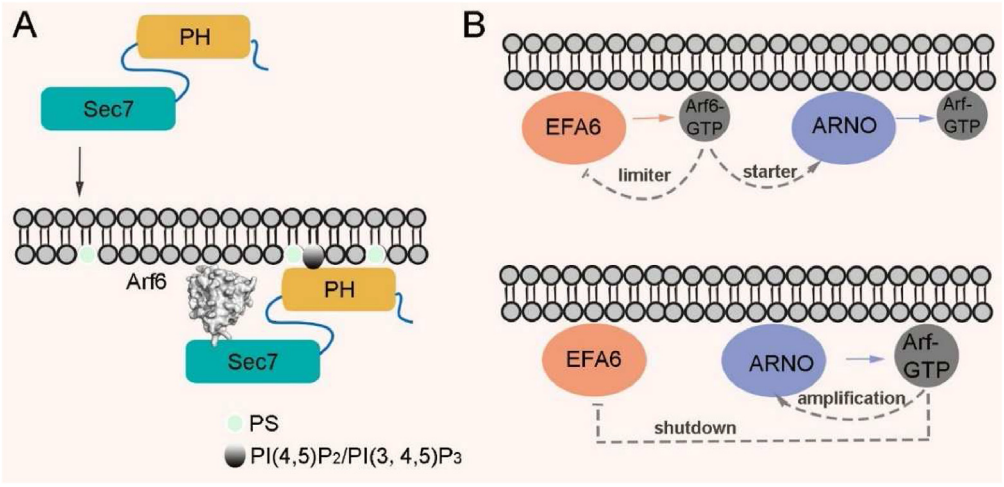


Figure 3 Model of activation of Arf6. (A) The Sec7 domain of EFA6 binds to and activates Arf6. The PH domain binds nonspecifically with PS and PI at the membrane. (B) The negative feedback loop of EFA6 family and the positive feedback loop of ARNO.

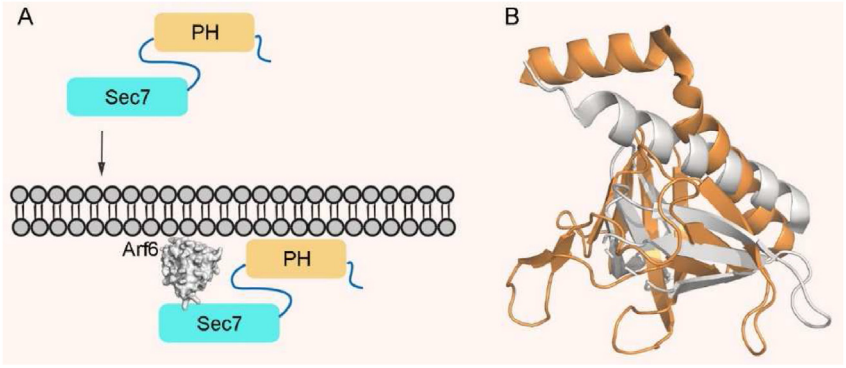


Figure 4 Model of activation of Arf6 by BRAGs and structural comparison of the PH domain of GEP100 and cytohesin 3. (A) The Sec7 domain of BRAGs binds with Arf6. (B) The PH domain of GEP100 (ID: 5NLV, gray). The PH domain of cytohesin 3 (ID: 2ROD, yellow).

functions by an Arf6-independent pathway, such as promoting the tight junction formation. Compared to EFA6, ARNO has less regulation of Arf6 activation. The PH domain, not Sec7 domain mediates the binding of ARNO and Arf6[20]. In BRAG, the PH domain is also not the state of autoinhibition. It has no marked phosphoinositides preference[36]. Both ARNO and GEP100 can activate Arf1 and Arf6. Interestingly, the two GEF possess different functions[36]. ARNO amplifies an initial Arf signal, and its PH domain and Sec7 domain interact with Arf6 and Arf1, respectively[44]. GEP100 completes sequential or simultaneous activation of Arf1 and Arf6. The following researches can explain the mechanism. The depletion of GEP100 and Arf6 are inconsistently shown to affect endocytosis of b1 integrins[39]. The Wnt/β-catenin pathway requiring GEP100 can be regulated by both Arf1 and Arf6[45]. Because the structure determines the function, it is essential to compare the differences in cytohesins and GEP100. The kinked C-terminal helix exists in PH domain of cytohesins. In GEP100, the helix is straight, which may be adverse effects on forming the autoinhibitory structure. Differences in recognizing phosphoinositides may be related to the altered sequence in lipid-binding pocket, such as the mentioned residue Glu639[42].

3. Roles and mechanisms of Arf6

3.1. Arf6 and membrane traffic

Malignant tumor cells acquire abnormal movement to intrude into the surrounding tissue parenchyma[46]. Polarized cellular response allows cancer cells to move forward and up the chemoattractant gradient, which is an effective motility of multiple tumor cells[47,48]. Arf6 regulates polarized morphology, endocytic trafficking and actin remodeling of cells. The researches have implicated Arf6 in determining polarized structures in the disassembly of polarized epithelium. Arf6 can regulate neurite outgrowth in PC12 cells[49]. FilGAP (also called ARHGAP24), a GAP for Rac1, is recruited to the plasma membrane, and regulates the formation of pseudopods in breast carcinoma cells[50]. FilGAP achieves Arf6-mediated action remodeling by a binding site of Arf6. The binding is necessary to regulate the length of pseudopods. Importantly, FilGAP combines with activated Arf6 Q67L but not with inactive Arf6 T27N[51]. Overexpression of FilGAP induces the polarity of breast cancer cells. The roles of FilGAP in cancer cells rely on its phosphatidylinositol 3,4,5-trisphosphate and Arf6 binding[52]. Arf6 is the important factor in dictating

structural organization of epithelial cysts[53]. Depletion of Arf6 in epithelial cysts causes a loss in cell polarity, a marked increase in Rac1 inactivation and Rho activation. Rac1 stimulation could rescue inverted polarity in MDCK^{Arf6si} cysts. Activation of Arf6 results in the disassembly of adherens junctions and membrane ruffling, which is mediated by PIP_2 generation (Fig. 5). E-cadherin can influence the effects by internalizing into cytoplasm. The disassembly of adherens junctions increases migratory capacity. Inactivation of Arf6 (T27N) regulates adherens junctions and enhances the epithelial phenotype[54]. E-cadherin regulated by Arf6 is responsible for the disassembly of adherens junctions[55]. Then, it interacts and recruits Nm23-H1, a nucleoside diphosphate kinase. In this process, the cellular levels of Rac1 active form are decreased. At later times, the cells begin to scatter. Arf6 is associated with endocytic trafficking and actin remodeling. In kidney proximal tubules, ARNO and Arf6 are recruited to endosomal membranes, which is related to the degree of intra-endosomal acidification[56].

3.2. *Arf6 and proliferation*

Arf6 promoting cell motility and invasion accelerates cancer development. In some tumor cells, active Arf6 gene expression stimulates cancer proliferation (Fig. 5), but does not affect cell movement. Arf6 is regarded as an integrator of signals in uveal melanoma[57]. Mutant GNAQ promotes tumorigenesis in uveal melanoma through activation of MAPK, PI3K/AKT, YAP and Rho/Rac signals. GNAQ activates the exchange factor Brag2, which increases Arf6·GTP level. The process promotes the levels of Rac1·GTP and Rho·GTP. Subsequently, it stimulates the activation of MAPK signal. Otherwise, YAP1 is activated by Arf6·GTP[58]. The inhibition of Arf6 activation suppresses tumor proliferation by regulating these pathways. Other mechanisms by which Arf6 regulating tumor is Arf6 activation promoting tumor progression and Arf6 overexpression is found in multiple cancer cells. miR-1299 is investigated about cells proliferation, apoptosis and metastasis. The research demonstrates miR-1299 targeting the 3′-untranslated region of Arf6 gene[59]. Overexpression of miR-1299 inhibits cell growth, and the effect can be attenuated by Arf6 overexpression. In general, it suggests that Arf6 is a tumor activator in the progression of gastric cancer. Active Arf6 significantly stimulates cell proliferation and induces the activation of phospholipase D (PLD)[60]. Arf6 N48I (a mutant Arf6) has no effect on the proliferation of cancer cells, but can promote motility, invasion, and matrix degradation. Mechanically, Arf6 stimulating cell proliferation is relative to Arf6 pro-mitogenic activity. Arf6 activates S6K1 kinase through PLD-mTORC1-dependent manner that regulates mitogen-stimulated translation initiation[60]. Otherwise, Arf6 activates the phosphorylation of ERK1/2 and p38MAP kinases. Interestingly, ERK1/2 significantly influences Arf6-dependent proliferation. These researches show the unique direction of Arf6 functioning in cell proliferation. In pancreatic cancer cell, silencing Arf6 down-regulates Rac-1 and p-ERK1/2 proteins and significantly inhibits tumor proliferation. Bisphenol A (BPA) reduces miR-149 expression to down-regulate tumor protein p53 and Arf6, and up-regulate CCNE2 expression, then interrupts cell cycle and induces cell proliferation. Furthermore, BPA-Arf6-inducing cell proliferation is attenuated by treatment with miR-107 inhibitor[61]. Arf6 is observed as a downstream target of Kras/ERK signaling pathway in pancreatic carcinoma cells[62]. Silencing Arf6 interrupts the signaling pathway, attenuates the Warburg effect and reduces cell proliferation[63]. EGF induces Arf6 expression in a dose-dependent manner in glioma cells. The levels of Arf6 and EGFR mRNA are elevated. Suppressing Arf6 gene expression by siRNA or the negative mutant significantly blocks EGF-induced cancer cell proliferation, demonstrating EGF-induced glioblastoma cell proliferation depending on Arf6.

3.3. *Arf6 and autophagy*

Arf6 plays a crucial role in the initiation of autophagosome formation. The mechanisms of Arf6 regulating autophagy involve in regulation of the phosphoinositide lipid, control of Atg-protein family, and membrane flow. Arf6 regulates autophagy by its contribution in the generation of phosphatidylinositol 4,5-bisphosphate[64]. It adjusts phagophores formation through interfering endocytic uptake of plasma membrane into small autophagosome precursors. ARF6 and phosphatidylinositol 4,5-bisphosphate locate with ATG12ATG5-ATG16L1 vesicles. Inhibition of ARF6 reduces phosphatidylinositol 4,5-bisphosphate level and breaks the formation of autophagosome. The plasma

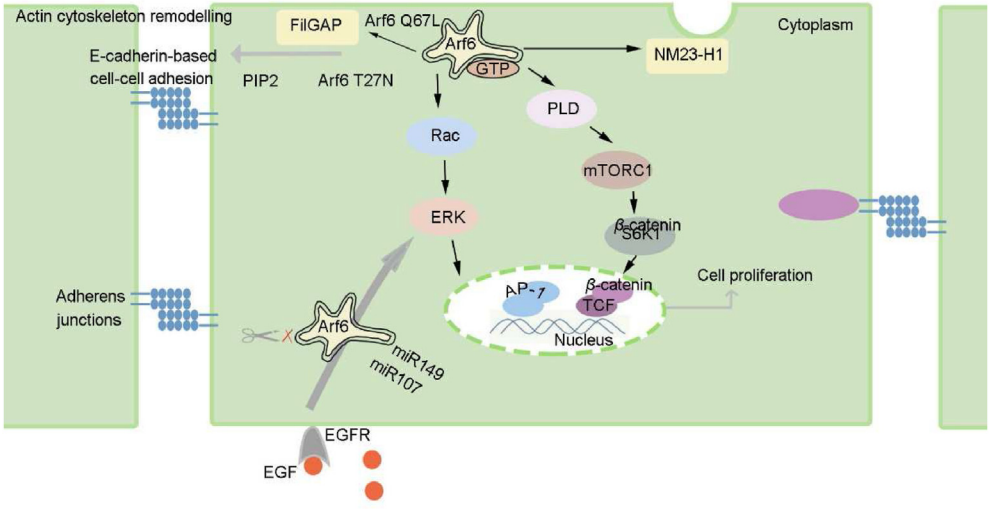


Figure 5 The roles and mechanisms of Arf6 in membrane traffic and cell proliferation.

membrane is essential for ATG5-ATG12-ATG16L1-positive phagophore precursor vesicles. Arf6 displays a co-localization with phagophore proteins Atg12 and Atg16L1 on autophagic vesicles. Knockdown of Arf6 decreases Atg16L1-and Atg12-positive vesicles in HeLa and CHO cells. Arf6 mutant (Arf6-N48R) decreases the LC3-II level of cancer cells[65]. This suggests that Arf6 plays a positive regulatory role in autophagy (Fig. 6). Otherwise, as mentioned earlier, Arf6 regulates membrane trafficking. During starvation-induced autophagy, phospholipase D1, the downstream of Arf6, can regulate the dynamics of autophagosome[66]. So, Arf6 might influence membrane flow to indirectly regulate autophagy.

3.4. *Arf6 and reactive oxygen species (ROS) production*

A novel association is found between cell movement and mitochondrial dynamics and is necessary for inhibiting detrimental reactive oxygen species production[67]. Blocking the Arf6 pathway inhibits β1-integrin recycling and the broken ILK located at FAs, which causes aggregation of mitochondria and increase of ROS level[67]. In Arf6 knockdown cells, the levels of γH_2AX and caspase-3 are detected. The repair of double-strand breaks is unfound, showing that Arf6 pathway induces mitochondrial ROS amplification (Fig. 6). AMAP1 (also called ASAP1 and DDEF1), the downstream effector of ARF6, promotes mitochondrial anterograde trafficking. Arf6-AMAP1 mediating mitochondrial distribution is reported in another research[67]. The pathway mediates integrin recycling, which promotes the formation of mature focal adhesions. Then, it recruits the complex of ILK and RhoT1, resulting in the increase of mitochondria anterograde trafficking and the amplification of ROS. Mitochondrial retrograde trafficking is controlled by RhoT1-TRAK2. The Arf6-based pathway blocks the association of RhoT1-TRAK2. Arf6 is involved in ROS-dependent vascular endothelial growth factor (VEGF) signaling[68]. A Rac-dependent NADPH oxidase is stimulated by VEGF, then increasing ROS production. Arf6 (T27N), the inactivation of Arf6 almost completely inhibits VEGF-induced responses and ROS production. Ang II enhances the expression of Arf6, accompanied by increase of Arf6 active form. After treatment of Ang II, ROS levels are dramatically increased and podocytes injury is induced. Nox4 is regarded as a pathway for producing intracellular ROS. The upregulation of Nox4 mediating oxidative stress was found in kidney injury[69]. The effect is regulated through Arf6-ERK1/2-Nox4 signaling, and can be prevented by Arf6 activity inhibitor or Arf6 knockdown[70]. These findings are confirmed by small molecular regulators. A novel benzyl indazole (CHS-111), inhibits $O_2^{\bullet-}$ generation and reduces the fMLP through interfering the interaction of PLD with Arf6. Another compound (Fal-002-2) inhibits fMLp-stimulated $O_2^{\bullet-}$ generation by destructing the activation of Arf6 and the interaction between PLD1 and Arf6[71].

3.5. *Arf6 and invasion/metastasis*

Arf6 regulates the invasion/metastasis of cancer cells by epithelial-mesenchymal transition (EMT) pathway. Investigating

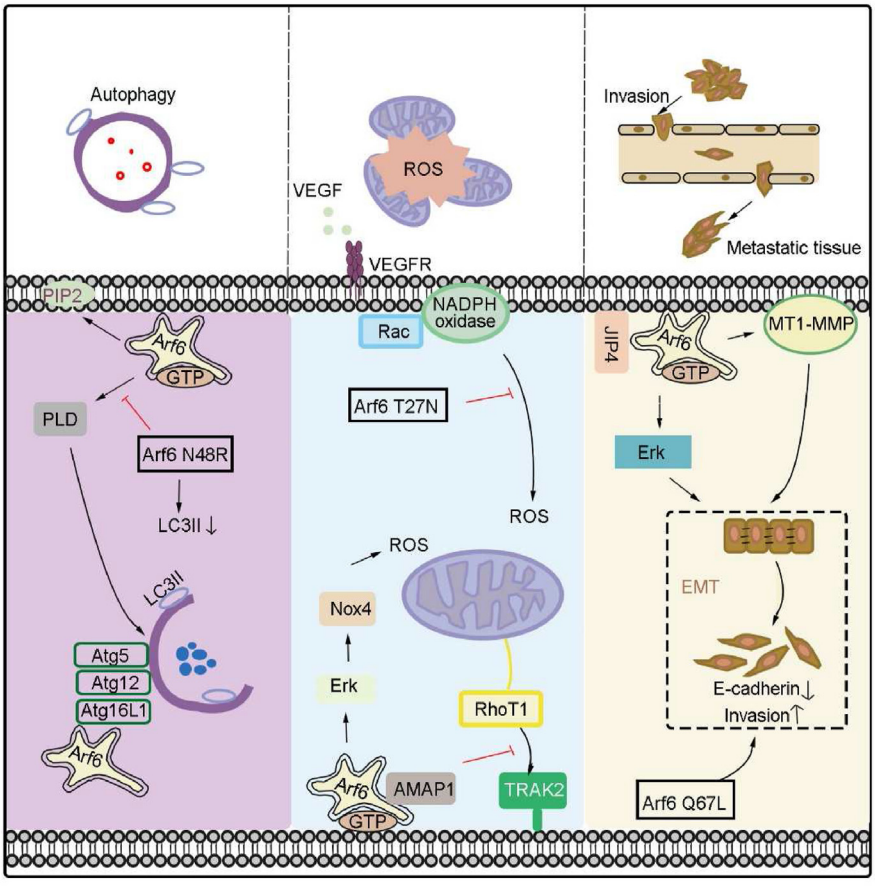


Figure 6 The roles and mechanisms of Arf6 in autophagy, ROS production and cell invasion/metastasis.

Arf6 in cancer metastasis focuses on local tissue invasion. Arf6 is involved in detach transformed epithelial cells through disassembling their cell−cell junction. Arf6 triggers the effects through regulating the internalization of E-cadherin[54]. Then, Arf6 recruits Nm-23-H1 to cell junctions to enhance E-cadherin internalization[55]. These results shows that Arf6 promotes E-cadherin internalization in cancer cells, and exerts pro-migratory action after EGF stimulated breast cancer cells. The effect of Arf6 on E-cadherin internalization facilitates EGF signaling stimulating EMT and dissolution of adherens junctions, which stimulates breast cancer cells epithelial-mesenchymal transition and migration[72].

Arf6 also regulates the invasion/metastasis of cancer cells by extracellular matrix (ECM) degradation. Cancer cells degrade the barriers of extracellular matrix to invade into surrounding stroma, after disassembly of cell−cell junction. Arf6 is involved in invadopodia formation degradation through regulating invadopodia formation and the release of tumor microvesicles[73]. Arf6 and AMAP1 are abnormally overexpressed in highly invasive MDA-MB-231 cells. Activation of Arf6 recruits AMAP1 together with paxillin and cortactin to the plasma membrane. The effects promote the formation of invadopodia[74]. Tumor-derived microvesicles are a class of extracellular vesicles with ECM degradative capacity, which promotes cell invasion. Arf6 activation increases the pre-miRNA cargo contained within tumor-derived microvesicles requiring the casein kinase 2-regulated RanGAP1 phosphorylation[73]. To release plasma membrane-derived microvesicles from cancer cells, activation of phospholipase D depending Arf6-GTP recruiting ERK to the plasma membrane. The release of microvesicle is influenced through phosphorylation of ERK and myosin light-chain kinase (MLC)[75]. Inhibition of Arf6 activation mediates phosphorylation of MLC and blocks microvesicle shedding. In addition, Arf6 and c-Jun NH2-terminal kinase-interacting protein 4 (JIP4) regulates membrane-tethered membrane type 1-matrix metalloproteinase (MT1-MMP)[76]. MT1-MMP is transported from the sorting endosome to invadopodia and is indispensable for ECM degradation. Arf6 is critical regulator of this process. Knockdown of Arf6 reduces MT1-MMP exocytosis and the invasion of breast cancer cells. MT1-MMP trafficking appears to require Arf6 activation.

In addition to the roles of Arf6 in cell invasion described above, the functions of Arf6 are also a critical cue in tumor microenvironment, especially tumor angiogenesis. Arf6 deletion abolishes HGF-stimulated β1 integrin recycling in endothelial cells[77]. Inhibiting Grp1, a GEF of Arf6, evidently suppresses vascularization and growth of tumor. The factor regulates HGF-stimulated β1 integrin recycling and tumor angiogenesis. Targeting the HGF-activated Arf6 shows the synergistic effect with anti-angiogenic drugs and thus improves clinical outcomes. Neovascularization supplies oxygen and nutrients to promote the grow of tumors. Consistent with above results, siRNA-mediated knockdown of Arf6 significantly suppresses their invasiveness and metastasis in multiple cancer cells, including breast cancer, melanoma cells[78,17]. In addition, Arf6 Q67L enhances invasive activity of cancer cells[79]. ARF6-AMAP1 pathway is related to the immune evasion of pancreatic ductal adenocarcinoma cells[80]. PDGF-induced recycling of PD-L1 can be suppressed by Arf6 knockdown, which remarkably affects cell viability and proliferation[80]. In general, Arf6 is a regulator for the invasion/metastasis (Fig. 6).

4. The structural features of Arf6 complexes

Arfs are ~20-kD guanine nucleotide-binding proteins and the structures can be termed the regions of switch I, switch II and interswitch[81]. Active Arf form, not unactive Arf form, can bind human effector proteins resulting from the different conformation of structural elements. Only GTP-bound ARF6 associates with effectors, and utilizes GTP binding energy to stabilize the switch regions[81]. Understanding the structure of Arf6 and Arf6-co complex is beneficial to the design of inhibitors.

Several regions are critical for the associate of Arf6 with effectors[82]. The superposition of Arf6 excludes the switch I (as Arf6 residues 32−48), the interswitch loop (as Arf6 residues 53−60) and the switch II (as Arf6 residues 65−80) (Fig. 7A). Switch I is also named as the “effector” loop. Switch II is the helix itself and the loop preceding a helix. Interswitch region comprises the loop connecting helix to strand. The regions of switches I and II are the major sites to regulate the interaction of Arf6 with effectors[83]. Ser38 coordinates the configuration of the nucleotide binding site, which drives Glu50 to form the hydrogen bond alternative interactions. Mutating Ser38 reduces the rate of GDP dissociation. The interaction of the switch I region and interswitch region forms an ordered β-strand. Arf6 activation are closely relative with rearrangements of the interswitch region (Fig. 7B). Thr53 and Lys58 in the interswitch regulate the switch I/II region by its N-terminal and C-terminal end, respectively[83]. The switch II region of Arf6−GDP is highly flexible and weak contacts near the switch II region.

The sequences of Arf1 and Arf6 are very similar. Surprisingly, Arf6−GDP localizes primarily to plasma membranes, while Arf1−GDP localizes cytosolic. The N-terminus of Arf1−GTP complex interacting with membranes, is shorter than Arf1, which raises the possibility that Arf1−GTP complex interacts with membranes[84]. Ser38 (Ile42 is equivalent residues in Arf1), controls the GDP-binding properties of Arf6, which is the explain of Arf GEFs distinguishing Arf1 and Arf6. The N-terminus is folded into an α-helix. The missing residues of Arf6 do not shorten the helix, but shorten the linker connecting the helix.

Several structures of Arf-GTP/effectors are determined. It is essential to summarize the characterization of the interaction with membranes. Structural feature shows that membranes are contributed by multipartite interactions of Arf and effector complexes. The complex models are helpful to understand the regulation of Arf6.

4.1. *Arf6 and Grp1*

The complex of Arf6-GTP with the Grp1 protein provides a guidance for the connection of Arf6/effectors and membranes. Grp1 is an Arf GEF family member that result in a positive feedback effect on the plasma membrane and endosomes[85] A pseudo-substrate mechanism leads to autoinhibition of Grp1. The Arf6 active form can partially reverse the autoinhibited mechanism[43]. Active Arf6 directly binds to the PH domain and coprecipitates Grp1 and (Fig. 8A and B)[20]. The exchange activity of the autoinhibited Grp163−399 construct is strongly stimulated by Arf6-GppNHp. The activation mechanism includes two states: “closed” state and “open” state. The “closed” state is the autoinhibited conformation. The “open” state is that sites are exposed for binding Arf6. It demonstrates the pseudo-substrate leading to autoinhibition of Grp1 family GEFs. It could occlude the Arf6 binding site. A phosphoinositide headgroup associates with the PH

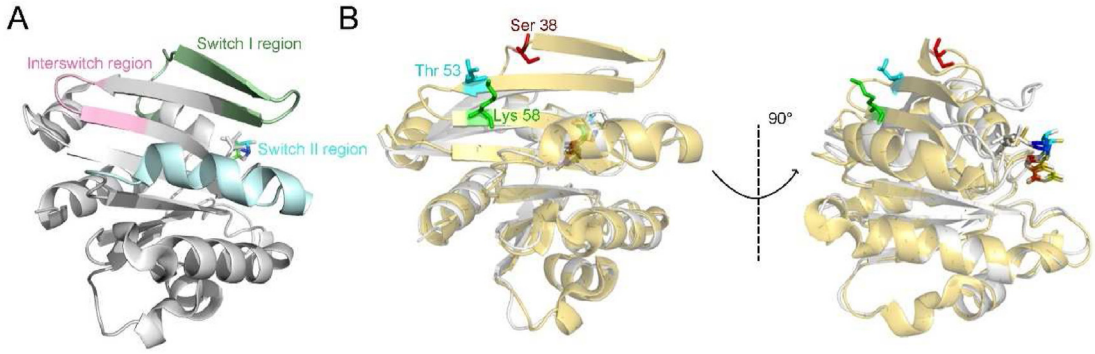


Figure 7 The structural features of Arf6. (A) The structure regions of Arf6 (PDB ID: 1E0S). (B) The comparison of structure between Arf6-GDP and Arf6-GTP.

domain of Grp1. It reveals that a contiguous surface is formed by Arf6 and the PH domain to contact with the membrane and precisely defines complex position[20]. The aromatic residues Phe47 and Tyr77 face the effectors, which plays a pivotal role in combination of Arf6 and different effectors. The comparison finds that the conformation of Tyr77 diverges in the complex of Arf6 and Grp1 compared to in Arf6 (Fig. 8C).

4.2. *Arf6 and JIP4*

The overall complex is organized with the JIP4-LZII (residue 392−462) homodimer and two Arf6 molecules, which forms an Arf6-$(JIP4)_2$-Arf6 heterotetramer[83]. Each JIP4 helix interacts with two opposing Arf6 molecules. The research does not observe the interaction of the two Arf6 molecules. The heterotetramer structure is including a twofold axis of symmetry that generates two interaction interfaces. The interaction of JIP4-LZII and with Arf6 mainly locate at switch II and the interswitch ($\beta2-\beta3$ strands) regions. The switch II and interswitch regions provide the specificity of JIP4-LZII binding Arf6 (Fig. 9A). Compared to Arf6, Phe47 and Tyr77 obviously diverge in the complex of Arf6 and JIP4 (Fig. 9B). JIP4 can interact with Arf6, not Arf1 or Arf5[86]. To survey JIP4's specificity, its interaction with Arf6 is analyzed by SPR assay. It confirms that JIP4 possesses a strong preference for Arf6[87]. The structural analysis suggests that four residues in Arf6 may play crucial roles in the specificity of JIP4 interaction, including Thr79 at the switch II region, Thr53, Lys58 and Asn60 in the interswitch region. These residues make hydrogen bonds with JIP4-LZII and are different in Arf1, which explains the lower affinity of JIP4 for ARF1 compared with Arf6. Four sequence differences between Arf6 and Arf1 also are experimentally explored, which verifies JIP4 discriminating between Arf6 and ARF1. The plasma membrane plays a critical role in orienting the Arf6−JIP4 complex[87]. At the plasma membrane, a heterotrimer is formed from a dimer of JIP4 interacting with one Arf6 molecule.

4.3. *Arf6 and cholera toxin*

Cholera toxin (CT), is composed of A1 (residues 1 to 192) and A2 domains (residues 193 to 240). A1 domain catalyzes the transfer of an ADP-ribose moiety and regulates enzymatic activity of toxin[88]. Structural studies report many cases where Arf-GTP allosterically regulating effectors. Such regulations are associated to conformational changes of the effector. Cholera toxin is allosterically regulated by binding of Arf6 GTP[89]. The CTA1 activation loop (residues 25 to 40), interacts with partners and is important in CT activation. An ordered coil is formed the loop region, but an amphipathic helix is rearranged and formed by residues 25 to 33 upon binding Arf6−GTP[90]. The structural analysis suggests that several residues in Arf6 may play crucial roles in the specificity of CT interaction by the switch I region (Fig. 9C and D). The residues Tyr30 and Phe31 of CT are toward to Arf6-GTP switch I. Arg25, Asp29, and Arg33 are toward to solvent. Here, CTA1 activation leads to a well-structured activation loop (residues 47 to 56)[91]. Arf6−GTP binding enables this

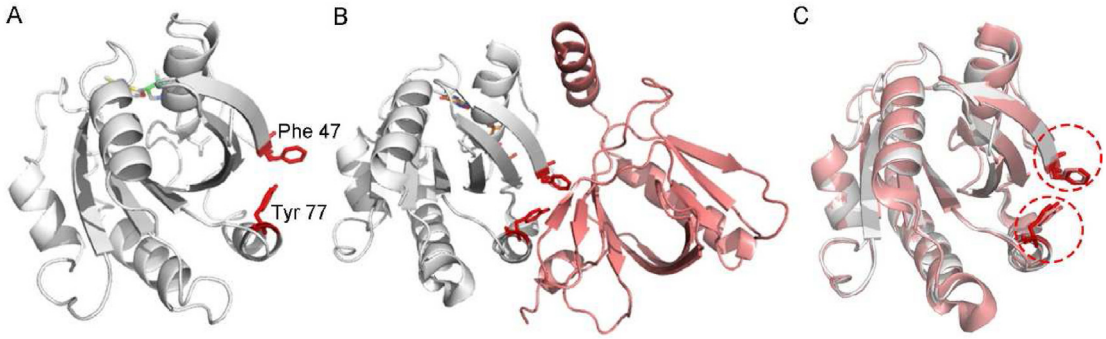


Figure 8 The structure characteristics of Arf6 and Grp1. (A) The structure of Arf6−GTP. (B) The structure of Arf6−GTP and Grp1 PH domain (PDB ID: 4KAX). (C) The comparison of structure between Arf6−GTP and Arf6−GTP in the complex of Arf6−GTP and Grp1 PH domain.

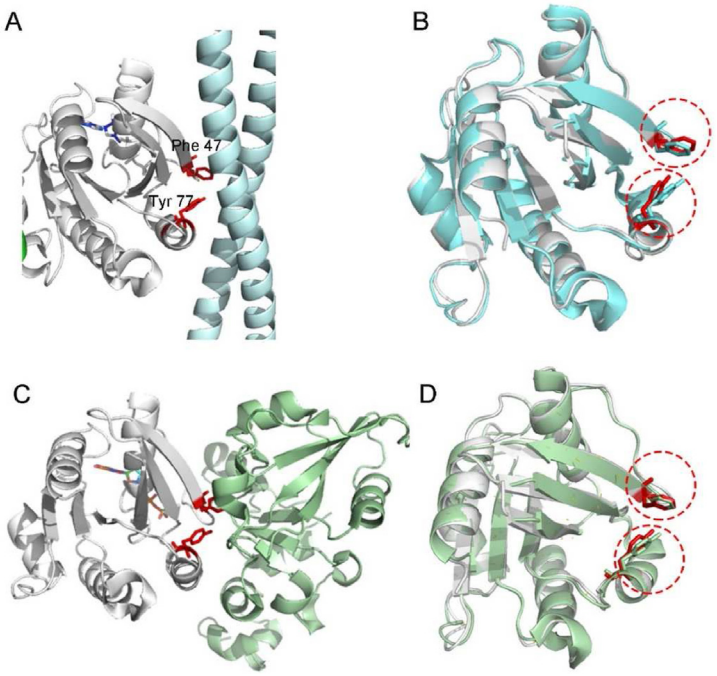


Figure 9 The structure characteristics of Arf6 complexes. (A) The structure of Arf6-GTP and JIP4 (PDB ID:2W83). (B) The comparison of structure between Arf6-GTP and Arf6-GTP in the complex of Arf6-GTP and JIP4. (C) The structure of Arf6-GTP and cholera toxin (PDB ID:2A5D). (D) The comparison of structure between Arf6-GTP and Arf6-GTP in the complex of Arf6-GTP and cholera toxin.

loop swinging out of the active site. The open conformation exposes residues Arg7, Ser61, Glu110, and Glu112, which implicates substrate binding and catalysis.

5. Development of Arf6 inhibitors and their potential on cancer treatment

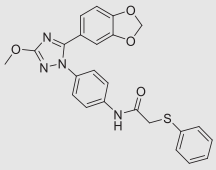
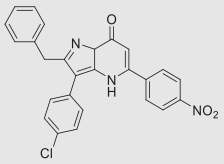
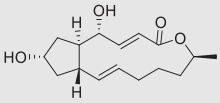
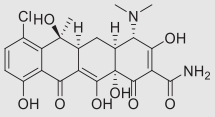
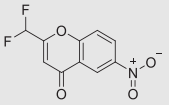
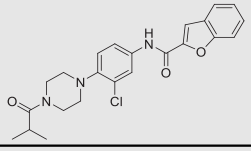
Arf6 overexpression regulates the progression and invasion of tumor, thus inhibiting the function of Arf6 could be useful for restraining cancer progression[90]. Presently, many studies have been completed to investigate the relation between Arf6 relative pathways and diseases. A number of inhibitors of Arf6 have been developed. These compounds mainly hinder the joining Arf6 and GEF. The inhibitors present at biological testing or preclinical phase, and we mainly introduce the inhibitory mechanism and pharmaceutical action. The relative inhibitors that are currently available to block the activation of Arf6 are listed in Table 1.

Sec7 inhibitor H3 (SecinH3), a non-specific Arf inhibitor, abrogates Arf6 signaling by binding and inhibiting the Sec7 catalytic domain of ARNO and deactivating ARNO[92]. The down-regulation of Arf6 hinders cell invasion. Moreover, SecinH3 can also reduce invasion by deactivating Arfs[93]. Otherwise, SecinH3 inhibits the proliferation of lung cancer cells by decreasing EGFR activation and promoting apoptosis *in vitro* and *in vivo*. Furthermore, the effects reduce lung cancer resistance to gefitinib[94]. Otherwise, the inhibitor reduces the migration, metastasis and tumor aggressiveness of multiple cancer cells and diminishes the growth of xenografts[95,96]. SecinH3 is employed as an Arf6 antagonist or a cytohesin inhibitor. Some SecinH3 derivatives are designed and synthesized (Fig. 10). The importance of several substituent groups is surveyed and documented, such as benzodioxan and sulfur bridge. The IC_{50} value for the GEF activity is determined. Compound **52** possesses twice inhibition potency as cytohesin antagonist activity compared to SecinH3[97]. Virtual screening is employed to identify the inhibitors of guanine nucleotide exchange. Secin**16** and Secin**69** possess three times inhibition efficacy compared to SecinH3, and can be used to investigate cytohesin signaling functions[98].

NAV-2729 directly binds to Arf6 and Arf6 GEF. The compound is found to bind to the binding area of Arf6 and its GEF through the analysis of the complex structure. NAV-2729 blocks GEP100- and ARNO-mediated guanine nucleotide exchange on Arf6, and is often served as an inhibitor of Arf6 activation. The inhibitor interferes with tumorigenesis and tumor growth[58]. NAV2729 inhibits proliferation of stromal cells and prostate smooth muscle contraction. In Arf6-expressing control clones, it inhibits proliferation (97%), and increases apoptosis (5-fold). The effects of NAV2729 are widely reduced, including decreases of apoptosis and cell death after Arf6 knockout[99].

The fungal metabolite brefeldin A (BFA) impairs Arf6 activation by influencing the association with Arf6 and its GEF. BFA molecule is planar and hydrophobic. The hydroxyl group in 13-membered ring is toward Arf and forms a hydrogen bond. The hydrophobic part of the molecule projects toward the Sec7 domain[100]. The 7-hydroxyl residue is essential to disrupt the form of Arf6−GEF complex, inactivating Arf6 action[101]. BFA can reduce cell proliferation through hindering Arf6 signal transducer and STAT3 phosphorylation[102]. The inhibitor presents obvious cytotoxic activity in several cancer cells, including in breast, lung, colorectal and prostate[103]. However, BFA possesses poor bioavailability and high toxicity, blocking the proceeding of phase 1 clinical trial. The derivatives of BFA are essential to increase the aqueous solubility, promote drug delivery and develop higher anticarcinogenic activity, thus improving its use in cancer therapy (Fig. 11). Furthermore, acetylated BFA derivatives can reduce the

Table 1 Small molecule inhibitors and their working mechanisms interfering with Arf6 signaling/cycling and cancer progression.

Compd.	Structure	Mechanism of action	Effect	Ref.
SecinH3		Inhibition of ArfGEF binding to Arf	Reduction of tumor growth, aggressiveness and metastasis	95,96
NAV-2729		Hindering of Arf6 and GEF joining	Inhibiting proliferation, increasing apoptosis and interfering with tumorigenesis and tumor growth	58,99
Brefeldin A		Hindering of Arf6 and GEF joining	Cytotoxic activity in several cancer cells	102
Chlortetracycline		Block of ArfGEF	Blocking both the Arf6-stimulated collective migration and cell invasion	105
Endosidin 4		Targets the Sec7 domain-containing Arf-GEFs	ES4 has higher affinity for Arf-GEFs than Brefeldin A	107
Rasarfin		The dual Ras and Arf6 inhibitor	Preventing cancer cell proliferation	108

viability by 500-times greater than BFA[104]. Otherwise, the excellent potency of ester derivatives is found in different cancer types[103].

Chlortetracycline (CTC) is obtained by a fluorescence-based high throughput screening[105]. The specificity of CTC on Arfs is evaluated. The assay presents that the compound inhibits GTP exchange. The computing simulation of CTC and Arf6 complex affords an association of the phenol diketone of CTC with Thr27 (localized in the P-loop, also interacted with the phosphate and Mg^{2+}) and Asp63 (a conserved residue in the switch II domain) of Arf6. Mechanically, CTC blocks Arf6 promoted cell migration and invasion in MDA-MB-231 cells. CTC shows phototoxic effect against melanoma cells with EC_{50} value of 250 μmol/L[106]. In the research, the effect of CTC is weaker than doxycycline, but it exhibits a greater safety because higher EC_{50} value is found in normal melanocytes. The mechanisms against melanoma cells involve in the decrease of GSH level and the stimulation of ROS production.

Identifying inhibitors of eukaryotic Arf-GEFs is carried out by a chemical genetics screen. Endosidin 4 (ES4) interferes with the activation of the Arf GTPases and has broad effects on intracellular trafficking[107]. The mode of ES4 inhibiting Arfs activation is similar to Brefeldin A. The complex structure is unreported, and the biochemical approaches and docking simulations confirm ES4 targeting the Sec7 domain. Endosidin 4 overcomes the effects of BFA, demonstrating that ES4 shows higher affinity than BFA. Combination of and ES4 and BFA achieves the synergistical effects. The research also verifies that ES4 widely inhibits trafficking processes[107].

Rasarfin, the dual Ras and Arf6 inhibitor, blocks GPCR activation through inhibition of Arf6[108]. Arf6 could regulate MAPK through inhibiting Ras pathway. The research initially seeks to identify receptor trafficking inhibitors by an endosomal BRET-based assay. The researchers have identified receptor trafficking inhibitors from approximately 115,000 small molecules. Present work reports the dual inhibitor, which effectively inhibits ERK1/2 signaling and MAPK and Akt signaling, further preventing cancer cell proliferation. The research data presents the possibility of targeting Ras and Arf6 to cancer therapy, and provides that new clues to develop the dual inhibitors.

6. Arf6 and therapy resistance

6.1. *Arf6 and breast cancer*

Increasing evidences confirm that Arf6 not only exerts a pivotal role in cancer development[62], but also is recognized as a negative

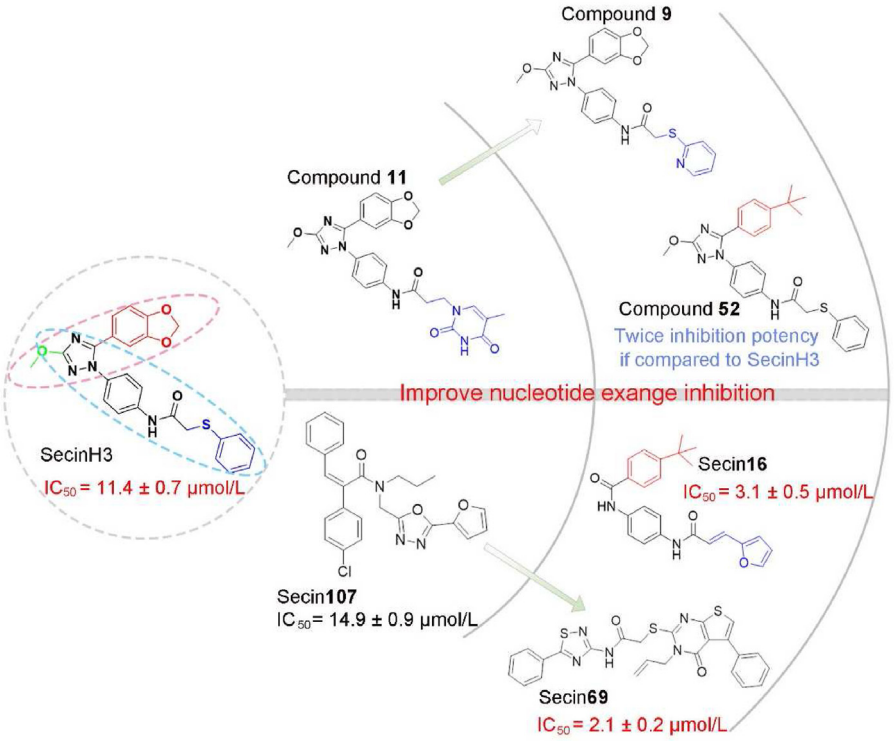


Figure 10 Chemical structure of SecinH3 and its derivative and the GEF activity.

prognostic factor in various types of human cancer, including breast cancer, gastric cancer, renal cell carcinoma and pancreatic cancer[80,109,110]. The association between the expression of mesenchymal genes and their acquisition of drug resistance is documented in breast cancer. EPB41L5, a partner of AMAP1, is a mesenchymal-specific protein in Arf6-based pathway and promotes focal adhesion dynamics[111,112]. Recent study in MDA-MB-231 cells shows that the expression of mesenchymal specific EPB41L5 is at high levels[113]. EPB41L5 may rely on E-cadherin signaling for regulating the drug resistance of cancer cells. Knockdown of EPB41L5 significantly improves the sensitivities of breast cancer cells to Gemcitabine and Temsirolimus.

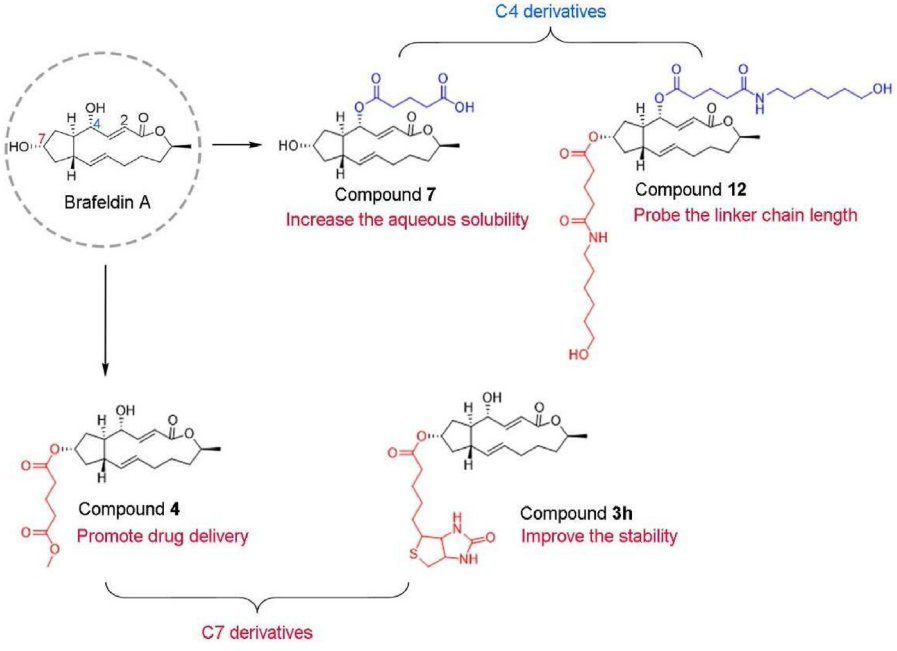


Figure 11 The inhibitor brefeldin A and its derivatives.

Simvastatin is proved to effectively improve the sensitivities of Gemcitabine and Temsirolimus through blocking TGFβ1-induced Arf6 activation. While the improvement by Simvastatin is not observed in several cells, which correlates with overexpress levels of the Arf6 pathway[114]. The Arf6-AMAP1-EPB41L5 mesenchymal pathway is great requirement for the therapy-resistant cancer cells. The silencing of EPB41L5 has been inversely correlated with treatment with Gemcitabine, Temsirolimus and Fluorouracil. Therefore, Arf6-based pathway is critical for the drug resistance[115].

6.2. *Arf6 and gastric cancer*

In glioblastoma cells, the study demonstrates that Arf6 is an absolute requirement for EGF-induced cell proliferation[16]. Arf6 plays critical roles in multiple cellular processes associated with tumorigenesis, including migration, invasion and EMT[116,117]. The EMT phenotype in cancers is closed relative with drug resistance[118,119]. The involvement of Arf6 in the drug resistance of glioblastoma cells is evaluated. The silencing of Arf6 exerts a pivotal role in the sensibility of SGC-7901 cells to 5-fluorouracil *in vitro*[120]. Inhibition of the ERK1/2 pathway increases 5-FU efficacy in glioblastoma cells. U0126, the specific ERK1/2 inhibitor, effectively increases knockdown of Arf6-mediated 5-FU sensitivity.

Otherwise, an association of Arf6 and ferroptosis is investigated in glioblastoma cells[121,122]. Arf6 affects the sensitivity of erastin-induced ferroptosis, which is not associated with the generation of lipid peroxidation. The study demonstrates that knockdown of Arf6 increases the level of GPX4 protein. Inactivating Arf6 increases the mRNA level of GPX4 and the downregulates GSH after the stimulation of erastin. Arf6 exerts the pivotal roles in erastin sensitivity, owing to inhibiting ACSL4. Furthermore, silencing of Arf6 shows lower elevation of ROS after the treatment of erastin. Arf6 pathway may regulate drug sensitivity in glioblastoma cells.

6.3. *Arf6 and renal cell carcinomas*

The origination of renal cell carcinomas is within epithelial ductal structures. The association between mesenchymal properties and malignant development is documented[123,124]. The research investigated whether renal cell carcinomas employ Arf6-associated pathway to survive, including drug resistance. Silencing of Arf6 effector AMAP1 in 786-O cells notably reduces cell survival on treatment with temsirolimus and sunitinib *in vitro*, whereas silencing of the gene does not significantly affect the viability. Silencing of EPB41L5, significantly reduces cells growth *in vivo* on treatment with Temsirolimus. Arf6-based mesenchymal pathway at high levels contributes to the drug resistance of renal cell carcinomas[125].

6.4. *Arf6 and pancreatic cancer*

In pancreatic cancer cells, the association of Arf6 and ferroptosis is reported[126]. It evaluates the sensitivity of Arf6 knockdown on RSL3 and erastin. Silencing Arf6 could robustly sensitize MIA PaCa-2 and PANC-1 cells to RSL3 induced ferroptosis, but not erastin-induced ferroptosis, indicating that Arf6 could regulate RSL3-induced ferroptosis. As for mechanism, Arf6 decreases the protein level of ACSL4 and aggravates gemcitabine resistance, which endows cancer cells to the status sensitizing to oxidative stress. These results present that Arf6 regulates ferroptosis and the effect exerts the significance for the development of pancreatic cancer.

7. Conclusions and prospectives

In recent decades, through technological advances, the understanding of ARF6 has progressed. Arf6 activation is regulated by several GEFs, including cytohesin, EFA6 and BRAG family. Arf6 initially is found to regulate cell motility and invasion/metastasis in tumor cells. It is also associated with other mechanisms of cancer cell growth and survival, including autophagy and ROS production. The structural features of Arf6 and its efforts are helpful to understand the actions of Arf6. Overexpression of Arf6 is observed in several cancers, such as breast cancer, gastric cancer, etc. Arf6 can be regarded as a promising target for therapy in cancer, due to the crucial roles of Arf6 in cancer. The status mentioned above can be suppressed by the inhibitors or small-interfering RNAs of Arf6. So, it attracted much attention to investigate some effective inhibitors.

Indeed, identification of Arf6 inhibitors is relative initiated. The inhibitory mechanism of the inhibitors mainly is to hinder the joining of Arf6 and GEF, which has nowadays become a major strategy using inhibitors that function as stabilizers. Future studies are needed to understand the lipid composition and dynamics of membrane, consider precise role of membrane to inhibition, and improve the efficiency of inhibitors. Lack of cocrystal structure of Arf6 and GEF is a main barrier. The binding modes guide rational design and improve the specificity of inhibitors. Knowledge of the three-dimensional structure is expected to develop new potent inhibitors through computer-aided drug design. Fragment-based drug design is the preferred strategy for protein−protein inhibitors. It will also be important to identify molecules disrupting the interface of protein/membrane without dissociation. However, low binding affinity of inhibitors and large contact surfaces of proteins remain a challenging to interfere protein-protein interfaces. Moreover, due to the high affinity of this nucleotide, it is difficult to develop inhibitors targeting the GTP binding site.

Moreover, Arf6 plays a vital role in drug resistance. Inhibition of Arf6 activation is important in cytotoxicity and tumor growth. Even though the detail mechanism remains unclear, studies that pharmacological or molecular blockage of Arf6 pathways improves the effectiveness of antitumor drugs. This view expands the use of Arf6 inhibitors to synergize the effects of therapeutic drugs and opens the perspectives for the design of dual target molecules. Although the research accidental discovers the first dual Arf6 and Ras inhibitor, the improved understanding of the Arf6 role is required to rationally choose the combination of dual targets.

Acknowledgments

The authors thank the National Natural Science Foundation of China (NSFC) (No. 81773594, 82204224), Chunhui Program-Cooperative Research Project of the Ministry of Education, Liaoning Province Natural Science Foundation (No. 2022-MS-241, China), China Postdoctoral Science Foundation (No. 2021M693957, China), Shenyang Young and Middle-aged Innovative Talents Support Program (RC210446, China), and Project of the Educational Department of Liaoning Province (No. LJKZ0919, China), for financial supports.

Author contributions

Dejuan Sun conceived the project and supervised the project. Yuanyuan Guo and Piyu Tang summed up the literature and drew the figures. Hua Li and Lixia Chen revised the manuscript. All authors approved the final manuscript.

Conflicts of interest

The authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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