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Targeting primary ciliogenesis with small-molecule inhibitors

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Abstract

The primary cilium is a generally non-motile solitary organelle that protrudes from a basal body at the cell surface in various cell types of multicellular organisms. This microtubule-based structure acts as a cell signaling platform to control key cellular processes including cell proliferation and differentiation in development and in adult tissues. Elongated and/or dysfunctional primary cilia cause developmental disorders termed ciliopathies and cancers. The genetic inhibition of ciliogenesis inducers can block the progression of these diseases in model organisms. Thus, pharmacological inhibition of primary ciliogenesis has emerged as a potential strategy to treat these pathological conditions. Pharmacological inhibitors that affect cilium assembly, in addition to having an impact on other cellular processes, have been identified. Here we review some of these tools and discuss their values and limitations in the study of the primary cilium biology, as well as for the treatment of some ciliopathies and cancers.

Keywords: Primary cilia, Primary ciliogenesis, Small-molecule inhibitors, Ciliopathies.

Main text

Background

The primary cilium. The primary cilium is an evolutionary conserved organelle that is assembled in various cell types from lower organisms to human (Rosenbaum and Witman, 2002). It is a microtubule-based structure which is generally non-motile and exists as a single structure per cell (Figure 1A). The core structure of primary cilia is called the axoneme, which is comprised of 9 parallel outer doublets of microtubules (A and B tubules, Figure 1B).

Primary ciliogenesis is the dynamic process of assembling the primary cilium (Ishikawa and Marshall, 2011). In proliferating cells, the primary cilium is transiently assembled on the cell surface during interphase. After cell division, each daughter cell inherits a centrosome composed of a mother and a daughter centriole. The mother centriole can rapidly differentiate to form the so-called basal body through maturation of subdistal and distal appendages (Figure 1C). Following actin and microtubule network reorganization, the centrosome migrates toward the plasma membrane where it anchors through the distal end of the basal body and nucleates microtubules for axoneme formation (Figure 1C). During the course of centrosome migration, a vesicle can associate with the distal end of the basal body to initiate axoneme formation prior to plasma membrane fusion in many but not all cell types (Figure 1C). Once docked to the plasma membrane, the distal end of the basal body forms a transition zone for the selective import of ciliary proteins that will enable complete cilium elongation at the cell surface (Nachury and Mick, 2019). Active vesicular trafficking at the cilium base constantly supplies essential components for axonemal elongation (Figure 1C). Continuous elongation occurs exclusively at the distal end of outer microtubule doublets. Constant import of ciliary proteins and transport of the cilium building blocks from the base to the tip of the axoneme is necessary to construct the primary cilium, and is mediated through a motor-driven process called intraflagellar transport (IFT) (Figure 2) (Rosenbaum and Witman, 2002). Upon completion of ciliary growth, a constant equilibrium between anterograde and retrograde IFT transport regulates axonemal assembly and disassembly to maintain primary cilium length (Figure 2) (Rosenbaum and Witman, 2002).

Primary cilium's function. The function of the primary cilium remained widely neglected until the discovery that it is essential for normal development (Pazour et al., 2000), through its function as a cell signaling platform, and notably for the regulation of Hedgehog signaling (Huangfu et al., 2003). Additional ciliary signaling pathways have now been identified and have been extensively reviewed elsewhere (Nachury and Mick, 2019). Given the key role of the primary cilium as a cell signaling hub, either loss, shortened or elongated/dysfunctional primary cilia cause severe developmental disorders, collectively termed ciliopathies (Reiter and Leroux, 2017). Ciliopathies are multisystemic diseases that are caused by mutations in genes that code for ciliogenesis and/or ciliary-signaling regulators, and are characterized by brain, skeletal, kidney developmental defects, among others. The most prominent ciliopathy is polycystic kidney disease (PKD), with the autosomal dominant form (ADPKD) being the most common of all inherited cystic kidney disease. It is caused by mutations of the *PKD1* or *PKD2* genes that encode key cilium components, which result in elongated/dysfunctional primary cilia (Reiter and Leroux, 2017). Strikingly, genetic inhibition of primary ciliogenesis inducers in the kidney of ADPKD mouse models is sufficient to block disease progression (Ma et al., 2013, Husson et al., 2016).

Beyond its requirement for proper tissue development, important work of the last decade has revealed that the primary cilium plays a key role in the formation and/or progression of various cancers (Eguether and Hahne, 2018). For instance, it can either repress the initiation and metastatic progression of melanoma, or promote the formation of Hedgehog-dependent basal cell carcinoma, medulloblastoma, choroid plexus tumors, as well as drive the formation and metastatic progression of non-luminal breast cancers (Guen et al., 2017a, Gencer et al., 2017). Additionally, primary cilia and ciliary signaling can mediate the sensitivity of medulloblastoma cells to Smoothed inhibitors (Zhao et al., 2017) and promote tumor cell resistance to therapy in rhabdomyosarcoma, non-luminal breast and lung cancer models (Jenks et al., 2018, Zhu et al., 2019).

Small molecule inhibitors of primary ciliogenesis.

Our understanding of the role of primary cilia in development and disease mainly comes from genetic invalidation strategies of essential ciliogenesis regulators in model organisms. However, these strategies have limitations for the study of a dynamic structure such as the primary cilium in

normal and pathological conditions. One emerging goal in the field is to develop highly specific small-molecule inhibitors of primary ciliogenesis to facilitate the study of primary cilia in a highly dynamic manner. Additionally, such compounds could be highly valuable pharmacological tools for the treatment of at least some ciliopathies and cancers, which are caused by dysfunctional primary cilia (Husson et al., 2016, Nikonova et al., 2018). Despite the fact that no specific small-molecule primary ciliogenesis inhibitor exist, here we review some classes of small-molecules that were found to be able to inhibit cilium assembly, while also impacting other cellular processes (Figure 3).

Cytoskeletal drugs. The first chemical compounds that were identified as ciliogenesis modulators constitute a class of cytoskeletal drugs.

Pioneering work revealed three decades ago that ciliary microtubules are more stable and more resistant to those compounds than cytoplasmic microtubules (Jensen et al., 1987). Neither the microtubule-depolymerizing drug colcemid nor the microtubule-stabilizer taxol affected the structure of primary cilia formed in cells in interphase prior treatment. Upon cell cycle progression and mitotic entry, the drugs also did not affect cilia disassembly. In 4N micronucleated G0/G1 cells that arose from spindle microtubule defects, only those treated with high doses of colcemid failed to re-assemble primary cilia. However, a mature basal body was able to dock at the plasma membrane in these cells. These results have indicated that microtubule-depolymerizing drugs can inhibit *de novo* primary ciliogenesis at late stages of cilium assembly when used at a high concentration.

More recently, the discovery that regulators of actin dynamics control primary ciliogenesis, prompted two independent groups to study the impact of actin-targeting drugs on primary ciliogenesis (Kim et al., 2010, Sharma et al., 2011). Both studies found that the actin polymerization inhibitor Cytochalasin D induces primary ciliogenesis. Hence, one group studied the putative inhibitory role of the actin stabilizer Jasplakinolide on primary ciliogenesis (Sharma et al., 2011). Unexpectedly, Jasplakinolide was also found to induce primary ciliogenesis. Sharma et al. found that both Cytochalasin D and Jasplakinolide induced an increase of soluble tubulin available for cilium elongation, which could be inhibited by taxol in this setting. These results

revealed that microtubule stabilizers may inhibit ciliogenesis that is induced by actin-targeting drugs.

Altogether, these data revealed that microtubule poisons can inhibit primary ciliogenesis in specific contexts. Whether this inhibitory role is the result of a direct inhibition of ciliogenesis through the perturbation of axonemal microtubule dynamics and/or of an indirect inhibition of ciliogenesis, through the perturbation of the cytoplasmic cytoskeleton, remain to be more thoroughly studied. In their study, Sharma et al. pointed-out that cytoskeletal drugs have major direct effects on membrane, vesicular transport, cell division, and cell spreading among other cellular processes, which can impact ciliogenesis through many indirect mechanisms. This finding highlights the limitations of using such compounds to target primary ciliogenesis.

Hedgehog pathway inhibitors/Molecular motor inhibitors. The primary cilium is specialized for Hedgehog signal transduction. Therefore, it is not surprising that initial studies aimed at identifying Hedgehog pathway inhibitors found primary ciliogenesis repressors.

A first study identified inhibitors of Hedgehog signaling in a large-scale screen (Hyman et al., 2009). Thorough characterization of the mechanism of action of those newly identified drugs revealed that the small molecule HPI-4 inhibits primary ciliogenesis and thereby represses Hedgehog signaling. In a subsequent study, it was revealed that HPI-4, renamed Ciliobrevin A, is a Dynein inhibitor (Firestone et al., 2012). It represses primary ciliogenesis through inhibition of Dynein-2-dependent retrograde IFT. However, it was also demonstrated that Ciliobrevin A can inhibit the ATPase activity of cytoplasmic Dynein, likely in a nucleotide competitive manner, thus regulating many other cilium-independent cellular processes such as the regulation of mitotic spindle formation (Firestone et al., 2012).

A second large-scale screen identified two Hedgehog pathway inhibitors, CA1 and CA2, as primary ciliogenesis repressors (Wu et al., 2012). To address the mechanism of action of these drugs, the authors studied the basal body and microtubule network organization of cilia-deficient cells upon treatment. CA1-treated cells exhibited reduced basal body γ -tubulin, dispersal of γ -tubulin into multiple foci, and a disorganized cytoplasmic microtubule cytoskeleton. CA-2-treated cells displayed a major loss of microtubules throughout the cell.

Collectively, these studies identified Hedgehog pathway inhibitors that repress primary ciliogenesis in a direct manner by perturbing IFT. However, these drugs also perturb many cytoplasmic cellular processes that indirectly influence primary ciliogenesis, including cytoplasmic dynein-dependent processes, centriole duplication, or more generally the microtubule cytoskeleton organization, in a similar manner to cytoskeletal drugs. Thus, such compounds also have limitations to specifically target primary ciliogenesis.

Protein kinase inhibitors. A few protein kinases have been identified as key regulators of ciliogenesis.

Fibroblast Growth Factor Receptor 1 (FGFR1) was identified a decade ago as a key positive regulator of ciliogenesis during embryonic development in lower organisms (Neugebauer et al., 2009). Pharmacological inhibition of FGFR1 *in vivo*, using the FGFR1 inhibitor SU5402, was found to repress ciliogenesis and perturb embryogenesis, at the shield stage of development, in the zebrafish embryo (Neugebauer et al., 2009). Nevertheless, treatment at later stages did not affect ciliogenesis but impacted normal development, indicating that FGFR1 has stage-specific, cilium-specific and independent roles in early embryonic development. More recently, an independent *in vitro* study suggested that in human cancers, FGFR1 promotes primary ciliogenesis in cells that are resistant to diverse kinase inhibitors (Jenks et al., 2018). Importantly, it was found that pharmacological inhibition of FGFR1, using BGJ398, represses primary ciliogenesis and sensitizes pre-resistant cells to treatment (Jenks et al., 2018). A recent study offered more insights into the mechanism by which inhibition of FGFR1 results in ciliogenesis repression (Honda et al., 2018). In this work, the authors began by demonstrating that FGFR1 positively controls ciliogenesis in the hair cells of the inner ear of the chick embryo. In this context, ciliogenesis could be repressed by SU5402. SU5402 inhibited FGFR1-mediated phosphorylation of Pcdh15, which resulted in a loss of its ciliary localization and of its interaction with IFT-B complex proteins, and led to inhibition of ciliogenesis. These data offered a straightforward explanation of the mechanism of action of FGFR1 inhibitors on cilia.

Altogether, these studies indicated that FGFR1 inhibitors can directly repress primary ciliogenesis but can also repress FGFR1 cilium-independent function(s). Notably, both SU5402 and BGJ398 are not specific to FGFR1 but can also target other FGF receptors, and kinases from independent

families, which have independent and widespread functions, emphasizing the limitations in using such compounds to specifically target primary ciliogenesis.

Cyclin-Dependent Kinases (CDK) have emerged as key primary ciliogenesis regulators (Maskey et al., 2015, Husson et al., 2016, Guen et al., 2016, Guen et al., 2017c, Guen et al., 2017b). In mammals, CDKs can either act as primary ciliogenesis inducers or inhibitors. CDK5 has emerged as a positive regulator of primary ciliogenesis and may do so by controlling a dynein-dependent IFT regulator Nde1 (Maskey et al., 2015) and/or a general microtubule network regulator CRMP2 (Husson et al., 2016). Importantly, earlier studies found that Roscovitine and its analogue (S)-CR8, two potent CDK5 inhibitors, dramatically suppress cystogenesis in PKD mouse models (Bukanov et al., 2006, Bukanov et al., 2012). This beneficial *in vivo* effect was later attributed to Roscovitine/(S)-CR8-dependent reduction of primary cilia length through the inhibition of CDK5 and of CRMP2 phosphorylation (Husson et al., 2016). Thus, Roscovitine and (S)-CR8 may repress ciliogenesis through direct inhibition of IFT and indirectly through a general perturbation of the microtubule cytoskeleton, as described above for cytoskeletal drugs. Additionally, the beneficial impact of Roscovitine and (S)-CR8 on cystogenesis is likely due to a combination of effects on primary ciliogenesis as well as on the cell cycle machinery, since abnormal proliferation occurs during cystogenesis in PKD.

Concluding remarks

In summary, these studies unveiled classes of small molecule inhibitors that repress primary ciliogenesis, in addition to having an impact on other cellular processes (Figure 3). Recent studies have identified drugs from different compound classes with unexpected inhibitory effects on primary ciliogenesis, including an HSP90 inhibitor (Ganetespib) (Nikonova et al., 2018), a Neddylation inhibitor (MLN4924) (Mao et al., 2019), and tyrosine kinase inhibitors (sunitinib, erlotinib) (Kiseleva et al., 2019). They all promote cilium disassembly. Again, all of these drugs can repress primary ciliogenesis as well as other independent cellular processes. Efficient and specific small molecule inhibitors of primary ciliogenesis remain to be discovered. Such inhibitors should inevitably target more specific regulators of ciliogenesis than those described above. For

example, one might predict that targeting protein-protein interactions, instead of targeting enzymes, within IFT multiprotein complexes could be an efficient and specific manner to perturb primary ciliogenesis.

The efficacy of some of the drugs described here in treating ciliopathies in mouse models suggests that primary ciliogenesis inhibition may be a valuable strategy to treat at least a subset of these diseases (Husson et al., 2016, Nikonova et al., 2018). Primary ciliogenesis inhibition may also be effectively used in combination therapies for the treatment of specific neoplasias. For both of these clinical applications, specific ciliogenesis inhibitors will be necessary to limit the side effects that such compounds may have for patients who receive these treatments long-term. The development of targeted drug delivery approaches to localize ciliogenesis inhibitors to the target tissue site where primary cilia display a detrimental role will also be critical to limiting the eventual side effects of the drugs. Although the clinical development of primary ciliogenesis inhibitors faces many challenges, such small-molecules will be of high interest for basic research on the biology of the primary cilium, as we still have much to learn about its role in development and disease.

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Conflicts of interest

The authors declare no competing interests.

Figure legends

Figure 1: The Primary Cilium and primary ciliogenesis (A) Transmission electron micrograph of the primary cilium protruding from the basal body at the cell surface of a human retinal pigment epithelial cell (hTERT-RPE1), Scale bar 250 nm. **(B)** Schematic representation of a cross section of the axoneme of a cilium. **(C)** Schematic representation of the mechanisms associated with the transition from the mother centriole to the basal body and primary ciliogenesis.

Figure 2: Intraflagellar Transport. Schematic representation of the bi-directional movement of cilium components essential for cilium assembly and function. Kinesin-2-driven anterograde transport enables the movement of the IFT complex B from the base to the tip of the cilium. Dynein-2 driven retrograde transport enables the movement of the IFT complex A, which transports cilium components from from the tip to the base of the cilium.

Figure 3: Schematic representation of the mechanism of action of small-molecules with direct anti-ciliogenesis properties. FGFR inhibitors SU5402, BGJ398 are both suspected to repress primary ciliogenesis through inhibition IFT-B-dependent anterograde IFT. Dynein-2 inhibitor, Ciliobrevin A also known as HPI-4, is thought to repress primary ciliogenesis through inhibition of Dynein-2-dependent retrograde IFT. CDK inhibitors, Roscovitine and (S)-CR8 are suspected to repress primary ciliogenesis through CDK5 inhibition. CDK5 has emerged as a positive regulator of Dynein-2 dependent retrograde IFT.

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