Pyrvinium Attenuates Hedgehog Signaling Downstream of Smoothened

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Introduction

The Hedgehog (HH) signaling pathway plays key instructional roles during embryonic development and adult tissue homeostasis. Consistent with this pivotal instructional role, the HH signaling pathway is commonly deregulated in many human cancers (1). The role HH plays in cancer was first identified in the inherited disorder Gorlin syndrome, which predisposes to basal cell carcinoma, medulloblastoma and rhabdomyosarcoma, and results from loss-of-function mutations in the gene encoding the HH core receptor component Patched1 (Ptch1; ref. 2). Spontaneous cases of these tumors were subsequently shown to result from mutations or amplifications of a number of HH signaling components, including Ptch1. Strong support for the role that HH signaling plays in these cancers was provided from a number of genetic mouse models of HH-driven medulloblastoma, in which mutations in HH signaling components lead to the genesis of the same tumors (3, 4). The growth of the tumors in these mice could also be abrogated by treatment with HH signaling inhibitors (5). The pharmaceutical industry is currently focused on developing small molecules targeting Smoothened (Smo), a key signaling effector of the HH pathway, pyrvinium is able to inhibit the activity of a clinically relevant, vismodegib-resistant Smo mutant, as well as the Gli activity resulting from loss of the negative regulator suppressor of fused. We go on to demonstrate the utility of this small molecule in vivo, against the HH-dependent cancer medulloblastoma, attenuating its growth and reducing the expression of HH biomarkers. Cancer Res; 74(17); 4811–21. ©2014 AACR.
regulates the activity, proteolytic processing, and stability of members of the Gli family of transcription factors, Gli1-3 (7). This regulation requires a number of protein kinases, including protein kinase A (PKA), glycogen synthase kinase 3 (GSK3), and casein kinase $\alpha$ (9–12), and the negative regulator suppressor of fused (Sufu; refs. 13, 14). Mammalian HH signaling requires trafficking through primary cilia, a membrane-encased microtubule-enriched organelle located on the apical side of polarized cells (8, 15). Many components of the HH signaling pathway transit through the primary cilia in their basal state and leave or enrich there in response to HH (8). During this trafficking through the specialized environment of the primary cilia, Gli proteins, likely through their interaction with Sufu, are converted into their repressor forms or in response to HH converted into their active forms (16–18). In the basal state, Gli2 and Gli3 are hyperphosphorylated at their Cul1-dependent degrons (10, 11, 19). Subsequent to ubiquitination, Gli2 and Gli3 are partially cleaved by proteasomes into their repressor degrons (10, 11, 19). Subsequent to ubiquitination, Gli2 and Gli3 become differentially phosphorylated by PKA, at a distinct amino-terminal domain, converting them into their activated nuclear forms (19–21). Nuclear-enriched Gli2 and Gli3 are labile and are quickly degraded by the proteasome through a Cul3-mediated ubiquitin proteasome system (17, 22).

We recently reported that the FDA-approved drug pyrvinium is a novel and potent small molecule inhibitor of the Wnt proteasome system (17, 22). Pyrvinium acts to regulate Gli activity and stability downstream of $\text{Smo}$, in a CK1 $\text{a}$ dependent manner, including attenuating HH signaling, and cellular binding studies demonstrated that pyrvinium acts as an allosteric activator of this protein kinase. Our $\text{in vitro}$ and cellular binding studies demonstrated that pyrvinium binds avidly to CK1 $\alpha$ (Kd, 1 mmol/L; ref. 23). Of the CK1 family members ($\alpha$, $\gamma$, $\delta$, and $\epsilon$), only CK1 $\alpha$ is activated by pyrvinium. Pyrvinium has no effect on the activities of a panel of other protein kinases representing all of the major branches of the kinase superfamily (23), demonstrating that pyrvinium selectively binds to and activates CK1 $\alpha$. As CK1 $\alpha$ is implicated as a negative regulator of the HH signaling pathway, we hypothesized that pyrvinium might inhibit HH signaling (24). Here, we show that pyrvinium does indeed attenuate HH signaling, and does so $\text{in vitro}$ and $\text{in vivo}$—attenuating the growth of a well-accepted HH-driven mouse tumor model. Furthermore, pyrvinium acts to regulate Gli activity and stability downstream of Smo, in a CK1 $\alpha$-dependent manner, including attenuating HH activity driven by a clinically relevant, vismodegib-resistant Smo mutation (6).

Materials and Methods

Cell culture

NIH-3T3, HEK 293T, and Light-II cells were purchased from the American Type Culture Collection (ATCC) and were grown in medium as indicated by ATCC’s instructions. Cerebellar granular precursor cells (GPC) were isolated as previously described (25). NIH-3T3 cells stably expressing HA-Gli2 were a gift of Dr. Philip Beachy (Stanford University, Stanford, CA). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Myc- and Flag-tagged Gli1 and Myc-tagged $\beta$-TREP were gifts of Dr. Anthony Oro (Stanford University, Stanford, CA). Myc-Gli2 and GFP-Smo were from Addgene. Various Gli-null MEF cells were gifts of Dr. Wade Bushman (University of Wisconsin, Madison, WI; ref. 26). shRNA constructs described here were purchased from Open Biosystems, and used to prepare lentivirus as described therein. shRNA-expressing cell lines were selected by 10 $\mu$g/mL puromycin. Smoothened agonist (SAG) and pyrvinium treatments were performed in the presence of 0.5% FBS.

Assays

Luciferase activity was determined using a luciferase detection kit (Promega), as previously described (27). Total RNA from cells or tissues was extracted using the RNAse kit (Qiagen), converted into cDNA (Applied Biosystems), then analyzed using real-time RT-PCR and the cognate Taqman probes, as per the manufacturer’s instructions (Invitrogen). Immunohistochemical analysis of Smo localization to primary cilia was performed as previously described (27). Immunohistochemical staining of CK1 $\alpha$ (Pierce) and Gli1 (27) were carried out using a Dako autostainer at the pathology core laboratory of the University of Miami. $\text{In vitro}$ phosphorylation of recombinant human Gli1 by CK1 $\alpha$ (Invitrogen) using radiolabeled [32P]ATP was performed as previously described (28). Hexahistidine-tagged human Gli1 was produced using the S9/ baculovirus system. Statistical analysis was determined by the Student two-tailed t test, unless other stated. P values $\leq$ 0.05 were considered statistically significant.

Biochemistry

Immunoblot analysis was carried out with the following primary antibodies: anti-Gli1 (Cell Signaling Technology), anti-Gli2 (R&D Systems), anti-Gli3 (9), anti-HA (Santa Cruz Biotechnology), anti-Myc (Santa Cruz Biotechnology), anti-Flag (Sigma), anti-Tubulin (Sigma), anti-Gapdh (Millipore), anti-ERK (Santa Cruz Biotechnology), anti-pAKT (Cell Signaling Technology), anti-pGSK3 (Cell Signaling Technology), anti-p-\beta-catenin (Cell Signaling Technology). CA-AKT plasmid (myrAkt; Addgene), Baflomycin A1 (Sigma), and MG132 (Calbiochem) were purchased. For immunoprecipitation, cells were lysed in a buffer containing 50 mmol/L Tris–HCl (pH, 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% NP-40, supplemented with cOmplete Mini Protease Inhibitor Cocktail (Roche). After centrifugation, the supernatants were incubated with the indicated antibodies overnight at 4°C, and the immunoprecipitates were extensively washed with lysis buffer, eluted with SDS sample buffer, and boiled for 5 minutes before analyses by immunoblotting.

Mice and drug administration

All mice were handled in accordance with the policies of the University of Miami Institutional Animal Care and Use Committee. Spontaneous medulloblastomas from $\text{P}t\text{ch}^{\text{1/2}}$ mice (The Jackson Laboratory; $\text{P}t\text{ch}^{\text{1tm1Mps/J}}$) were gifted onto CD-1 nude mice (Charles River Laboratories) subcutaneously. Drug treatment started when the tumors reached a size of approximately 100 mm$^3$. For acute treatment, pyrvinium was dissolved in DMSO and delivered through intraperitoneal injection.
injection for the indicated times. For local peritumoral delivery, pyrvinium chloride was resuspended in PBS containing 10% (2-Hydroxypropyl)-cyclodextrin and injected close to the tumor nodule every other day.

**Results**

We recently identified CK1α as the critical cellular target of pyrvinium and showed that pyrvinium acts as a novel allosteric activator of its protein kinase activity (23). As CK1α is also a key regulator of HH signaling (24), we hypothesized that pyrvinium would function as an inhibitor of this important developmental signaling pathway. To test this hypothesis, we evaluated pyrvinium’s ability to attenuate HH signaling. We stimulated HH activity in a HH reporter cell line (Light-II cells), using either SHH or the Smo agonist SAG, and in both cases pyrvinium attenuated HH activity in a potent (IC₅₀ of ~10 nmol/L), dose-dependent manner (Fig. 1A). Pyrvinium also inhibited HH-dependent expression of Gli1 and Ptch1, two well-established biomarkers of HH activity (Fig. 1B; ref. 1). In contrast, the inactive structural analog of pyrvinium VU211 (23) was unable to attenuate the expression of these HH biomarkers. We next evaluated the ability of pyrvinium to inhibit the proliferation of HH-dependent primary GPC, another well-established readout of HH activity (25, 29). Primary GPC were isolated and treated with SHH plus pyrvinium or the Smo antagonist cyclopamine. Pyrvinium attenuated GPC proliferation, and did so in a manner similar to cyclopamine (Fig. 1C). These data indicate that pyrvinium is a potent inhibitor of HH signaling.

Pyrvinium has been reported to exhibit biologic effects via mechanisms distinct from CK1α activation (30-33). Most...
recently, it has been suggested that pyrvinium attenuates Wnt activity by blocking activation of AKT, preventing GSK3β phosphorylation and its subsequent inactivation, which results in the phosphorylation and destabilization of β-catenin (34). However, we show that the expression of a constitutive active AKT construct did not abolish pyrvinium’s suppression of HH signaling (Supplementary Fig. S1A). Furthermore, we recently determined pyrvinium’s ability to bind to the active site of a large panel of protein kinases (442 purified kinases) using Ambit’s scanMAX kinase profiling service (data not shown). This screen examined the ability of pyrvinium to outcompete kinases bound to a set of immobilized active site ligands. These ligands bind to the active site of one or more kinases with high affinity ($K_d < 1 \text{nmol/L}$). Different concentrations of pyrvinium were used to outcompete the individual kinases from the immobilized ligands. Our data suggest that pyrvinium does not bind to the active site of CK1α, as pyrvinium did not compete with CK1α binding to its immobilized ligand in this assay. Consistent with this result, we did not observe the effects of ATP on pyrvinium activity. We did, however, observe effects of pyrvinium on the conformation of CK1α as detected by an altered trypsin proteolysis pattern (23). Significantly, the scanMAX kinase screen failed to detect inhibition of any of the kinases tested (using 1 μmol/L of pyrvinium), which included AKT1-3 isoforms and all known PI3-kinases. Furthermore, consistent with pyrvinium acting through CK1α, we show that the addition of pyrvinium to cells results in the time-dependent phosphorylation of a known CK1α substrate (Supplementary Fig. S1B).

To more directly show that pyrvinium’s ability to inhibit HH signaling is CK1α dependent, we identified two shRNAs capable of reducing CK1α protein levels (Supplementary Fig. S2A). Light-Ii cells infected with this lentivirus were treated with SAG to stimulate HH activity, followed by pyrvinium treatment. CK1α knockdown reduced the capability of pyrvinium to attenuate the expression of Gli1, relative to cells infected with control shRNA lentivirus (Fig. 1D). We also observed that knockdown of CK1α reduced the overall levels of SAG-induced HH target gene expression, as might be expected, given the positive role that CK1α also plays in Smo activation (35).

The HH and Wnt signaling pathways negatively regulate or positively reinforce each other depending on cell context (36). Thus, CK1α agonists could potentially attenuate HH signaling indirectly via inhibition of Wnt signaling. To test this possibility, we asked whether the Wnt inhibitors IWR-1 or XAV939, which mediate their activity via mechanisms distinct from pyrvinium, similarly inhibit HH signaling. We found that IWR-1 and XAV939 did not suppress SAG-stimulated HH activation, nor did they potentiate the capacity of pyrvinium to inhibit HH signaling (Fig. 1E and data not shown). We then treated Gli2$^{−/−}$; Gli3$^{−/−}$ immortalized mouse embryonic fibroblasts (MEF), which are not capable of responding to HH (26), with Wnt3a in the presence and absence of pyrvinium. Although these MEFs did not respond to SAG, Wnt3a-induced signaling was still observed. Furthermore, Wnt signaling remained pyrvinium sensitive in the absence of any HH signaling (Fig. 1F). Thus, in these contexts, pyrvinium’s ability to inhibit HH or Wnt signaling is independent of each other.

Pyrvinium potently inhibits HH signaling stimulated by the oncogenic Smo-M2 mutant (data not shown). Given the emergence of vismodegib resistance in the clinic (6), due to mutation of its binding site on Smo, we tested whether pyrvinium could bypass a vismodegib-resistant Smo mutant (Smo-M2-D473H; ref. 6) to inhibit HH signaling. Pyrvinium potently inhibited HH signaling activated by Smo-M2-D473H; vismodegib had no observable effect (Fig. 2A). Thus, pyrvinium acts to inhibit HH signaling via a mechanism distinct from vismodegib. We next determined the impact of pyrvinium treatment on the localization of Smo to the primary cilium of cells, which is an indication of Smo activation (35). Our results show that although pyrvinium treatment did result in a slight increase in Smo localization, in the absence of SAG treatment, this increase was not statistically significant (Fig. 2B). In addition, pyrvinium treatment had no impact on the increased Smo localization observed in response to activation by SAG, which suggests that pyrvinium acts downstream of Smo. Consistent with this hypothesis, pyrvinium was able to inhibit constitutive HH pathway activity in MEFs lacking Sfu, a negative regulator of HH signaling (14), whereas the Smo antagonist cyclopamine was unable to inhibit this activity (Fig. 2C).

Ultimately, HH signaling is mediated by Gli transcription factors, whose activity and stability are regulated by a number of protein kinases, including CK1α (7). We, therefore, determined whether pyrvinium’s ability to inhibit the expression of a HH biomarker is Gli dependent. MEFs lacking Gli1, Gli2, or Gli3 were all capable of responding to SAG in a manner that was attenuated by pyrvinium (Fig. 2D). Consistent with Gli2 and Gli3 playing redundant roles as HH-stimulated transcription factors, immortalized MEFs lacking both Gli2 and Gli3 did not respond to SAG to promote HH pathway activation (26). A low level of SAG-induced activity was observed in MEFs lacking both Gli1 and Gli2, and this low-level activation was mediated by the activator form of Gli3 (26). Pyrvinium did not attenuate this low level of activity, suggesting that pyrvinium does not regulate Gli3A-mediated transcriptional activity (Fig. 2D, right). Pyrvinium attenuated constitutive HH activity in cells overexpressing Gli1 (Fig. 2E) or Gli2 (data not shown), in contrast with the Smo antagonist vismodegib. These results suggest that pyrvinium is capable of attenuating the transcriptional activator function of Gli1 and Gli2, which are the major drivers of HH activity in cancer.

As CK1α has been implicated in the proteolysis of the Drosophila Gli homolog Ci (8), we tested the hypothesis that pyrvinium attenuates Gli activity by regulating its stability. We transfected the NIH-3T3 cells with a plasmid expressing Gli1 and treated them with increasing doses of pyrvinium. Immunoblotting of these cell lysates showed a dose-dependent decrease in the levels of Gli1 relative to control (Fig. 3A). This decrease in Gli1 protein levels occurred at doses of pyrvinium as low as 10 nmol/L, and was not a general consequence of inhibiting HH activity because two structurally distinct Smo antagonists did not affect Gli1 protein levels (Fig. 3A, right). Similar results were obtained with Gli2, although in this case, pyrvinium-mediated destabilization was observed only when HH signaling was activated (Fig. 3B). We also analyzed the levels of Gli3 protein in 3T3 cells treated with or without...
Figure 2. Pyrvinium inhibits HH signaling downstream of Smo. A, cells expressing a vismodegib-resistant, oncogenic Smo mutant (D473-M2 Smo) were treated with the indicated drugs and then assayed for luciferase activity (left). The effectiveness of vismodegib was validated on Light-II cells treated with the Smo agonist SAG (right). B, NIH-3T3 cells stably expressing Smo–GFP were treated with vehicle, 100 nmol/L SAG, 100 nmol/L pyrvinium, or SAG plus pyrvinium, for 24 hours. Primary cilia localization of Smo was revealed by immunocytochemistry. Smo localization was manually quantitated over five random images. Representative images are shown at the bottom (green, Smo–GFP; blue, DAPI; red, primary cilia). C, Sufu−/− or Sufu+/+ MEFs were treated with the indicated agents (5 μmol/L cyclopamine) for 3 days. The expression of Gli1, Ptch1, and Hip was determined and normalized to that of Gapdh. D, the indicated MEFs were treated with the Smo agonist SAG in the presence or absence of pyrvinium (10 nmol/L). Twenty-four hours later, the expression of Gli1 (left) or Ptch1 (right) was determined. E, Light-II cells expressing Myc-Gli1 were treated with the indicated drugs, followed by quantitation of luciferase activity a day later. Error bars, SEM (n = 3); *, P < 0.05.
pyrvinium, and did not observe decreased levels of the full-length Gli3 protein or increased levels of Gli3-R (Fig. 3C). In response to HH, both full-length Gli3 and Gli3-R levels were reduced regardless of pyrvinium treatment (Fig. 3C). This result is consistent with our analysis of pyrvinium’s effect in Gli1−/−;Gli2−/− double-knockout MEF cells, in which pyrvinium has no effect on HH signaling (Fig. 2D, right). We do not know why activation of CK1α by pyrvinium is insufficient to promote additional Gli3 processing, but speculate that the basal levels of CK1α activity are sufficient to promote maximum processing or that priming by PKA is the rate-limiting event in Gli3 processing (19).

Consistent with pyrvinium acting through CK1α to regulate Gli stability, CK1α overexpression also resulted in destabilization of Gli transcription factors. A, NIH-3T3 cells expressing Flag-Gli1 were treated with the indicated agents (vismodegib, 100 nmol/L; cyclopamine, 5 μmol/L), followed by immunoblotting of these cellular lysates. B, NIH-3T3 cells expressing HA-Gli2 were treated with the indicated agents (SAG, 50 nmol/L; pyrvinium, 100 nmol/L), followed by immunoblotting of immunoprecipitated Gli2. C, NIH-3T3 were treated with indicated agents (SAG, 50 nmol/L; pyrvinium, 100 nmol/L), followed by immunoblotting of endogenous Gli3. Quantification of multiple replicates (left) and a representative immunoblot (right) are shown for A–C. Error bars, SEM; NS, not statistically different; *, P < 0.05. D, NIH-3T3 cells stably expressing the indicated shRNA were transfected with a plasmid expressing Myc-Gli1 (1 μg in control shRNA–infected cells and 0.5 μg in CK1α-knockdown cells). These cells were subsequently treated with 10 or 100 nmol/L of pyrvinium, lysed, and then analyzed by immunoblotting. E, HEK 293T cells expressing Flag-Gli1 were treated with 200 nmol/L pyrvinium and 5 μmol/L of MG132 overnight. Anti-Flag immunoprecipitates, from lysates of these cells, were immunoblotted using the indicated antibodies. F, NIH-3T3 cells expressing Myc-Gli1 were treated with 100 μg/mL cycloheximide (CHX) at the indicated time points, in the presence or absence of 100 nmol/L pyrvinium. Immunoblotting was then performed to detect Gli1 and Gapdh levels. Error bars, SEM (n = 3); *, P < 0.05.
of the exogenous Gli1 protein (Supplementary Fig. S3A, left). In contrast, knockdown of CK1α attenuated pyrvinium’s ability to degrade Gli1 (Fig. 3D), whereas treatment with a CK1 antagonist (Supplementary Fig. S3A, right) increased Gli protein levels. To test the hypothesis that decreased stability of Glis results from ubiquitin-mediated proteosomal and lysosomal-mediated degradation pathways, we applied pharmacologic inhibitors to block specific protein degradation pathways and determined their effect on pyrvinium-mediated Gli destabilization. The proteosomal inhibitor MG-132, but not the lysosomal inhibitor Bafilomycin A1, blocks pyrvinium-induced Gli1 destabilization (Supplementary Fig. S3B), suggesting that pyrvinium regulates Gli levels via proteosome-mediated degradation. Consistent with this, pyrvinium greatly increased the level of Gli1 ubiquitination (Fig. 3E) and decreased the half-life of Gli1 protein in cells treated with the protein synthesis inhibitor cycloheximide (Fig. 3F and Supplementary Fig. S3C).

We hypothesized that CK1α might regulate Gli stability through direct association, in a manner analogous to the association described between the Drosophila Gli homolog Ci and CK1α (8). To test this possibility, we lysed HEK 293T cells expressing Flag-tagged Gli1, Myc-tagged Gli2, or both as indicated by the indicated antibodies. C, pcDNA3-Flag (Ctrl) or pcDNA3-Myc-Gli2 (B) were subjected to immunoprecipitation and immunoblotting with the indicated antibodies. D, immunohistochemical staining of CK1α and Gli1 proteins in Ptc-homa-derived medulloblastoma tissue. Scale bar, 100 μm. E, endogenous CK1α (left) or Gli1 (right) was immunoprecipitated from homogenized medulloblastoma tissue using the indicated antibodies (2 μg). These immunoprecipitates were then immunoblotted for CK1α or Gli1.

Figure 4. CK1α associates with Gli proteins in HEK 293T cells transfected with pcDNA3-Flag-Gli1 (Ctrl) or pcDNA-Myc-Gli2 (B). These cells were subjected to immunoprecipitation and immunoblotting with the indicated antibodies. C, pcDNA3-Flag (Ctrl) or Flag-Gli1-transfected HEK 293T cells were treated with vehicle or 200 mM pyrvinium for 1 hour, before any detectable changes in Gli1 protein levels. Samples were immunoprecipitated with Flag M2 beads and subject to immunoblot analysis for CK1α or Gli1.

Consistent with this observation, purine phosphorylation of CK1α is regulated by pyrvinium (Supplementary Fig. S3C). To demonstrate the capacity of pyrvinium to attenuate the growth of HH activity–dependent cancers, we focused on a well-established mouse model of HH-dependent medulloblastoma that is routinely used to determine the efficacy of HH inhibitors, the Ptc-homa medulloblastoma model (2). We dissected a spontaneous medulloblastoma from a Ptc-homa mouse, and serially passaged the tumor as an allograft in CD-1 nude mice. We validated the dependency of HH signaling for these tumors as HH (Gli1, Ptc-homa) but not Wnt (Axin2, Dkk1, and Lgr5) biomarkers were aberrantly activated (Fig. 5A). Acute treatment of pyrvinium in this mouse model attenuated the expression of the HH biomarkers Gli1 and Ptc-homa (Fig. 5B). Chronic treatment of such mice, using animals subcutaneously injected with pyrvinium adjacent to the approximately 100-mm3 tumors, dramatically reduced the growth of medulloblastoma allografts (Fig. 5C). Hematoxylin and eosin (H&E) staining demonstrated that tumors from pyrvinium-treated animals showed decreased cancer cells and increased fibrotic tissue,
compared with that from the vehicle-treated controls (Fig. 5D), consistent with a reduction in the size of the tumor driven by decreased numbers of tumor cells. In the pyrvinium-treated tumors, the expression of HH target genes was decreased relative to the vehicle control group (Fig. 5E). However, Wnt target genes, which are not hyperactivated in this subtype of medulloblastoma (see Fig. 5A), were not different between pyrvinium and vehicle-treated tissue (Fig. 5E). These observations are inconsistent with a generalized, nonspecific effect on tumor cells, in which a reduction in total gene expression might be expected. Thus, the CK1α agonist pyrvinium attenuates HH pathway activity and tumor growth in vivo in a well-established model of HH-driven medulloblastoma.

Discussion

We show here that the FDA-approved anthelmintic drug pyrvinium can be repurposed as a potent HH inhibitor.
Consistent with pyrvinium acting as a CK1ε agonist (23), we show that it attenuates HH signaling in a CK1ε-dependent manner. We further show that CK1ε and Gli proteins associate and propose a model by which pyrvinium induces Gli destabilization downstream of Smo. Unlike vismodegib, the only Smo inhibitor currently FDA approved (2), pyrvinium is able to attenuate increased Gli activity resulting from loss of Sufu, overexpression of a Gli protein, or a Smo protein harboring a clinically relevant vismodegib-resistant mutation. Pyrvinium was also able to attenuate the growth of a well-established HH-driven cancer model, a Ptch−/−-driven medulloblastoma allograft mouse model (5, 37). Interestingly, a significant number of human medulloblastomas result from loss of Sufu or amplification of Gli2 (1), and thus would be resistant to vismodegib but might conceivably respond to a CK1ε agonist such as pyrvinium. In its current method of dosing and formulation, pyrvinium likely lacks the pharmacokinetic properties to be used in the clinic against HH-driven tumors. However, improvements in its formulation or delivery, such as perhaps direct ventricular delivery into the brain, might rapidly be adapted to treat such patients with late-stage cancer for whom few therapeutic options remain.

Importantly, given the emergence of noncanonical Gli activation in human cancers (1), pyrvinium’s effects on HH signaling occurred downstream of Smo, where it induces the destabilization of Gli proteins. A number of other Gli inhibitors have now been described (2). For example, although Gli antagonists (GANT) are now widely used Gli inhibitors in the laboratory setting, their IC50 values are more than 100-times higher than the CK1ε agonists described here. Similar potency concerns also exist for another class of HH inhibitor HPI (IC50, 10 μmol/L), which perturb Gli processing and stability. Arsenic trioxide is another FDA-approved drug that has been repurposed as a Gli inhibitor in vitro and in vivo. Although this drug is already clinically available, its limited potency and well-described dose-limiting toxicities in humans, which occur at doses similar to those required to inhibit HH signaling, may limit its usefulness in the clinic.

Although CK1ε was initially thought to be constitutively active, recent evidence suggests that individual members of this protein kinase family are regulated via an array of transcriptional and posttranslational mechanisms (38). For CK1ε, such mechanisms include the frequent silencing of CK1ε in melanoma and loss-of-heterozygosity of the CK1ε gene in approximately 30% of all human tumors (http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghHome.cgi)—consistent with CK1ε activity being growth suppressing in many human cancers (39, 40). The identification of pyrvinium as an allosteric activator of CK1ε led to the speculation that pyrvinium may be mimicking an endogenous regulator of CK1ε. This was confirmed with the recent identification of a novel family of proteins that regulate the activity of CK1 kinases in vivo, and the demonstration that CK1ε activity can be attenuated by extracellular ligands, such as Wnts (41, 42). These newly identified levels of regulation could potentially be usurped for the development of novel anticancer therapeutics that target individual CK1 isoforms. The excitement of a class of anticancer agents such as pyrvinium comes from the recognition that drugs that act via allosteric mechanisms are not constrained by active site chemistry and are much more selective (sites may not be conserved even between proteins in the same family) in their mechanism of action.

CK1ε participates in a number of cellular processes other than HH and Wnt signaling, including retinoid X receptor regulation (43). Interestingly, similar to its role in HH signaling, CK1ε plays a dual function in NF-κB signaling, both promoting and repressing receptor-induced NF-κB activity (44). However, this is unlikely to be an impediment for its further development as a target for treating cancer. In fact, inhibitors that target other distinct CK1 isozymes (δ and ε) are currently in preclinical development in both academia and industry, to treat a variety of human pathologies (45, 46). In addition, there is significant precedence for the development of very effective anticancer agents that target proteins involved in basic cellular processes, which takes advantage of a tumor’s increased sensitivity to such agents. Many of these have already been approved by the FDA or are in clinical trials. These include paclitaxel (microtubule stabilizer), vorinostat (HDAC inhibitor), velcade (proteasome inhibitor), temsirolimus (mTOR inhibitor), and geldanamycin derivatives (targets HSP90 and currently in phase II clinical trials).

Disclosure of Potential Conflicts of Interest

D. Orton received a commercial research grant from and has ownership interest (including patents) in StemSynergy Therapeutics, Inc. E. Lee is the founder of and has ownership interest (including patents) in StemSynergy Therapeutics, Inc. D.J. Robbins has ownership interest (including patents) in and is a consultant/advisory board member for StemSynergy Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

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