Repurposing the Antihelmintic Mebendazole as a Hedgehog Inhibitor

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Abstract

The hedgehog (Hh) signaling pathway is activated in many types of cancer and therefore presents an attractive target for new anticancer agents. Here, we show that mebendazole, a benzimidazole with a long history of safe use against nematode infestations and hydatid disease, potently inhibited Hh signaling and slowed the growth of Hh-driven human medulloblastoma cells at clinically attainable concentrations. As an antiparasitic, mebendazole avidly binds nematode tubulin and causes inhibition of intestinal microtubule synthesis. In human cells, mebendazole suppressed the formation of the primary cilium, a microtubule-based organelle that functions as a signaling hub for Hh pathway activation. The inhibition of Hh signaling by mebendazole was unaffected by mutants in the gene that encodes human Smoothed (SMO), which are selectively propagated in cell clones that survive treatment with the Hh inhibitor vismodegib. Combination of vismodegib and mebendazole resulted in additive Hh signaling inhibition. Because mebendazole can be safely administered to adults and children at high doses over extended time periods, we propose that mebendazole could be rapidly repurposed and clinically tested as a prospective therapeutic agent for many tumors that are dependent on Hh signaling. Mol Cancer Ther; 14(1): 3–13. ©2014 AACR.

Introduction

Activation of the hedgehog (Hh) signaling pathway is required for developmental morphogenesis and is frequently observed in human cancers (1, 2). Canonical Hh signals are initiated by the interaction of Hh ligands with the receptor PTCH1. In the unbound state, PTCH1 prevents SMO activation in the primary cilium, an organelle required for the transduction of various chemical and mechanical signals (3). In the presence of ligand, PTCH1 disappears from the cilium and SMO activates downstream effectors, including the GLI family of transcription factors (4). Several types of cancer, including basal cell carcinoma and medulloblastoma, are frequently caused by germline or somatic mutations in PTCH1 or by less common alterations within the pathway that lead to constitutive signaling by SMO (1, 2). Alternative modes of Hh pathway activation in some of the most common types of cancer are suggested by the widespread presence of Hh ligands and evidence of elevated GLI activity in many tumors that lack pathway-activating mutations (5, 6).

SMO antagonism has proven to be an effective strategy for treating tumors with active Hh signaling (7). The first SMO antagonist to be approved for clinical use is vismodegib (Erivedge; also known as GDC-0449). Vismodegib has been used successfully for the treatment of locally advanced and metastatic basal cell carcinomas (8), and is currently being tested for use in adults and children with many diverse types of tumors, including medulloblastomas and gliomas, which are often refractory to conventional therapies (9). When used as a monotherapy, vismodegib is associated with adverse effects that include fatigue, vomiting, weight loss, decreased appetite, dysgeusia, dehydration, and muscle spasm (10). Such low-grade toxicities have contributed to treatment discontinuation and appear to be potentially problematic when vismodegib is combined with conventional agents (11). When used to treat a patient with metastatic medulloblastoma, vismodegib caused a response that was impressive but transient (12). Recurrent tumors in this patient were found to harbor a novel SMO mutation that caused drug resistance (13). Selection for SMO-mutant tumor cell populations can similarly be caused by vismodegib therapy in mouse models of medulloblastoma (13). Alternative strategies to inhibit Hh signaling have been explored for the prevention or treatment of such recurrent tumors (14).

Benzimidazoles approved by the U.S. Food and Drug Administration for the treatment of nematode infestations have been reported to have antiproliferative effects in diverse types of cancer cells, including those derived from melanoma, non–small cell lung cancer, ovarian cancer, adrenocortical carcinoma, and colorectal cancers (15–20). Case reports have documented responses of a metastatic adrenocortical carcinoma (21) and a metastatic colorectal carcinoma (22) to mebendazole (methyl N-[6-(benzyl)-1H-benzamidazol-2-yl] carbamate). Our group recently found that experimental brain tumors were highly sensitive to...
benzimidazole therapy that was administered to a mouse colony for control of a pinworm infestation. Follow-up studies of this serendipitous observation showed that mebendazole inhibited the growth of glia-derived neurospheres in vitro, and among the benzimidazoles most effectively entered the central nervous system and slowed the growth of orthotopically implanted gliomas, which are characteristically resistant to standard modes of therapy (23).

The anticancer effect of mebendazole defies a simple explanation. Mebendazole and related compounds have been reported to cause growth arrest and induce apoptosis in cultured cancer cells at doses that have little effect on noncancer immortalized cells (15–17, 19, 20). It is unclear why such nonspecific antiproliferative effects would preferentially target tumor cells over the cells in normal renewing tissues. Like all benzimidazoles used for treatment of helminth infestations, mebendazole binds tubulin at a binding site also recognized by colchicine, and inhibits microtubule polymerization. The rate of dissociation of mebendazole from nematode tubulin is an order of magnitude lower than from human tubulin (24). Inhibition of microtubule formation in the gut of the nematode prevents the absorption of glucose and thereby leads to elimination of the parasite (25), whereas human cells and tissues are apparently minimally affected by the less avid mebendazole–tubulin interaction. Mebendazole is therefore well tolerated, even at high doses administered over lengthy time periods for treatment of cystic echinococcosis (26).

A growing body of evidence suggests that activated Hh signaling contributes to the diverse cancers that are preclinically responsive to mebendazole and structurally related benzimidazoles. Recently, Hh signaling has been shown to be active in many gliomas (27, 28), while Hh ligands or markers of downstream pathway activity have been detected in melanomas, lung cancers, ovarian cancers, adrenocortical cancers, and colorectal cancers (5, 6, 29–32), which are all responsive to mebendazole (15–21). Notably, unbiased screens for novel Hh inhibitors have previously identified drugs that interact with microtubules, including vinblastine, vincristine, and paclitaxel (33). These drugs potentially inhibit mitosis, are highly toxic, and therefore unsuitable for long-term therapy. The specific effects of these compounds on components of the Hh pathway have not been reported.

In this study, we evaluated the effect of mebendazole on the Hh pathway. Mebendazole treatment prevented the formation of the primary cilium, decreased expression of downstream Hh pathway effectors, and decreased the proliferation and survival of human medulloblastoma cells with constitutive Hh activation. Mebendazole inhibited the activation of SMO-mutant proteins that give rise to disease recurrence. A combination of mebendazole and vismodegib achieved additive inhibition of canonical Hh signaling. These results support the repurposing of mebendazole for use in the many types of cancers that are initiated or maintained by active Hh signaling, and suggest combinations of drugs that could facilitate the achievement of durable responses.

**Materials and Methods**

**Cell lines and cell cultures**

Cultures of 293T and hTERT-RPE1 cells and Smo<sup>−/−</sup> mouse embryonic fibroblasts (MEF) were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Hyclone) and penicillin/streptomycin. DAOY and CH110T1/2 mouse fibroblast cells were grown in Eagle's MEM (Life Technologies) supplemented with 10% FBS and penicillin/streptomycin. NIH3T3 cells were grown in DMEM supplemented with 10% calf serum. Shh-Light2 cells were grown in DMEM with 10% calf serum and 0.4 mg/mL geneticin and 0.15 mg/mL zeocin, both purchased from Life Technologies. All cell lines were obtained from the ATCC within 6 months of the beginning of the project and validated by the supplier, except for Smo<sup>−/−</sup> MEFs that were a gift from James Kim (University of Texas Southwestern Medical Center) and were not genetically authenticated upon their receipt in July 2013.

**Orthotopic tumors**

Syngeneic GL261 glioma tumors were grown intracranially in 4- to 6-week-old female nu/nu athymic mice (NCl-Fredrick) and treated with mebendazole as previously described (23). Each brain was snap-frozen after extraction and stored in liquid nitrogen until further analysis. Orthotopic medulloblastoma xenografts were generated in female athymic mice, 5 to 6 weeks of age (NCl). DAOY cells were infected with a lentivirus carrying a firefly luciferase DNA (23) before implantation. For the implantation procedure, mice were anesthetized and 200,000 DAOY cells were injected through a burr hole drilled 1 mm lateral to the right of the sagittal suture and 1 mm posterior to the lambda at a depth of 2.5 mm below the dura, with the guidance of a stereotactic frame, at a rate of 1 μL/min. Treatment was initiated at 5 days after implantation, with a daily dose of mebendazole of 25 or 50 mg/kg delivered with 50% (v/v) sesame oil and PBS, by gavage. Intracranial luciferase activity was determined with a bioluminescence imager (Xenogen) following intraperitoneal injection of 2 mg o-luciferin potassium salt (Gold Biotechnology). Animals were scanned 15 min after injection for 1 m at a distance of 20 cm. Mice were euthanized when they exhibited signs of increased intracranial pressure. All animal protocols and procedures were performed under an approved protocol and in accordance with the Johns Hopkins Animal Care and Use Committee guidelines. For RNA preparation, each brain was thawed on ice before removing the right anterior cerebral cortex and the contralateral brain section. Each tissue sample was suspended in 1 mL of TRIzol (Life Technologies) per 0.1 g of material, and the RNA fraction was purified according to the manufacturer’s recommendations.

**Plasmids and cell transfections**

ShhN-conditioned media was generated by transfection of pDNA3 ShhN (provided by Pao Tien Chuang, University of California San Francisco) into 293T cells. Control media was obtained from mock-transfected 293T cells. For localization of SMO, hTERT RPE-1 cells grown in chamber slides (Nunc) were transfected with pDNA3 Smo–FLAG (provided by Chen Ming Fan, Carnegie Institute of Science). GLI1 reporter assays were conducted by cotransfecting the firefly luciferase reporter pBVLuc8xGfi (provided by Craig Peacock, Cleveland Clinic) and TKRenilla luciferase reporter pGL4 74 (Promega). Overexpression of Hh components was achieved by transient transfection of pRK-SmoM2 (35), pCMV5 hGLI1 FLAG (provided by Peter Zahiroupolos, Karolinska Institute) and pCS2-M hGLI2 FL (Addgene plasmid 17648; ref. 36). Mutant-Smo expression constructs were provided by James Kim. All transfections were performed with FuGENE HD (Promega).

**GLI-reporter assays and drug treatment**

Subconfluent Shh-Light2 cells were routinely incubated in low serum conditions (0.25% FBS, 5-mm HEPES), to optimize Hh
responsiveness, during a 48-hour period of drug treatment. For ShhN ligand stimulation, cells were incubated with ShhN-conditioned media or control media, diluted at 1:5, during the treatment period. Final DMSO concentration in all cultures was 1%. For experiments involving overexpression of Hh pathway components, cells were transfected 24 hours before low serum and drug treatment. Cell lysates were analyzed using the Dual-Luciferase Assay Reporter System (Promega). GLI-dependent luciferase was measured on a Victor3 V 1420 Multilabel Counter (PerkinElmer) and standardized against Renilla luciferase activity. The IC₅₀ was calculated by Prism 5 software package (GraphPad).

Cell proliferation, viability, and survival assays
Bromodeoxyuridine (BrdUrd) incorporation during DNA synthesis was measured using the Cell Proliferation ELISA Kit (Roche). Cell viability was measured with the CellTiter-Blue Cell Viability Assay Kit (Promega). Colorimetric signals were measured on a SpectraMax M5 (Molecular Devices). For each assay, the IC₅₀ was calculated with the Prism 5 software package (GraphPad). For assessment of clonogenic survival, cells were drug-treated under low serum conditions in 12-well plates. After 48 hours, cells were harvested in Trypsin–EDTA (Life Technologies), diluted at 1:4,000 in standard growth media, and seeded in 10-cm plates in triplicate. After 10 days of growth, plates were stained with 0.2% crystal violet in 50% MeOH and destained in water. Colonies containing more than 50 cells were scored. For the quantitation of apoptosis, cells were detached and stained with a fluorescent antibody directed against Annexin V with the Dead Cell Apoptosis Kit (Life Technologies). The fraction of stained cells was determined with a FACSAria II flow cytometer (BD) in the Sidney Kimmel Comprehensive Cancer Center Flow Cytometry Core.

Immunoblots and immunofluorescence
Proteins were separated on Bis–Tris gels (Life Technologies) and transferred onto Immobilon-P nylon membranes (Millipore). Following overnight incubation with primary antibodies under standard conditions, blots were developed with horseradish peroxidase (HRP)–conjugated secondary antibodies and visualized by chemiluminescence (Amersham). Band intensities were quantified by Image Lab software and standardized to the intensity of the loading controls anti–α-tubulin or anti–β-actin. To assess tubulin polymerization, polymerized and unpolymerized tubulin fractions were separated on the basis of solubility as reported previously (37).

For analysis of primary cilia by immunofluorescence, cells were grown on poly-ν-lysine (Sigma)–coated chamber slides (Nunc) and fixed in 0.4% paraformaldehyde at 37°C for 5 minutes, permeabilized in 0.5% Triton X-100 at 37°C for 2 minutes, washed in PBS, then sequentially incubated in 4% paraformaldehyde, 37°C for 5 minutes, and methanol, –20°C for 5 minutes, as previously described (38). Nonspecific proteins were blocked with 2% bovine serum albumin in PBS for 30 minutes at room temperature. Slides were incubated with primary antibody in blocking buffer overnight at 4°C, then washed with PBS, and incubated with either biotinylated (Santa Cruz Biotechnology) or Alexa Fluor 594–conjugated (Life Technologies) secondary antibodies in blocking buffer for 20 minutes at room temperature. Cells were washed with PBS before adding an Alexa Fluor 488–streptavidin conjugate (Life Technologies). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Stained cells were visualized with an Axioimager Z1 (Carl Zeiss) and images were captured with Axiovision Rel 4.6 software.

The following primary antibodies were used: anti–GLI1 (C66H3; Cell Signaling Technology), anti–caspase-3 (9662; Cell Signaling Technology) anti–caspase-3 (D175; Cell Signaling Technology), anti–α-tubulin (TU-02; Santa Cruz Biotechnology), HRP-conjugated anti–β-actin (Santa Cruz Biotechnology), anti–acetylated α-tubulin (6–11 B-1; Sigma), anti–FLAG (anti–DYKDDDDK; Cell Signaling Technology).

Quantitative real time RT-PCR
RNA was isolated and purified with the TRizol reagent (Life Technologies), treated with DNase I (Thermo Scientific), and assayed by spectrophotometry. cDNA was synthesized with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative, real-time reverse transcription PCR (qRT-PCR) was performed using both Maxima Probe and Maxima SYBR Green qPCR Master Mixes (Thermo Scientific) with standard cycling conditions on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Prime Time qPCR probes and primers were used to assay the mouse genes: Pch1: probe (5′-ATTCGGACCTCGAAGCATCAGT-3′), forward primer (5′-CTTGTGGCTCCGCCTTG-3′), reverse primer (5′-AACCAGTCCATGGAACCC-3′), Gli1: probe (5′-CTGGGACCTGACATAAGTGCC-3′); forward primer (5′-CTTCTTGTATTTTGACTGAACTCCTC-3′); reverse primer (5′-TCTTTGTGTCCTGCTTGG-3′); Hrpt1: probe (5′-CTTCTCTAGGAGATAACGGGAATCCAAGG-3′), forward primer (5′-AGAAGTTGACCTCGCATGAGA-3′); reverse primer (5′-TGATAACATATGTCGCTCAATC-3′); Human transcripts were analyzed with SYBR green. Gli1: forward primer (5′-CCACCGGGAGCCGGAGGAAGG-3′); reverse primer (5′-ACTGCGATTCGTCAGGATTTACTG-3′). PTC1: forward primer (5′-CCCAAGAGGAGCCTCCTACA-3′); reverse primer (5′-CTGTAATTCGTCGGCCTTTCC-3′). PTC2: forward primer (5′-GGAATGATTGCGAGATGATGAAG-3′); reverse primer (5′-CCCCGCGTCCCTGTTAGCC-3′). GAPDH: forward primer (5′-CCGGATCAACGGAGTGTGCTTAC-3′); reverse primer (5′-AGCCTTCCCTCAGGGTGTAAGAC-3′). All results were analyzed using SDS RQ Manager (Applied Biosystems).

Results
Inhibition of Hh signaling in vitro and in vivo
To directly assess the effect of mebendazole on canonical Hh signaling, we incubated the drug with murine Shh-Light2 cells, which have stably incorporated a GLI-activated luciferase, during a 48-hour period of drug treatment. For ShhN ligand stimulation, cells were incubated with ShhN-conditioned media or control media, diluted at 1:5, during the treatment period. Final DMSO concentration in all cultures was 1%. For experiments involving overexpression of Hh pathway components, cells were transfected 24 hours before low serum and drug treatment. Cell lysates were analyzed using the Dual-Luciferase Assay Reporter System (Promega). GLI-dependent luciferase was measured on a Victor3 V 1420 Multilabel Counter (PerkinElmer) and standardized against Renilla luciferase activity. The IC₅₀ was calculated by Prism 5 software package (GraphPad).
Gli1 expression was significantly elevated in untreated tumors compared with nonaffected tissue from the contralateral side of the brain (Fig. 1D), suggesting a role for Hh signaling in the growth of these tumors. Gli1 transcript and protein expression in tumor tissues was decreased by mebendazole treatment (Fig. 1E and F).

Figure 1.
Mebendazole (MBZ) inhibits Hh signaling. A, Shh-Light2 cells maintained in low-serum conditions were incubated in ShhN-conditioned medium or control medium, in the presence of mebendazole at the indicated concentrations. The activity of the stably integrated Gli-luc reporter was measured after 48 hours of treatment. The effect on this assay of 0.2 μmol/L vismodegib (Vis) is indicated by the dashed line. B, C3H10T1/2 mouse fibroblasts were cotransfected with the Gli-luc and Renilla luciferase reporters. After 24 hours, mebendazole was added for an additional 48 hours in low-serum media before cell lysis and measurement of luciferase activity. The effect of 0.2 μmol/L vismodegib (Vis) is indicated by the dashed line. C, the effect of mebendazole on Gli-luc reporter activity in Shh-Light2 cells was compared with that of the structurally related benzimidazoles albendazole, fenbendazole, and tiabendazole. Treatment times and conditions were as in A. D, endogenous levels of Gli1 transcripts in syngeneic GL261 gliomas and in normal brain tissue from the contralateral region were measured by qRT-PCR. E, Gli1 expression was measured by qRT-PCR in untreated and mebendazole-treated GL261 tumors. Each measurement was standardized to a parallel measurement from a contralateral brain section that did not contain tumor tissue. F, relative protein levels of Gli1 in three untreated and three mebendazole-treated GL261 tumors were assessed by immunoblot. Immortalized hTERT-RPE1 cells (G) or DAOY medulloblastoma cells (H) growing in low serum were treated with ShhN-conditioned or control medium for 48 hours. Mebendazole was included during this treatment period at the concentrations indicated. GLI1 and PTCH1 transcript levels were assessed by qRT-PCR.
To extend our analysis to human cells, we tested the effects of mebendazole on Hh-mediated gene expression in the immortalized human retinal pigment epithelial cell line hTERT-RPE1. Unlike the majority of human cancer cell lines, this noncancer cell line is responsive to ShhN (Fig. 1G). A concentration of 0.1 μmol/L mebendazole was sufficient to reduce ShhN-induced expression of endogenous GLI1 and PTCH1 to basal levels (Fig. 1G). Further Hh pathway inhibition was observed at higher mebendazole concentrations. Using the same conditions, we assessed the effect of mebendazole on the human medulloblastoma cell line DAOY. In both the presence and absence of ShhN ligand, there was partial reduction in GLI1 and PTCH1 transcripts at 0.1 μmol/L mebendazole and almost complete suppression at 1 μmol/L mebendazole, similar to the effect of 0.2 μmol/L vismodegib (Fig. 1H).
IC50 mebendazole caused a reduction in and accordingly exhibit elevated Hh signaling (40). In these cells, proliferation and survival. DAOY cells have a gene expression tumor growth

Figure 3.

Accordingly, a significant proportion of DAOY cells stained with (Fig. 2E) and morphologic (Fig. 2F) evidence of apoptosis.

We next assessed the effects of mebendazole on Hh-dependent cell proliferation and survival, DAOY cells have a gene expression profile consistent with a type II (Hh-subtype) medulloblastoma and accordingly exhibit elevated Hh signaling (40). In these cells, mebendazole caused a reduction in GLI1 expression with an IC50 = 516 ± 81 nmol/L (SEM; Fig. 2A). At similar concentrations, mebendazole markedly inhibited DAOY cell proliferation (Fig. 2B). Clonogenic survival was affected by mebendazole at a concentration as low as 100 nmol/L (Fig. 2C). The viability of DAOY cells, as assessed by a metabolic assay, was significantly impaired by mebendazole at concentrations approaching 1 μmol/L. In contrast, hTERT RPE-1 cells, which are Hh-responsive but not dependent on Hh signals for growth, were only modestly affected by this treatment (Fig. 2D). The expression of GLI1 protein was similarly reduced in both cell types by increasing concentrations of mebendazole, but only DAOY cells exhibited biochemical (Fig. 2E) and morphologic (Fig. 2F) evidence of apoptosis. Accordingly, a significant proportion of DAOY cells stained with Annexin V after mebendazole treatment (Fig. 2G), and this response was dose dependent (Fig. 2H).

When injected into the cerebella of nude mice to form orthotopic xenograft tumors, DAOY cells were responsive to 50 mg/kg mebendazole administered by daily gavage. Mebendazole treatment extended the median survival of tumor-bearing mice by 38 days (Fig. 3A). Levels of GLI1 and PTCH2 transcripts were reduced in the DAOY-derived tumors at the time of death (Fig. 3B), while bioluminescence imaging demonstrated a marked effect on tumor cell proliferation in asymptomatic mice (Fig. 3C).

Inhibition of Hh-dependent cell proliferation and survival, and tumor growth

We next tested the ability of mebendazole to counteract Hh signaling induced by individual components of the pathway. NIH3T3 cells strongly activated the Gliluc reporter in response to ShhN-conditioned medium; this upregulation of Hh activity was suppressed by mebendazole and by vismodegib (Fig. 4A). Hh signaling could also be strongly stimulated in these cells by transient overexpression of the Ptc1-resistant SmoM2-mutant protein (35), and by GLI1 or GLI2 (Fig. 4B). The stimulatory effect of SmoM2 on Gliluc activation could be suppressed by mebendazole, whereas reporter activation by the downstream effectors GLI1 and GLI2 was resistant to mebendazole (Fig. 4C).

To assess the effect of mebendazole on upstream signaling by SMO, we examined the cellular localization of a Smo–FLAG fusion protein (42) by immunofluorescence. Under low-serum conditions that favor primary cilium formation and robust SMO activation, Smo–FLAG was predominantly localized to the
primary cilia (Fig. 5A). Mebendazole-treated cells exhibited a notable paucity of primary cilia, and in nonciliated cells Smo-FLAG was distributed throughout the cytoplasm (Fig. 5A). The suppressive effect of mebendazole on ciliogenesis was quantified in larger cell populations (Fig. 5B and C). The proportion of ciliated cells decreased in response to mebendazole within a concentration range that also inhibited the polymerization of human α-tubulin in vitro (Fig. 5D). We next tested whether allowing primary cilia to pre-form before mebendazole addition could alter the effect of mebendazole on pathway activity. Temporally separating serum starvation from mebendazole administration partially restored GLI1 expression (Fig. 5E) and viability (Fig. 5F) in mebendazole-sensitive DAOY cells.

Distinct modes of Hh-pathway inhibition by vismodegib and mebendazole

As mebendazole exerts an indirect effect on SMO, we reasoned that mebendazole might inhibit the activity of mutant SMO proteins that have been found to confer vismodegib resistance. To test this idea, we assayed the ability of mebendazole to inhibit Gli-luc activation by wild-type and vismodegib-resistant mutant Smo proteins exogenously expressed in NIH3T3 cells 48 hours after treatment with ShhN ligand, or 72 hours after cotransfection with plasmids that drive exogenous expression of the Ptch1-resistant SMO-mutant SmoM2, GLI1 or GLI2 in low-serum media. C, mebendazole or vismodegib was added to SmoM2-, GLI1-, or GLI2-transfected NIH3T3 cells during 48 hours of incubation in low-serum conditions. The activation of a cotransfected Gli-luc reporter by each overexpressed gene, in the absence of drug treatment, was normalized to 100.

Figure 4.
Mebendazole (MBZ) inhibits activation of SMO. A, NIH3T3 fibroblasts cotransfected with Gli-luc and Renilla reporter plasmids were maintained in low-serum conditions for 48 hours in the presence of ShhN-conditioned or control media. During this period, mebendazole or vismodegib were added at the indicated concentrations. B, a direct comparison of Gli-luc activation in NIH3T3 cells 48 hours after treatment with ShhN ligand, or 72 hours after cotransfection with plasmids that drive exogenous expression of the Ptch1-resistant SMO-mutant SmoM2, GLI1 or GLI2 in low-serum media. C, mebendazole or vismodegib was added to SmoM2-, GLI1- or GLI2-transfected NIH3T3 cells during 48 hours of incubation in low-serum conditions. The activation of a cotransfected Gli-luc reporter by each overexpressed gene, in the absence of drug treatment, was normalized to 100.

Discussion

The development of new drugs for the treatment of cancers is a time-consuming and expensive process. Unanticipated toxicities at all stages of development are a major cause of failure (43). Repurposing well-characterized compounds for new uses can minimize such uncertainty, save time and markedly reduce costs. There are over 4,000 unique molecular entities approved worldwide for human or veterinary use (44). Exploring new indications for this rich compound library is a particularly attractive strategy to find new treatments for rare and neglected diseases, for which there are few financial incentives to justify the traditional process of commercial drug discovery. Brain tumors are relatively rare, but highly lethal. The incidence of medulloblastoma, for example, is approximately 1.5 per million in the United States and the majority of these cases occur in children (45). Economic constraints and the unique vulnerability of pediatric patients are obstacles to the traditional drug discovery process. New strategies to treat such diseases will likely involve agents that are first approved for other purposes.
Mebendazole (MBZ) inhibits formation of primary cilia. A, a Smo-FLAG fusion protein was expressed in hTERT-RPE1 cells by transient transfection. After 48-hour incubation in low serum and treatment with 1 μmol/L mebendazole or vehicle, cells were fixed, permeabilized, and stained with antibodies directed against acetyl-α-tubulin (green) and FLAG (red). Nuclei were counterstained with DAPI. Scale bar, 10 μm.

B, primary cilia (indicated by arrows) were numerically assessed for ciliated cells. C, the effects of mebendazole or 0.2 μmol/L vismodegib on the proportion of ciliated cells. D, hTERT-RPE1 cells were treated with mebendazole at the indicated concentrations or with 10 mmol/L paclitaxel (red line) for 48 hours under low-serum conditions. Polymmerized and unpolymerized tubulin fractions were quantified by immunoblotting, normalized to the loading control β-actin, and expressed as the proportion of polymerized tubulin compared with the combined polymerized and unpolymerized tubulin.

E, GLUT1/GAPDH mRNA expression was assessed in DAOY cells that were incubated with mebendazole for 48 hours under the low-serum conditions that allow formation of the primary cilium (“Treat in low serum”), or that were first maintained in low serum for 20 hours before adding mebendazole for an additional 48 hours (“Preincubate in low serum”). F, cell viability was assessed by CellTiter-Blue after the treatments described in E.

Mebendazole was developed in 1968 for use against a broad spectrum of gastrointestinal helminth pathogens, and remains among the most widely used agents in infected adults, including pregnant women, and children (46). Doses as high as 200 mg/kg/d have been safely and effectively administered for up to 48 weeks for the treatment of internal hydatid cysts (47, 48).

Although systemic absorption is low, serum levels of mebendazole peak 1 to 3 hours after intake, at reported levels that range from 0.3 to 1.6 μmol/L (48, 49). We observed significant levels of Hh pathway inhibition within this dose range. The only significant side effect of these high, chronic doses is bone marrow suppression, which has been observed in fewer than 5% of patients. Case reports suggest that this adverse effect is associated with increased therapeutic effect, and is therefore probably caused by higher blood levels of mebendazole in these patients. In supportive clinical settings, this untoward effect could be straightforwardly monitored and was reversible upon cessation of treatment (50).

We show here that the low-avidity binding of mebendazole to human tubulin can inhibit the assembly of the primary cilium, a tubulin-based structure. Hh pathway components are concentrated in the primary cilium (4, 51), and mutations in murine genes necessary for cilium formation cause phenotypes consistent with deficiencies in Hh signaling (52). In mice, primary cilia are dispensable for the growth of fibroblasts and other cell types, but essential for the ongoing proliferation of cultured medulloblastoma cells and the growth of established tumors (53). The differences in mebendazole sensitivity that we observed between human medulloblastoma cells and immortalized cells (Fig. 2D and E) are consistent with these dependencies.

The efficiency with which mebendazole can inhibit the formation of the primary cilium suggests that this anti-Hh effect might account for much of its activity against brain tumors and other tumor types thus far tested. Effects on other targets may also be relevant. For example, signals arising from the Wnt signaling pathway and platelet-derived growth factor (PDGF) ligands travel through the primary cilium (3). However, the developmental abnormalities that arise in mutant mice deficient for ciliogenesis most clearly overlap with those caused by Hh signaling defects (3). Beyond the primary cilium, microtubule assembly plays a fundamental role in cell division. Our results cannot rule out effects of mebendazole on the mitotic spindle as a cause of cytotoxicity. However, this type of antiproliferative activity would not be highly specific for cancer cells. Indeed, mitotic spindle checkpoint defects that are found in many cancers have been proposed to confer relative resistance to microtubule inhibitors (54). Interestingly, mebendazole-treated xenografts have been found to exhibit reduced vascularity (17).

VEGF-A has accordingly been proposed as a target of benzimidazole therapy (18). Inhibition of Hh activation potently stimulates angiogenesis by the paracrine induction of VEGF-A and other angiogenic factors (55), and could therefore account for these seemingly disparate observations.

The central role of the primary cilium in Hh pathway activation suggests that interfering with cilia formation should be an effective strategy for targeting Hh-driven tumors. The inhibitory effect of microtubule inhibitors on Hh signaling has been demonstrated by unbiased screens that have identified such agents indirectly, as inhibitors of GLI activity, or by direct inhibition of cilia formation and function (33, 56, 57). Thus...
far, the antimicrotubule agents identified in these screens are either highly toxic, or at early stages of characterization. Our data suggest that mebendazole might represent a simple path forward for this promising therapeutic strategy.

A burgeoning body of evidence suggests that Hh signaling is involved in the initiation and/or maintenance of a large proportion of human cancers (2, 5, 6, 29). The repurposing of mebendazole as an anticancer therapeutic will be guided by our rapidly growing understanding of the Hh pathway and how it contributes to tumor growth. It will be important to understand how mebendazole interacts with other agents, including other Hh inhibitors. In particular, the additive effect of mebendazole on vismodegib that we observed in vitro suggests a combinatorial strategy that could potentially alleviate the untoward effects associated with vismodegib and also suppress recurrence. Clinical trials will be the next step in exploring these new possibilities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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