The antihelmintic flubendazole inhibits microtubule function through a mechanism distinct from vinca-alkaloids and displays preclinical activity in leukemia and myeloma

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Abstract

On-patent and off-patent drugs with previously unrecognized anti-cancer activity could be rapidly repurposed for this new indication given their prior toxicity testing. To identify such compounds, we conducted chemical screens and identified the antihelmintic flubendazole. Flubendazole induced cell death in leukemia and myeloma cell lines and primary patient samples at nanomolar concentrations. Moreover, it delayed tumor growth in leukemia and myeloma xenografts without evidence of toxicity. Mechanistically, flubendazole inhibited tubulin polymerization by binding tubulin at a site distinct from vinblastine. In addition, cells resistant to vinblastine due to over-expression of p-glycoprotein remained fully sensitive to flubendazole, indicating that flubendazole can overcome some forms of vinblastine resistance. Given the different mechanisms of action, we evaluated the combination of flubendazole and vinblastine in vitro and in vivo. Flubendazole synergized with vinblastine to reduce the viability of OCI-AML2 cells. In addition, combinations of flubendazole with vinblastine or vincristine in a leukemia xenograft model delayed tumor growth more than either drug alone. Therefore, flubendazole is a novel microtubule inhibitor that displays preclinical activity in leukemia and myeloma.
Introduction

Drugs approved or tested experimentally for indications other than cancer that possess previously unrecognized cytotoxicity towards malignant cells could be rapidly repurposed for this new indication given their prior toxicity testing in humans and animals. For example, the oral hypoglycemic metformin activates the AMP-activated protein kinase pathway through a mechanism dependent on the tumor suppressor LKB1.\textsuperscript{1} At pharmacologically achievable concentrations, metformin inhibited the growth of prostate and colon cancer cells and delayed tumor growth in xenografts.\textsuperscript{2} Moreover, patients with diabetes receiving metformin had a reduced risk of pancreatic cancer compared to diabetics not receiving metformin.\textsuperscript{3} Given data such as these, metformin is currently being evaluated in clinical trials for the treatment of patients with advanced solid tumors.\textsuperscript{4} Likewise, thalidomide was discovered to possess anti-myeloma activity\textsuperscript{5} and was repurposed for this new indication. The identification of thalidomide, and its derivative lenolidamide, as anti-cancer agents improved standard care for patients with this hematological disease.\textsuperscript{6}

To date, the identification of drugs with unanticipated anti-cancer effects has been largely serendipitous. Here we used a systematic approach to identify compounds with unanticipated anti-cancer activity by testing an in-house chemical library of on-patent and off-patent drugs for their ability to reduce the growth and viability of leukemia cell lines. From these screens, we identified flubendazole, a member of the benzimidazole family of antihelmintic drugs, with potential anti-leukemia and anti-myeloma activity. Flubendazole has been extensively evaluated in humans and animals for the treatment of intestinal parasites as well as for the treatment of systemic worm infections. In these studies, patients have received up to 50mg/kg orally daily for 24 months without serious adverse effects.\textsuperscript{7-9} Healthy volunteers have also received single oral
doses up to 2000 mg without toxicity. In mice receiving a single dose of 5mg/kg of flubendazole, a Cmax of 1.12 ug/mL (3.6µM) and an area under the curve (AUC) of 2.17 µg.h/mL with no evidence of toxicity was recorded.

While selected members of the benzimidazole family have recently been reported to induce cell death in solid tumor cell lines, the anti-tumor properties of flubendazole have not been previously reported. Moreover, the mechanism by which benzimidazoles exert their effects as antihelmintics and by which they induce cell death in malignant cells is not fully understood and several cellular responses have been described. For example, benzimidazoles have been shown to inhibit amino peptidase activity and glutamate catabolism, reduce glucose uptake, increase intracellular calcium levels, and inhibit microtubule formation.

Here we demonstrate that flubendazole displays anti-leukemia and anti-myeloma activity in vitro and in vivo at pharmacologically achievable concentrations. Mechanistically, flubendazole alters tubulin structure and function by interacting with a site on tubulin distinct from vinca-alkaloid tubulin inhibitors.

Material and Methods

Reagents

Colchicine, taxol, and benzimidazole compounds were purchased from Sigma Chemical (St. Louis, MO). Vinblastine was purchased from Calbiochem (San Diego, CA). Drugs were prepared in dimethyl sulfoxide (DMSO).

Cell Culture

Culture of cells was carried out as previously described. For a detailed description of cell culture see the supplemental materials and methods.

Cell Growth and Viability Assays
Cell growth and viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) reduction assay (Promega, Madison, WI) according to the manufacture’s protocol and as previously described\textsuperscript{18}. Cells were seeded in 96 well plates and treated with drug for 72 hours. Optical density (OD) was measured at 490nm. Cell viability was also assessed by the Trypan blue exclusion assay and by Annexin V and PI staining (Biovision, Mountainview, CA), as previously described\textsuperscript{17}.

Clonogeneic growth assays with fresh primary AML patient and normal hematopoietic stem cells were performed as previously described\textsuperscript{17}. For a detailed description of the clonogenic growth assays see the supplemental materials and methods.

Mitotic catastrophe was measured by enumerating the number of multinucleated cells similar to the method previously described\textsuperscript{19}. A more detailed description of the assessment of mitotic catastrophe is presented in the supplemental materials and methods.

**Analysis of gene expression**

Changes in gene expression were measured in U937 leukemia cells treated with 1µM flubendazole or buffer control for 4h using Ingenuity Pathways Analysis (www.ingenuity.com) and the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov). For a detailed description of gene expression analysis see the supplemental materials and methods.

**Leukemia and myeloma xenograft models**

Sub-lethally irradiated SCID mice were injected subcutaneously in the left flank with leukemia OCI-AML2 (2.0x10\textsuperscript{6}) or myeloma OPM2 (1.0x10\textsuperscript{7}) cells. Mice were then randomly assigned to receive flubendazole (in 0.9%NaCl and 0.01% tween-80) or vehicle control (0.9%NaCl and 0.01% tween-80) intraperitoneally. When the combination of flubendazole and
vinblastine or vincristine was evaluated, mice were randomly assigned to receive flubendazole (in 0.9%NaCl and 0.01% tween-80), vinblastine (in PBS and 0.01% tween-20) or vincristine (in PBS and 0.01% tween-20), the combination of flubendazole and vinblastine or vincristine, or vehicle control intraperitoneally. Tumor volumes (tumor length x width$^2$ x 0.5236) were monitored daily using calipers. During drug treatment animal body weights were measured and recorded every 2-3 days. Changes in mouse behavior, including feeding pattern, posture, and overall activity were monitored and recorded daily. At the end of the experiment (16-18d), mice were sacrificed, tumors excised and tumor volume and weight measured. In addition, at the conclusion of the experiment, a necropsy was performed and the organs were examined for gross morphological alterations. Animal studies were carried out according to the regulations of the Canadian Council on Animal Care and with the approval of the Princess Margaret Hospital ethics review board.

Assessment of Glucose Uptake

The effect of flubendazole on the uptake of 2-deoxy-D-glucose in OCI-AML2 cells was performed using a radioactive glucose uptake assay as described by Wood et al$^{20}$. For a detailed description of the assessment of glucose uptake see the supplemental materials and methods.

Tubulin polymerization assay

Polymerization of bovine tubulin was measured according to Beyer et al$^{21}$. Briefly, bovine tubulin (1.8 mg/ml; Cytoskeleton; Denver, USA) was added to ice cold polymerization buffer (PEM; 80mM PIPES, 0.5mM EGTA, 2mM MgCl$_2$, 10% glycerol and 1mM GTP) and centrifuged at top speed in a microcentrifuge for 5 min at 4°C. Supernatant (100µL/well) was immediately added to a 96 well plate, which contained colchicine, taxol, flubendazole, or buffer/DMSO control in PEM -buffer. Final drug concentrations were similar to those
previously described\textsuperscript{13}. Following addition of tubulin, the plate was immediately placed in the spectrophotometer, which was maintained at 37°C, and the absorbance measured every 3 minutes for 2.5h at 340 nm.

\textbf{Measurement of tubulin sulfhydryl groups}

The ability of microtubule target drugs to alter tubulin structure was assessed by measuring the number of reactive cysteine residues using the sulfhydryl reagent 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB; Sigma) as previously described\textsuperscript{13, 22}. Bovine tubulin (1.5µM) was incubated with 10µM vinblastine, 10µM colchicine, 100µM flubendazole or a control for 15 min at 4°C. After incubation, DTNB was added (100µM final concentration) and the absorbance was measured in a 1cm path length cuvette at 412nm for 60 min at 37°C. The number of sulfhydryl groups were determined by using a molar extinction coefficient for DTNB of 13 600 (M\textsuperscript{-1},cm\textsuperscript{-1}).\textsuperscript{22}

\textbf{Determining the site of tubulin binding}

The site of flubendazole binding was determined similar to Gupta et al\textsuperscript{13}. Bovine tubulin (5µM) was incubated with 100µM flubendazole, 100µM vinblastine or buffer control for 30 min at 37°C. Colchicine (10µM) was then added and incubated for an additional 60 min at 37°C. Fluorescence of the colchicine-tubulin complex was subsequently measured at excitation and emission wavelengths of 360 nm and 430 nm, respectively.

\textbf{Determining effect of flubendazole on microtubules in cultured cells}

The morphology of PPC-1 cells incubated with vehicle control or 1µM flubendazole were examined by confocal microscopy. For a detailed description of confocal microscopy see the supplemental materials and methods.

\textbf{Cell migration assays}
HeLa cell migration was measured as previously described\textsuperscript{23}. For a detailed description of cell migration assays see the supplemental materials and methods.

\textit{Cell cycle analysis}

Cell cycle analysis was performed as previously described\textsuperscript{24}. For a detailed description of cell cycle analysis see the supplemental materials and methods.

\textit{Assessment of sensory function with the tail-flick assay}

Sensory function was assessed with the tail-flick assay by Chempartners Co. (Shanghai, China) similar to previously described\textsuperscript{25}. Briefly, 50 experimentally-naïve, male, adult C57BL/6J mice from Shanghai SLAC Co. Ltd were treated with 50, 100, 200 mg/kg of flubendazole in 0.9\%NaCl and 0.01\% tween-80 or vehicle control (n=10 per group). Before and after 14 days of treatment, tail flick latency was measured by applying a high-intensity, noxious, radiant heat stimulus 20 mm from the tip of the tail. When a withdrawal tail-flick response occurred, the thermal stimulus was terminated automatically and the response latency was measured electronically.

\textit{Drug combination studies}

The combination index (CI) was used to evaluate the interaction between flubendazole and other therapeutic agents. OCI-AML2 cells were treated with increasing concentrations of flubendazole, vinblastine, colchicine, daunorubicin, or cytarabine. Seventy-two hours after incubation cell viability was measured by the MTS assay. The Calcusyn median effect model was used to calculate the CI values and evaluate whether the combination of flubendazole with vinblastine, cytarabine, daunorubicin, or colchicine was synergistic, antagonistic or additive. CI values of <1 indicate synergism, CI=1 indicate additivity and CI>1 indicate antagonism\textsuperscript{26}.

\textit{Statistical Analysis}
Unless otherwise stated, the results are presented as mean ± SD. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software, USA). p<0.05 was accepted as being statistically significant. Drug combination data were analyzed using CalcuSyn software (Biosoft, UK).

Results

A screen of on-patent and off-patent drugs for compounds with novel anti-cancer activity identifies flubendazole

To identify drugs with unanticipated anti-cancer activity, we compiled a library of 110 on-patent and off-patent drugs focused on anti-microbials and metabolic regulators with a wide therapeutic index and well characterized pharmacokinetics that were available from the Canadian and United States drug formularies. We then screened this library at increasing concentrations, using the MTS assay, to identify compounds that reduced the growth and viability of three leukemia cell lines after 72 hours of incubation27. From these screens, we identified several cytotoxic agents including mebendazole. Mebendazole is a member of the benzimidazole family of antihelmintics, so we investigated the cytotoxicity of this drug class. OCI-AML2 leukemia cells were treated with increasing concentrations of 8 benzimidazole family members. Seventy two hours after incubation, cell growth and viability was measured by the MTS assay. The most potent benzimidazole in this panel was flubendazole (Figure 1A).

Flubendazole is cytotoxic to leukemia and myeloma cell lines

Having identified flubendazole as a potential anti-cancer agent, we evaluated its effects in a panel of malignant cell lines. Leukemia and myeloma cell lines were treated with increasing concentrations of flubendazole. Seventy two hours after incubation, cell growth and viability was measured by the MTS assay. Flubendazole reduced cell viability with an IC$_{50}$ < 1µM in 8/8
myeloma and 4/6 leukemia cell lines, including MDAY-D2 cells with an IC50 of 3nM (Figure 1B). Of note, the remaining 2 cell lines (OCI-AML-2 and CEM) had IC50 values of 1.1 ± 0.6 and 1.9 ± 0.9 μM respectively. Concentrations of 1μM appear pharmacologically achievable, based on prior studies in mice that demonstrated a dose of 5 mg/kg produced a Cmax of 1.12 μg/Ml (3.6μM) and an AUC of 2.17 μg.h/mL without toxicity. Cell death was confirmed by PI staining and trypan blue exclusion assay (ANN-/PI-: 0µM: 94.2±1.9; 1.5µM: 59.4±1.0; 2M: 45.8±1.1; and Supplemental figure 1). We also evaluated the effects of flubendazole in primary normal hematopoietic cells obtained from donors of GCSF-mobilized peripheral blood for allotransplant (PBSC). In contrast to the effects observed in leukemia cell lines, treatment of PBSCs with 2µM of flubendazole for 24 hours induced minimal cell death(ANN-/PI-: 0µM: 93.0±4.5; 2µM: 89.0±4.9).

Flubendazole was also evaluated in clonogenic growth assays with primary AML and normal hematopoietic samples. Flubendazole, reduced the clonogenic growth of primary AML blasts (n=3) from peripheral blood samples obtained from patients with AML [intermediate risk cytogenetics (n=2) and poor risk cytogenetics (n=1)] and complete loss of clonogenic growth was observed after treatment with 1µM of flubendazole (Figure 1C). However, similar reductions in clonogenic growth were also observed when PBSCs were treated with flubendazole with complete loss of clonogenic growth with 1µM of flubendazole (Figure 1D). Thus, flubendazole displays activity against leukemia and myeloma cells at nanomolar concentrations, but there is a narrow difference between primary AML and normal hematopoietic cells in clonogenic growth assays.

Flubendazole delays tumor growth in leukemia and myeloma xenografts
As flubendazole was cytotoxic to malignant cells in vitro, we evaluated its anti-tumor effects in leukemia and myeloma xenografts. OCI-AML2 leukemia cells were injected subcutaneously into sub-lethally irradiated SCID mice. Mice were then treated with flubendazole (20 or 50 mg/kg) daily or vehicle control intraperitoneally. Compared to mice treated with vehicle control, flubendazole significantly delayed tumor growth and reduced tumor weights (p<0.0001; Figures 2A-B). Likewise, sub-lethally irradiated SCID mice were injected subcutaneously with OPM2 myeloma cells. Mice were then treated daily with 50 mg/kg of flubendazole or buffer control for 17 days intraperitoneally. Flubendazole significantly delayed tumor growth and reduced tumor weights compared to mice treated with vehicle control (p<0.05; Figures 2C).

In both leukemia and myeloma models, no significant differences in body weight were observed in flubendazole-treated mice compared to control (Supplemental Figure 2). Likewise, flubendazole treatment did not alter the behavior of the mice or produce gross organ changes upon necroscopy. Thus, flubendazole displays novel pre-clinical activity against leukemia and myeloma at concentrations that appear pharmacologically achievable.

**Flubendazole does not alter glucose uptake**

In studies with the parasite *Trichuris globulosa*, the antihelmintic effects of the benzimidazoles thiabendazole and fenbendazole were related to inhibition of glucose uptake with resultant alterations in glucose metabolism. Therefore, we tested the effects of flubendazole on glucose uptake in malignant cells. OCI-AML2 cells were treated with increasing concentrations of flubendazole for 16 hours and uptake of 3H-deoxy-D-glucose was measured (Supplemental figure 3A). In contrast to the observations in the parasite, flubendazole at concentrations up to 4µM did not alter glucose uptake. Likewise, culturing cells with varying concentrations of
glucose did not alter flubendazole-induced death (Supplementary figure 3B). Thus, flubendazole-induced cell death does not appear related to inhibition of glucose uptake.

**Flubendazole alters microtubule structure and function**

To better understand the mechanism by which flubendazole induced death of malignant cells we examined changes in gene expression following 4h of flubendazole treatment. By gene ontology and pathway analysis, 196 genes were identified to be deregulated >4 fold with flubendazole treatment (ArrayExpress accession: E-MEXP-2352; Supplemental table 1). Of these, 179 genes were annotated and 58/179 fell within 8 functional annotations associated with chromosomal segregation and cytoskeleton regulation; genes involved in chromosomal segregation were the most affected by flubendazole treatment (Supplemental table 2). Moreover, by Connectivity Map analysis, changes in gene expression were found to be similar to gene signatures induced by the known tubulin inhibitors nocodazole and colchicine (data not shown).

Given these findings and previous observations that benzimidazoles such as mebendazole exert an antihelmintic effect at least in part by inhibiting tubulin polymerization, we evaluated the effects of flubendazole on tubulin structure and polymerization. To determine whether flubendazole alters tubulin structure, we measured changes in the number of reactive cysteine residues on tubulin following incubation with flubendazole. Treatment with flubendazole reduced the number of reactive cysteines by 5.2 ± 2.6 compared to buffer control (p<0.05). In comparison, vinblastine decreased the number of reactive cysteines by 8.6 ± 2.5 (p<0.01). Thus, these results suggest that flubendazole interacts with tubulin to alter its structure (Figure 3A).

Drugs that alter microtubules can either promote or inhibit tubulin polymerization. Therefore, we evaluated the effects of flubendazole on tubulin polymerization. Flubendazole was incubated with purified bovine tubulin and tubulin polymerization was recorded over time. As
controls, bovine tubulin was incubated with colchicine which is known to inhibit tubulin polymerization and taxol which is known to promote tubulin polymerization. In this assay, flubendazole inhibited tubulin polymerization (Figure 3B).

As flubendazole altered the structure and polymerization of tubulin, we compared its binding site to that of vinblastine, a member of the vinca-alkaloid family of microtubule inhibitors that is currently used for the treatment of leukemia and myeloma. Purified bovine tubulin was incubated with flubendazole or vinblastine followed by the addition of colchicine. The interaction of colchicine with tubulin was then assayed by measuring the fluorescence of colchicine bound to tubulin. The addition of flubendazole decreased colchicine fluorescence, but the addition of vinblastine had no effect (Figure 3C), indicating that flubendazole but not vinblastine blocks colchicine from binding tubulin. Thus, flubendazole interacts with tubulin through a mechanism distinct from vinblastine.

Since flubendazole altered tubulin structure and function in cell-free assays, we evaluated the effects of flubendazole on microtubule formation in intact cells. PPC-1 prostate cancer cells were treated with flubendazole (1µM) or vehicle control for 24 hours, and then stained with anti-tubulin and DAPI. Microtubule architecture was visualized by confocal microscopy (Figure 3D). PPC-1 cells treated with vehicle control exhibited an organized network of elongated microtubules. In contrast, cells treated with flubendazole were rounded with contracted and disorganized microtubules. Thus, flubendazole disrupts the microtubule architecture in intact cells.

Microtubules mediate cell migration, so we investigated the effects of flubendazole on cell migration with a wound healing assay. HeLa cells were seeded in 4 well chambers and after adhering overnight, the cell monolayer was scratched to create a wound. Cells were treated with
flubendazole or buffer control and migration of cells to heal the wound was measured overtime. Treatment with flubendazole impaired cell migration and delayed wound healing (Figure 3E). Of note, at the concentrations and times tested in these assays, the flubendazole-treated cells were more than 89% viable as measured by MTS assay.

**Flubendazole arrests cells in cell cycle and induces mitotic catastrophe**

Inhibition of tubulin polymerization can inhibit cell cycle progression and induce mitotic catastrophe\(^3\), so we assessed the effects of flubendazole on the cell cycle by flow cytometry and on chromosomal segregation by enumerating the number of multi-nucleated cells in OCI-AML2 and PPC-1 cells. Flubendazole arrested cells in the G2 phase of the cell cycle (Figures 4A-B) and increased the number of multi-nucleated cells (Figure 4C). Thus, flubendazole produces cell cycle arrest and mitotic catastrophe, consistent with its effects as a microtubule inhibitor.

**Inhibition of microtubules is functionally important for flubendazole’s cytotoxicity**

Next, we determined whether inhibition of microtubule formation was functionally important for flubendazole’s cytotoxicity. KB-4.0-HTI cells have a single nucleotide change in α-tubulin that renders it resistant to microtubule inhibitors.\(^3\) Therefore, we treated KB-4.0-HTI and KB-3-1 wild type controls with increasing concentrations of flubendazole and colchicine. Consistent with flubendazole’s effects on tubulin, KB-4.0-HTI cells were more resistant to flubendazole with an IC\(_{50}\) 7-fold higher than the non-mutated KB-3-1 wild type cells (Table 1). Similarly, KB-4.0-HTI cells were also resistant to colchicine with an IC\(_{50}\) 2.5-fold higher than the KB-3-1 control cells (Table 1), consistent with previous observations with this cell line.\(^3\) Flubendazole was also tested in A549.EpoB40 cells, which are more sensitive to microtubule inhibitors due to a point mutation in β-tubulin at residue 292.\(^3\) A549.EpoB40 cells were more sensitive to flubendazole with an IC\(_{50}\) 5-fold lower than the non-mutated A549 control cells.
Similarly, A549.EpoB40 cells were sensitive to colchicine with an IC$_{50}$ 1.5-fold lower than the A549 control cells (Table 1), consistent with previous observations with this cell line.$^{37}$

As further evidence that flubendazole’s cytotoxicity was related to microtubule inhibition, the benzimidazoles thiabendazole and 1,3-benzidiazole that did not induce cell death in OCI-AML2 cells (Figure 1A) also did not inhibit microtubule formation in our cell-free polymerization assays (data not shown). Thus, taken together, flubendazole induces cell death through a mechanism that appears related to its inhibition of microtubule polymerization.

Over-expression of P-glycoprotein does not alter flubendazole’s cytotoxicity.

Over-expression of P-glycoprotein (Pgp, MDR1) renders cells resistant to vinca-alkaloid microtubule inhibitors.$^{38}$ Therefore, we tested the effects of Pgp over-expression on flubendazole’s cytotoxicity. CEM-VBL cells over-express Pgp,$^{39}$ which was confirmed with a rhodamine dye exclusion assay (data not shown). CEM wild type and CEM-VBL cells were treated with increasing concentrations of flubendazole or vinblastine and cell viability measured by the MTS assay (Table 1). CEM-VBL cells remained fully sensitive to flubendazole compared to the wild type controls. In contrast, CEM-VBL cells were resistant to vinblastine at concentrations greater than 5µM (Table 1). Therefore, as over-expression of Pgp does not abrogate the cytotoxicity of flubendazole, this drug is capable of overcoming some forms of resistance to vinca-alkaloids.

Flubendazole does not induce neuropathy

Neuropathy is a dose limiting toxicity of vinca-alkaloid microtubule inhibitors such as vincristine. Therefore, we tested the effects of flubendazole on neurologic function in mice. Mice (n=10/group) were treated with 50, 100, and 200mg/kg of flubendazole or vehicle control intraperitoneally daily for 14 days, and sensory function was assessed with the tail-flick assay.
No changes in tail-flick latency were observed at doses up to 200 mg/kg compared to controls, a dose 10-fold higher than the dose required for an anti-tumor effect (mean + SD tail-flick latency: control 3.04 + 0.52 seconds vs. 200 mg/kg flubendazole 2.91 + 0.50 seconds, p=0.58 by Student’s t-test). However, doses of 200mg/kg resulted in 5% decrease in body weight compared to vehicle controls (p=0.03, Student’s t-test).

**Flubendazole synergizes with vinblastine and enhances vinblastine and vincristine activity in vivo**

Flubendazole interacts with tubulin through a mechanism distinct from vinblastine. Therefore, we evaluated the cytotoxicity of flubendazole and vinblastine in combination. OCI-AML2 cells were treated with increasing concentrations of flubendazole and vinblastine and 72 hours after incubation cell growth and viability was measured by the MTS assay. Combinations were assessed based on CI values where CI values <1, equal to 1 or >1 are considered synergistic, additive or antagonistic, respectively.\(^{26, 40}\) The combination of flubendazole and vinblastine synergistically induced cell death with CI values of 0.09, 0.017, 0.003 and 0.001 at the EC 50, 25, 10 and 5, respectively (Figure 5A). In contrast, cell death produced by the combination of flubendazole and colchicine was closer to additive with CI values of 0.54, 0.70, 0.90 and 1.07 at EC 50, 25, 10 and 5, respectively (Figure 5B). Combinations of flubendazole and cytarabine or daunorubicin were also closer to additive (Figures 5C-D).

Given the synergy of flubendazole and vinblastine in cell culture, we evaluated the combination of flubendazole and vinblastine and vincristine in vivo. OCI-AML2 cells were injected subcutaneously into SCID mice and treated intraperitoneally with flubendazole (15 mg/kg), vinblastine (0.3 mg/kg) or vincristine (0.25mg/kg or 0.35mg/kg), or the combination of the two agents. The combination of flubendazole and vinblastine decreased tumor weight greater
than either agent alone (p<0.01) (Figure 6A). Similarly, the combination of flubendazole and vincristine decreased tumor weight and volume greater than either agent alone (p<0.001) (Figures 6B). Moreover, there were no observed behavioral changes, weight loss, or gross organ toxicity from either combination treatment (Supplemental figure 2). Of note, however, despite dramatic reductions in tumor growth even with established tumors, neither flubendazole, vincristine, or the combination of these agents caused tumor regression in this mouse model (Figure 6C). Thus, flubendazole synergizes with vinblastine and vincristine and could be used in combination with these vinca-alkaloids to achieve a greater anti-tumor effect.

**Discussion**

Through screens of libraries of on-patent and off-patent drugs we identified flubendazole as having previously unrecognized anti-leukemia and anti-myeloma activity. At pharmacologically achievable concentrations, flubendazole induced cell death in malignant cells and delayed tumor growth in vivo. Mechanistically, flubendazole altered microtubule structure and inhibited tubulin polymerization by interacting with a site on tubulin similar to colchicine and distinct from vinblastine.

As part of its development as an antihelmintic, flubendazole has been studied extensively in animals and humans, where it has displayed favorable toxicology profiles. For example, in rats, mice and guinea-pigs the LD$_{50}$ is >5000 mg/kg and >400 mg/kg after oral and intraperitoneal administration, respectively.$^{10}$ No toxicity was noted in rats that received up to 150mg/kg/day for 3 months, while chickens receiving up to 180 mg/kg flubendazole daily for 7 days developed anemia and reduction of red cells in the spleen.$^{10}$ In humans, doses of 40-50 mg/kg/day for 10 days have been administered for the treatment of neurocysticercosis and no toxicity from the drug was reported.$^9$ Likewise, patients received up to 50 mg/kg/day of
flubendazole for up to 24 months for the treatment of alveolar echinococcosis without adverse effect.8

The pharmacokinetics of flubendazole are also well characterized. For example, in sheep the estimated half life for flubendazole after oral administration is 6.5 hours and the main metabolic pathways are carbamate hydrolysis and ketone reduction.10 After intravenous administration, an AUC of 6.53µg.h/mL is achieved over 36 hours after a single intravenous dose of 5 mg/kg. However, only 18% of flubendazole is absorbed, so after a single oral dose of 5mg/kg the AUC over 36 hours was 1.17 µg.h/mL.41 The oral bioavailability of flubendazole is better, however, in mice. After oral administration of 5mg/kg of flubendazole to mice, an AUC of 2.17 µg.h/mL and a Cmax of 1.12 µg/mL (3.6µM) was achieved without toxicity11. While the extent to which flubendazole is bound to proteins in the plasma is unclear, albendazole, a compound with similar absorption properties and structure as flubendazole42, has an estimated plasma protein binding of 70%. If the plasma protein binding was similar for flubendazole, then a Cmax of 1.12 µg/ml would equate to 0.34 µg/mL (1.1µM) of free flubendazole. This value compares very favorably to our reported sub-micromolar IC₅₀ values for flubendazole’s cytotoxicity in malignant cells.

It is unknown whether the current oral formulation of flubendazole would be suitable for the treatment of patients with malignancy as the oral bioavailability of the current formulation is low in humans10. However, since large doses can be safely administered and are sufficient to treat systemic worm infections the current formulation might be suitable for the treatment of hematologic malignancies. Alternatively, an intravenous formulation or an oral formulation with improved bioavailability could be developed, but might require additional pharmacokinetic and/or toxicology testing prior to clinical trial.
Our clonogenic assays did not demonstrate a difference between the cytotoxicity of flubendazole for primary AML and normal hematopoietic cells and this finding raises concerns regarding the potential therapeutic window of flubendazole. However, it is important to note that results of colony formation assays do not always predict clinical toxicity. For example, cytarabine and m-AMSA, which are routinely used in the treatment of AML, show little or no selectivity for malignant cells over normal cells in colony formation assays. Likewise, the microtubule inhibitor vincristine does not show preferential cytotoxicity to malignant cells over normal hematopoietic cells in vitro. Moreover, we observed that oral flubendazole delayed tumor growth in mouse models of leukemia and myeloma without toxicity. Finally, toxicology studies with flubendazole conducted in mice and sheep did not report hematologic toxicity. Nonetheless, the lack of difference between primary AML and normal hematopoietic cells in the clonogenic assay raises concerns about the potential hematologic toxicity and its safety will have to be carefully evaluated in phase I clinical trials.

Vinca-alkaloids are currently used in the treatment of myeloma, but are not part of standard induction chemotherapy for patients with AML. It is interesting to note, however that induction chemotherapy for AML in the 1970’s used vincristine in combination with cyclophosphamide and cytarabine or cyclophosphamide and daunorubicin. In the 1980’s this regimen was replaced with daunorubicin and cytarabine and vincristine was dropped from the protocol. However, it has never been determined whether addition of vincristine to daunorubicin and cytarabine would provide any additional benefit. These early studies, nonetheless, demonstrate the efficacy of tubulin inhibitors in AML and suggest that they could be beneficial in some patients, possibly in the setting of relapsed disease.
In support of the evaluation of flubendazole for the treatment of patients with refractory hematologic malignancies, a Phase I clinical trial of the related benzimidazole, albendazole was recently conducted in patients with advanced solid tumors.\textsuperscript{48} In 2 of 7 patients, albendazole reduced levels of the tumor markers AFP and CEA. However, albendazole caused severe neutropenia in 3 of these patients and the development of albendazole as an anti-tumor agent has not been pursued to date.

In our study, flubendazole inhibited tubulin polymerization and function which were functionally important for its cytotoxicity. Microtubules are cytoskeleton components that are required for cell division, cellular transport and in the maintenance of cellular integrity.\textsuperscript{28} Microtubules are comprised of $\alpha$- and $\beta$- tubulin heterodimers that assemble into linear protofilaments and polymerize into hollow, cylindrical structures.\textsuperscript{31} The gain or loss of tubulin heterodimers leads to elongation or shortening of the microtubules.\textsuperscript{49} Drugs that alter the polymerization of microtubules are well validated therapeutic agents for the treatment of malignancies. For example, vinca-alkaloids inhibit microtubule polymerization by binding to $\beta$-tubulin near the GTP-binding site.\textsuperscript{31} In contrast, colchicine inhibits polymerization by binding the interface of the $\alpha/\beta$-tubulin heterodimer and taxol promotes tubulin polymerization by binding in the lumen of the polymer.\textsuperscript{31} In our study, we demonstrated that flubendazole interacts with tubulin at a site similar to that of colchicine and distinct from vinca-alkaloids. A similar interaction with tubulin has been reported for the benzimidazole mebendazole.\textsuperscript{50} However, the benzimidazole benomyl has been reported to inhibit tubulin polymerization by interacting at a site distinct from both the colchicine site and the vinca domain.\textsuperscript{13} Thus, the mechanism by which benzimidazoles inhibit tubulin formation appears to vary among family members.
As flubendazole and vinca-alkaloids inhibited tubulin through distinct mechanisms, we evaluated the combination of these drugs. Flubendazole synergized with the vinca-alkaloids vinblastine and vincristine in vitro and in vivo. Therefore, these drugs could be used in combination to enhance the efficacy of standard therapy for these diseases or potentially reduce their toxicity.

Finally, as flubendazole inhibits tubulin function through a mechanism distinct from vinca-alkaloids, flubendazole could be useful in overcoming some forms of resistance to vinca-alkaloids. For example, as over-expression of Pgp does not render cells resistant to flubendazole, it could also overcome this specific mechanism of resistance to vinca-alkaloids.

In summary, flubendazole is a novel microtubule inhibitor that acts through a mechanism distinct from vinca-alkaloids. Given its prior safety record in humans and animals coupled with its pre-clinical efficacy in hematological malignancies demonstrated here, flubendazole could be repurposed for evaluation in these diseases.

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Author Contribution

PAS designed research, analyzed data, performed research and wrote the paper. JH designed research, analyzed data and performed research. RH, MG and MS designed and performed research and analyzed data. AD, JB and IA performed research and analyzed data. RBZ designed
research, performed research and analyzed data. NF designed research. CDS performed research and analyzed data. RR designed research. ADS designed research, analyzed data, performed research and wrote the paper. The authors reviewed and edited the paper and declare no conflict of interest.
References


Table 1: Flubendazole reduces cell growth and viability in malignant cells

KB-4.0-HT1 cells with a β-tubulin mutation and KB-3-1 wild type controls, A549.EpoB40 cells with an α-tubulin mutation and the A549 wild type controls, CEM VBL cells over-expressing PgP and CEM wild type controls were treated with increasing concentrations of flubendazole for 72 hours. Data represent IC$_{50}$ values, as measured by the MTS assay.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Flubendazole (µM)</th>
<th>Colchicine (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB-3-1</td>
<td>1.9 ± 1.1</td>
<td>14.9 ± 4.5</td>
</tr>
<tr>
<td>KB-4.0-HT1</td>
<td>12.5 ± 1.8</td>
<td>41.6 ± 5.5</td>
</tr>
<tr>
<td>A549</td>
<td>4.1 ± 1.3</td>
<td>0.09 ±0.01</td>
</tr>
<tr>
<td>A549.EpoB40</td>
<td>0.8 ± 0.2</td>
<td>0.06 ± 0.01</td>
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</table>

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Flubendazole (µM)</th>
<th>Vinblastine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>1.9 ± 0.9</td>
<td>0.14 ± 0.01</td>
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<tr>
<td>CEM-VBL</td>
<td>2.7 ± 1.2</td>
<td>&gt;5.0</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1: Flubendazole induces cell death in malignant cell lines

(A) OCI-AML2 cells were treated for 72 h with increasing concentrations of benzimidazoles. After incubation, cell growth and viability was measured by the MTS assay. Data represent the mean percentage of viable cells + SD from a representative experiment.

(B) Leukemia and myeloma cell lines were treated with increasing concentrations of flubendazole. Seventy two hours after incubation, cell growth and viability was measured by the MTS assay. Data represent the mean percentage of viable cells + SD from representative experiments.

(C) Primary AML patient samples or (D) primary normal hematopoietic cells (1 x 10^5 cells) (n = 3) were plated in a methylcellulose colony forming assay with increasing concentrations of flubendazole. Colonies were counted 7 days after plating as described in the supplemental method section on colony formation assay. Data represent the mean + SD from 3 independent experiments performed in duplicate.

Figure 2: Flubendazole delays tumor growth and reduces tumor weight in leukemia and myeloma mouse xenografts

Sub-lethally irradiated SCID mice were injected subcutaneously with OCI-AML2 cells (n=20; 10 per group). After implantation, mice were treated with 50 mg/kg flubendazole (A), 20mg/kg flubendazole (B), or vehicle control by intraperitoneal injection daily. Tumor volume was measured over time. After 16 days (20mg/kg dose) or 18 days (50 mg/kg dose), mice were sacrificed and tumors were excised, measured and weighted.

(C) Sub-lethally irradiated SCID mice were injected subcutaneously with OPM2 cells (n=20; 10 per group). One week after implantation, when tumors were palpable, mice were treated with 50
mg/kg flubendazole or vehicle control by intraperitoneal injection twice daily. Tumor volume and body weight was measured over time. After 17 days, mice were sacrificed and tumors were excised, measured and weighted.

Data are presented as means ± SEM. Differences in tumor volume and tumor weight were analyzed by an unpaired t-test: *** p<0.0001; **p<0.001; *p<0.05.

**Figure 3: Flubendazole inhibits tubulin structure, polymerization and function**

(A) Flubendazole (100µM) and vinblastine (10µM) were incubated with bovine tubulin (1.5µM) and the conformational changes were monitored spectrophotometrically by measuring the decrease in the number of reactive cysteine residues at an absorbance of 412 nm as described in the materials and methods. A representative figure is shown.

(B) Flubendazole (100µM), colchicine (6µM) and taxol (6µM) were incubated with bovine tubulin (1.8 mg/ml) and the effects on polymerization were monitored spectrophotometrically by measuring turbidity at 340 nm as described in the materials and methods. A representative figure is shown.

(C) Tubulin (5µM) was incubated for 30 min with 100µM vinblastine, 100µM flubendazole, or buffer control. After incubation, colchicine (10µM) was added and incubated for 60 minutes. Fluorescence of the tubulin-colchicine complex was measured with excitation and emission wavelengths of 360 nm and 430 nm, respectively. Reduced fluorescence indicates binding at the colchicine site. *p<0.01 (ANOVA, bonferoni post hoc). A representative figure is shown.

(D) PPC-1 cells were treated with 1.0µM flubendazole for 24 h and stained with DAPI and an anti α-tubulin AlexaFluor 488nm antibody. Images were captured using an Olympus Fluorview confocal microscope at room temperature. Representative confocal micrographs at 40X are shown.
(E) HeLa cells were grown to confluence and a wound created on the cell monolayer using a 200µL pipette. Cells were treated with increasing concentrations of flubendazole and imaged every 2 h for 8 h. Wound healing was measured as described in the materials and methods. Representative data is shown and are presented as % wound recovery. *p<0.05 (ANOVA, bonferroni post hoc).

**Figure 4: Flubendazole induces cell cycle arrest and mitotic catastrophe**

OCI-AML2 cells (A) or PPC-1 cells (B) were incubated with flubendazole or buffer control for 24 h. Cells were then stained with PI and the DNA content was measured by flow cytometry. A representative figure is shown.

(C) PPC-1 cells were treated as above and stained with anti α-tubulin antibody and DAPI, as described in the materials and methods. Cells were imaged by confocal microscopy and the number of multi-nucleated cells was enumerated. Data represent the mean + SD percent of multinucleated cells from a representative experiment *p<0.0001 (unpaired t test). **Insert:** a representative multi-nucleated cell.

**Figure 5: Flubendazole synergizes with vinblastine**

The effects of increasing concentrations of flubendazole in combination with vinblastine (A), colchicine (B), cytarabine (C), or daunorubicin (D) on the viability of OCI-AML2 cells. Cell viability was measured by the MTS assay after 72 h incubation. Data were analyzed with CalcuSyn software as described in ‘Materials and Methods’. Combination index (CI) versus Fractional effect (Fa) plot showing the effect of the combination of flubendazole and vinblastine colchicine, cytarabine or daunorubicin. CI < 1 indicates synergism. One of two representative isobologram experiments performed in triplicate is shown.
Figure 6: Flubendazole enhances the activity of vinca-alkaloids in vivo

Sub-lethally irradiated SCID mice were injected subcutaneously with OCI-AML2 cells (n = 40; 10 per group). After implantation, mice were treated with (A) 15 mg/kg flubendazole, 0.3 mg/kg vinblastine, a combination of flubendazole and vinblastine, or vehicle control; or (B) 20 mg/kg flubendazole, 0.25 mg/kg vincristine, a combination of flubendazole and vincristine, or vehicle control. After 16 (A) or 18 (B) days, mice were sacrificed and tumors were excised, measured and weighted. (C) Sub-lethally irradiated SCID mice were injected subcutaneously with OCI-AML2 cells (n = 40; 10 per group). Eleven days after injection, when tumors were established (e.g., tumor volume = 32 mm³), mice were treated with 50 mg/kg flubendazole, 0.25 mg/kg vincristine (VCR) the combination of flubendazole and VCR, or vehicle control. Tumor volume was measured over time with calipers. Data represent the mean + SD tumor weight. A representative experiment is shown. # p<0.05, * p<0.01, ** p<0.001 compared to controls (Unpaired t-test).
Figure 1

Panel A: Graph showing cell viability (% control) against drug concentration (μM) for Myeloma. The graph includes lines for various compounds such as 1,3-Benzidiazole, Thiabendazole, Albendazole Sulfoxide, Mebendazole, Oxibendazole, Flubendazole, and Methyl 1-2-benzimidazole carbamate.

Panel B: Graph showing % viable for various leukemia cell lines including KMS11, KSM18, OCIMY5, KMS12, H929, OPM2, LP1, and JJN3 against Flubendazole concentration [μM].

Panel C: Bar graph showing the number of colonies for AML patient samples with different concentrations of flubendazole (0μM, 0.5μM, and 1μM).

Panel D: Bar graph showing the number of colonies for PBSC patients with different concentrations of flubendazole (0μM, 0.5μM, and 1.0μM).
Figure 2

A. OCIAML-2 (50mg/kg)

B. OCIAML-2 (20mg/kg)

C. OPM2 (50mg/kg)

**Figure 2**
Figure 3

A. Absorbance over time for Control, Flubendazole, and Vinblastine.

B. Absorbance over time for Control, Taxol, Flubendazole, and Colchicine.

C. Fluorescence (as % control) for Control, Vinblastine, and Flubendazole.

D. Images showing cell morphology changes under different treatments.

E. % Wound recovery for different Flubendazole concentrations.
Figure 4
Figure 5
Figure 6
The antihelmintic flubendazole inhibits microtubule function through a mechanism distinct from vinca-alkaloids and displays preclinical activity in leukemia and myeloma

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