6 α -Acetoxyanopterin: A Novel Structure Class of Mitotic Inhibitor Disrupting Microtubule Dynamics in Prostate Cancer Cells

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Abstract

The lack of a cure for metastatic castrate-resistant prostate cancer (mCRPC) highlights the urgent need for more efficient drugs to fight this disease. Here, we report the mechanism of action of the natural product 6 α -acetoxyanopterin (6-AA) in prostate cancer cells. At low nanomolar doses, this potent cytotoxic alkaloid from the Australian endemic tree *Anopterus macleayanus* induced a strong accumulation of LNCaP and PC-3 (prostate cancer) cells as well as HeLa (cervical cancer) cells in mitosis, severe mitotic spindle defects and asymmetric cell divisions, ultimately leading to mitotic catastrophe accompanied by cell death through apoptosis. DNA microarray of 6-AA-treated LNCaP cells combined with pathway analysis identified very similar transcriptional changes when compared to the anticancer drug vinblastine, which included pathways involved in mitosis, microtubule spindle organization and microtubule binding. Like vinblastine, 6-AA inhibited microtubule polymerization in a cell-free system and reduced cellular microtubule polymer mass. Yet, microtubule alterations that are associated with resistance to microtubule-destabilizing drugs like *vinca* alkaloids (vinblastine/vincristine) or 2-methoxyestradiol did not confer resistance to 6-AA, suggesting a different mechanism of microtubule interaction. 6-AA is a first-in-class microtubule inhibitor that features the unique anopterin scaffold. This study provides a strong rationale to further develop this novel structure class of microtubule inhibitor for the treatment of malignant disease.

Introduction

Prostate cancer is the second most diagnosed cancer in men in developed countries and the fifth most common cause for cancer-related death (1). Metastatic castrate-resistant prostate cancer (mCRPC) remains an incurable disease despite the recent approvals of new and more efficacious therapeutics (2). This highlights the need for additional prostate cancer therapies, e.g., drugs that inhibit proven targets, yet remain unchallenged by known resistance pathways (2). The majority of anticancer drugs currently in use are natural products, natural product derivatives, or compounds developed on the basis of a natural product pharmacophore (3). Numerous organisms of various biota independently developed a great diversity of natural products throughout evolution as antipredatory defenses based on targeting microtubule dynamics (4). Microtubules are crucial for cellular processes such as cell division, mobility, intracellular organelle transport, and endothelial cell biology (angiogenesis) (5). Microtubules are highly dynamic protein structures which continuously undergo growth through polymerization and shrinkage through depolymerization of α - and β -tubulin heterodimers (microtubule dynamics) (6). In mitosis, microtubules play a vital role in the assembly of the spindle apparatus and proper segregation of chromosomes to both daughter cells (6). This critical function has been successfully exploited for the development of anticancer drugs and is exemplified by the semi-synthetic taxanes docetaxel and cabazitaxel, which are the FDA-approved mainstay therapies of mCRPC (2). *Vinca* alkaloids (e.g., the natural products vinblastine, vincristine and semi-synthetic vinorelbine) are another class of microtubule inhibitors currently used as gold standards in chemotherapy for Hodgkin's disease, non-Hodgkin's lymphoma and breast cancer (6). At clinically relevant doses (low nanomolar), both drug classes kinetically stabilize the microtubules, without changing microtubule polymer mass (7-9). This leads to mitotic arrest at the metaphase-anaphase transition, chromosome mis-segregation, genome instability and ultimately mitotic catastrophe and cell

death (6). At higher concentrations (micromolar), taxanes stabilize microtubules through binding to the taxane binding site on β -tubulin (10). *Vinca* alkaloids bind to the *vinca* domain on β -tubulin and induce microtubule depolymerization (11). Another class of microtubule destabilizers includes the natural products colchicine, combretastatins and 2-methoxyestradiol (2ME2), which all interact with the colchicine domain located at the intradimer interface between α/β -tubulin heterodimers, leading to microtubule depolymerization (12). Since the development of vinblastine and paclitaxel, other natural product-derived microtubule inhibitors have reached the market. Sagopilone (epothilone analogue, stabilizer) has shown very promising results in phase II clinical trial in mCRPC (13). The FDA-approved microtubule depolymerizing agent Eribulin has shown activity in a phase II clinical trial (14). Despite their initial therapeutic success, resistance to these agents develops. Known resistance mechanisms induced by microtubule inhibitors include drug efflux through increased expression of multidrug resistance transporters, tubulin isoform expression and mutations (6). We have previously reported the identification and cytotoxicity analysis of eight C₂₀ diterpenoid alkaloids that feature the unique anopterine scaffold from the Australian endemic rainforest plant *Anopterus macleayanus* (15). The anopterine analogs induced cell death in a panel of prostate cancer cell lines at nanomolar concentrations (15). Here, we present the mechanism of action studies of the most potent anopterine analog, 6 α -acetoxyanopterine (6-AA) and provide evidence that the anopterine scaffold represents a novel structure class of microtubule inhibitors with a mechanism of interaction that is distinct to taxanes, *vinca* alkaloids and 2-methoxyestradiol. This is the first time that such activity has been described for this structure class.

Material and methods

Reagents

Vinblastine, colchicine, nocodazole, verapamil, 2-methoxyestradiol (all Sigma Aldrich), vincristine, doxorubicin, MNL8237, BI2536 (all Selleckchem), paclitaxel (Cytoskeleton), 6-AA (15), were resuspended in DMSO (Sigma Aldrich) and stock solutions were stored at -20°C.

Cell lines

LNCaP and PC-3 cells were obtained in 2010 from the American Type Culture Collection (ATCC) and cultured in phenol-red free RPMI-1640 medium supplemented with 5% FBS (Thermo Fisher Scientific). Cells were genotyped in December 2013 by STR analysis at the DNA Diagnostic Center (Cincinnati, USA). HeLa cells were obtained in 1993 and authenticated by STR fingerprinting in 2015. HeLa-H2B-GFP (pBOS-H2BGFP, BD Biosciences) and HeLa-EB1-GFP (EB1-GFP (JB131), Addgene) were made by Prof. Gabrielli (16) and cultured in DMEM medium supplemented with 10% FBS. Non-adherent human T-cell acute lymphoblastic leukemia (T-ALL) cell line CCRF-CEM (CEM) and its drug-resistant sublines CEM/VCR-R (17, 18), CEM/2ME2-14.4R and CEM/2ME2-28.8R (19) were a kind gift from the Kanamatsu lab (Sydney University, Australia) in 1989 and validated by STR profiling in May 2016 by the Molecular Genetics Facility at the Garvan Institute of Medical Research. CCRF-CEM (CEM) and its drug-resistant sublines were cultured in RPMI-1640 medium supplemented with 5% FBS. All cell lines were kept at 37°C in an atmosphere containing 5% CO₂, maintained in log phase growth and were routinely screened for mycoplasma.

Assessment of cell viability and proliferation

LNCaP (4,000 cells/well), HeLa (2,000 cells/well), and PC-3 (3,000 cells/well) cells were

seeded in 96-well plates and allowed to attach for 24 h. Cell viability as a function of metabolic activity was measured by alamarBlue endpoint assay (Thermo Fisher Scientific) after 72 h of treatment (20). Non-adherent T-ALL cell lines (20,000 cells/well) were simultaneously seeded in 96-well plates and treated with the indicated compounds for 72 h. Cell proliferation as a function of cell confluence was evaluated using live-cell imaging (IncuCyte, Essen BioScience) as described before (20).

Cell cycle analysis by flow cytometry

LNCaP cells were plated in 6-well plates (150,000 cells/well), allowed to attach for 24 h, and treated with the indicated compounds for 24 h. Cell cycle experiments were conducted as described previously (21). Samples were analyzed on a FACSCanto (BD Biosciences). DNA histograms were analyzed using ModFit LT software (Verity Software House).

Wound closure assay

PC-3 cells (30,000 cells/well) were seeded in 96-well ImageLock plates (Essen BioScience) and allowed to attach for 24 h. Wounds were generated with a 96-well mechanical Woundmaker (Essen BioScience); cells were treated with the indicated compounds for 16 h. Wound closure was measured using a live-cell imaging system (IncuCyte) according to the manufacturer's instructions (Essen BioScience).

Microarray gene expression profiling

LNCaP were seeded and allowed to attach for 24 h in a 6-well plate (150,000 cells/well) then treated with 6-AA (10 nM) or vinblastine (3.25 nM) for 24 h before RNA extraction and processing as described previously (22). For gene expression profiling, 3-4 repeats of each treatment were analysed on a custom 180k Agilent oligo microarray (ID032034, GPL16604). Raw data were processed using the Agilent Feature Extraction Software (v10.7) and the LIMMA package in R as described before (22). Differential expression between treatment

and vehicle control (DMSO) was classified based on an adjusted *P*-value of ≤ 0.05 and an average fold-change of ≥ 1.5 . Expression data are 'Minimum Information About a Microarray Experiment' (MIAME) compliant and have been submitted to Gene Expression Omnibus (GEO) with the accession number GSE81277. Differentially expressed genes were examined using GeneGo MetaCore (Thomson Reuters) and GOrilla (Gene Ontology enRIchment anaLysis and visuaLizAtion tool) (23) for functional annotation and network analysis.

Quantitative real time polymerase chain reaction (qRT-PCR)

RNA samples were generated as described above and processed as reported previously (21). qRT-PCR was performed with SYBR Green PCR Master Mix (Thermo Fisher Scientific) using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Changes in mRNA expression levels were calculated based on the $\Delta\Delta C_t$ method, normalized relative to RPL32 expression and expressed as fold-change relative to control (DMSO). Primer sequences are listed in Supp. Table S1.

Western blotting

LNCaP cells were seeded, grown for 24 h in a 6-well plate (150,000 cells/well) and treated with the indicated compounds for 24 h before cell lysis in modified RIPA buffer (21). Cell lysates (30 μ g/lane) were separated by SDS-polyacrylamide gel electrophoresis, and proteins blotted to PVDF membrane (Millipore). Primary antibodies used were: PARP (46D11, 9532, Cell Signaling, 1:1,000), phospho-Ser10 histone H3 (ab5176, Abcam, 1:1,000), and β -Actin antibody (sc-47778, Santa Cruz Biotechnology, 1:2,500). Primary antibodies were probed with the appropriate horseradish peroxidase-conjugated secondary antibody (GE Healthcare) and visualized on a ChemiDoc XRS system (Bio Rad) by chemiluminescence (Immobilion).

Immunofluorescence microscopy

Optical 96-well plates (ibidi) were coated with poly-L-ornithine (Sigma-Aldrich) as described

previously (24). LNCaP cells (5,000 cells/well) were seeded and allowed to attach for 24 h. After the indicated times and concentrations of treatment, cells were fixed with ice-cold methanol for 3 min, incubated in blocking buffer [2% bovine serum albumin (Sigma Aldrich) in TBS with 0.1% Triton X-100] for 30 min at room temperature before immunostaining for 1 h at room temperature with primary antibodies against phospho-Ser10 histone H3 (ab5176, Abcam, 1:1,000) and α -tubulin (DM1A, ab7291, Abcam, 1:500), pericentrin (ab448, Abcam, 1:100), and Aurora A kinase (610398, BD Biosciences, 1:100). Cells were washed twice in TBS-T and incubated with the appropriate secondary, fluorophore-labelled antibody for 1h at room temperature in the dark. DNA was counterstained with 1 μ g/mL DAPI [4',6-diamidino-2-phenylindole, Sigma Aldrich]. Methanol fixation and permeabilization with Triton X-100 were performed to wash out soluble tubulin subunits (25, 26).

Images were acquired on a Cytell or INCell 2200 automated imaging systems (GE Healthcare) at 10 $\times$, 20 $\times$ or 40 $\times$ magnifications. Image segmentation of $\sim$ 4500 cells/treatment and quantitation were performed with CellProfiler software (Broad Institute, Cambridge, USA) (27). For detailed segmentation method, see Supp. Fig. S1. For detailed segmentation method, see Supp. Fig. S1. Since the total tubulin level is not affected by known microtubule-targeting agents (28, 29), and most of soluble tubulin subunits were extracted during wash steps, mean α -tubulin staining intensity was correlated to α -tubulin polymer mass (29, 30). Analysis of spindle organization ($\sim$ 800 cells/treatment) and the distance between spindle poles ($\sim$ 120 cells/treatment) were performed using Fiji software (31).

Washout experiments

LNCaP and HeLa cells were seeded as described above in poly-L-ornithine coated plates. Cells were treated with the indicated concentration of compounds for 8 h, 24 h or 72 h. For washout experiments, media was carefully removed after 8 h or 24 h of treatment as indicated and cells were left to recover in fresh media until completion of the experiment.

Assessment of apoptosis

The CellEvent Caspase-3/7 reagent (Thermo Fisher Scientific) was used to quantitatively evaluate apoptosis. CEM (10,000 cells/well) and CEM/VCR-R (10,000 cells/well) cells were simultaneously seeded and treated in optical 96-well plates pre-coated with poly-L-ornithine. After 24 h, cells were processed with CellEvent Caspase-3/7 reagent following the manufacturer's instructions. DNA was stained with Hoechst 33342 (Thermo Fisher Scientific). Images were acquired on an INCell 2200 imaging system (GE Healthcare) at 20× and 40× magnifications. Image analysis of 3,000 cells/treatment was performed using CellProfiler software. To quantify apoptotic cells, all nuclei (based on DAPI staining) were scored for caspase 3/7-positive and -negative staining.

Time-lapse microscopy

HeLa-H2B-GFP cells (2,000 cells/well) were seeded and allowed to attach for 48 h in optical 96-well plates then treated with the indicated compounds. Images were immediately acquired with Cell R software on an Olympus IX81 live-cell microscope using a 10× objective at 37°C, 5% CO₂. Images were captured every 5min for 24 h. Quantification of the time in mitosis and cell fate of ~120 cells/treatment was performed using Fiji software (31).

Spinning disk microscopy

HeLa-EB1-GFP cells (20,000 cells/well) were seeded and allowed to attach for 48 h in a 35 mm 4-chambers glass bottom dish (Cellvis). After treatment with the specified compounds for 2 h, cells were imaged on a Nikon Ti-E Motorized Inverted Microscope. Images (60×) were taken every second up to 2 min. EB1 comets were tracked and analyzed using Imaris software (8.2 version).

***In vitro* tubulin polymerization assay**

Microtubule assembly was studied using the CytoDYNAMIX Screen kit (BK006P; Cytoskeleton Inc.) according to the manufacturer's instructions and polymerization was monitored using a FLUOstar Omega plate reader (BMG LABTECH) at 340 nm every 1 min for 30 min at 37°C.

Statistical analysis

All data points were performed in technical duplicates or triplicates, and experiments were repeated independently at least twice and reported as the mean $\pm$ standard deviation (SD) of the biological replicates. For all experiments, one-way ANOVA with Dunnet's multiple comparisons test was used (ns=non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$) unless stated otherwise. Half-maximal inhibitory concentration (IC_{50}) and statistical significance were analyzed using GraphPad Prism 6 software (GraphPad Software).

Results

6-AA inhibits cell proliferation and induces apoptosis

In our previous study, we showed that 6-AA (Fig. 1A) inhibited cell growth of five prostate cell lines (IC_{50} =3.1–11.5 nM) (15). We have further tested 6-AA in HeLa (cervical cancer) and PC-3 cells (metastatic prostate cancer), where 6-AA was also potently cytotoxic (IC_{50} : LNCaP=3.1nM, HeLa=3.2nM, PC-3=5.1nM, Fig. 1B, Supp. Table S2) with a potency comparable to the microtubule inhibitor vinblastine (IC_{50} : LNCaP=3.2nM, HeLa=11.6nM, PC-3=2.5 nM, Supp. Table S2). 6-AA inhibited cell proliferation in a concentration-dependent manner in LNCaP (Fig. 1C) and HeLa (Supp. Fig. S2A) cells and significantly inhibited cell migration of PC-3 cells at 1.25 nM (Supp. Fig. S2B).

Cell cycle analysis by flow cytometry showed that 6-AA led to a significant increase in the population of LNCaP cells in the G₂-M phase in a concentration-dependent (Fig. 1D) and

time-dependent manner (Supp. Fig. S2D). At 5 nM, the G₂-M arrest of LNCaP cells reached significance ($P < 0.01$) as early as 14h after treatment with 6-AA and preceded the induction of significant levels of cell death (24 h), as indicated by the increased number of cells in the sub G₀-G₁ phase (Fig. 1E and Supp. Fig. S2D). Caspase-dependent cleavage of PARP, a marker of apoptosis (32), was detected in 6-AA-treated LNCaP cells after 24 h, similar to the microtubule inhibitors paclitaxel and nocodazole (33, 34) (Fig. 1F). Consistent with this, 6-AA and vinblastine-treated LNCaP (Fig. 1C) and HeLa cells (Supp. Fig. S2C) showed membrane blebbing, chromatin condensation (visible in HeLa-H2B-GFP, Supp. Fig. S2C), and cell disintegration, which are typical signs of apoptosis (35, 36).

Transcriptional networks of mitosis and spindle microtubules are affected by 6-AA

For target discovery, we performed microarray analysis of LNCaP cells treated for 24 h with 6-AA (10 nM), or vinblastine (3.25 nM) (Supp. Fig. S3A). Pathway analysis (MetaCore) of the 6-AA and vinblastine data sets identified a strong overlap of networks enriched in differentially expressed genes that were shared between both treatments (Fig. 2A), with mitosis as deregulated core network and the top three process subnetworks being “cell cycle-mitosis”, “cytoskeleton-spindle microtubules” and “cell cycle-G₂-M”. Several critical regulatory genes of mitotic entry, spindle formation and mitotic exit were up-regulated (Supp. Table S3) such as mitotic kinases (AURKA, AURKB, PLK1), mitotic checkpoint components (BUB1, CDC20, CENPF), mitotic kinesins (KIF18B, KIF20A) and other important mitotic regulators (CCNB1, CDK1, NEK2, CDC25B, BIRC5, INCENP and NUF2). Furthermore, GOrilla analysis revealed that the genes deregulated by 6-AA and vinblastine corresponded to the “microtubule binding” function (Supp. Fig. S3C). 6-AA-mediated differential expression of CCNB1, CDC25B, PLK1, HMMR, PTTG1, and CDKN3 was confirmed by qRT-PCR (Fig. 2B).

6-AA is a reversible inhibitor of mitosis

The observed accumulation of cells in G₂-M and deregulation of mitotic pathways prompted us to investigate if 6-AA is an inhibitor of mitosis. Similar to the mitotic inhibitors paclitaxel and nocodazole, Western blotting revealed that 6-AA strongly increased the level of phospho-Ser10 histone 3 protein (PHH3) in LNCaP cells, a marker of mitosis (Fig. 3A) (37). Fluorescence microscopy of PHH3 demonstrated that, like vinblastine, 6-AA caused a concentration-dependent accumulation of PHH3-positive LNCaP cells after 24 h (Fig. 3B), which was detectable as early as 8 h (1.25–10 nM, Supp. Fig. S4A). Consistent with this, these cells displayed the morphological hallmarks of mitosis (cell rounding and condensation of chromatin, Supp. Fig. S1).

Notably, like vinblastine (38), the 6-AA-mediated mitotic block and growth inhibition (up to 2.5 nM) was reversible when cells were treated for 8 h, then washed with fresh media and left to recover for 16 h (Supp. Fig. S4). However, treatment with 6-AA for longer than 8 h before inhibitor removal was associated with a visibly reduced rate of proliferation and viability, which was also observed for vinblastine (Supp. Figs. S4B, S4C, S4F).

6-AA induced mitotic arrest leads to asymmetric division and cell death

A detailed analysis of 6-AA-mediated inhibition of mitosis and its impact on cell fate was performed using time-lapse microscopy of HeLa cells expressing H2B-GFP (Fig. 3C). HeLa cells normally progressed through mitosis with a mean time of 67.4 ± 32.4 min, followed by cytokinesis (87.1% bipolar division leading to two daughter cells). Like vinblastine (10 nM: 398.8 ± 270.3 min), 6-AA caused a significant, concentration-dependant increase in the duration of mitosis (Fig. 3C), prolonging the time spent in mitosis from 216.3 ± 192.0 min (0.62 nM) to 687.8 ± 207.7 min (10 nM). Close inspection of the images further revealed that cells treated with increasing concentrations of 6-AA failed to properly congress and align their chromosomes at the metaphase plate and displayed misaligned chromosomes and

patterns of chromosome alignment that are evidence of monopolar and multipolar spindles. These phenotypes were also seen in vinblastine-treated cells (Supp. Videos S1-S2). Evaluation of the cellular fate (number of daughter cells and cell death) revealed a concentration-dependent decrease in bipolar divisions and concomitant increase in asymmetric cell divisions leading to more than two daughter cells (mostly 3–4) when cells were treated with 0.62 nM to 5 nM 6-AA as well as a substantial rise in cell death (Fig. 3C), culminating at 10 nM 6-AA with 100% cell death. Similar effects were observed with vinblastine (Fig. 3C). Anaphases with completed karyokinesis and cytokinesis (into three or more daughter cells) were considered as asymmetric divisions (39). Furthermore, daughter cells derived from these asymmetric divisions commonly merged together into a single multinuclear cell (40) (Fig. 3D, Supp. Videos S1–S2). Immunofluorescence microscopy of LNCaP cells treated with 6-AA (2.5 nM) or vinblastine (10 nM) also showed multinucleated cells (data not shown).

We next tracked HeLa-H2B-GFP cells treated with 6-AA (2.5 nM) for 144 h by time-lapse microscopy to study the fate of the cells derived from asymmetric cell division. We observed that most of the daughter cells failed to divide further and died in an interphase-like state (Fig. 3D, left panel) or during the next cell cycle (Fig. 3D, right panel). These results are consistent with a study by Ganem *et al.*(41) and could partially explain the long term cell killing effect of transient treatment with 6-AA.

Mitotic spindle organization is perturbed by 6-AA

Given the almost identical transcriptional and phenotypic effects of 6-AA and vinblastine, we investigated whether 6-AA interfered with the mitotic spindle organization, in particular microtubule dynamics, the major target of vinblastine (8). Immunofluorescence microscopy of PHH3 and α -tubulin showed that LNCaP cells treated for 24 h with 2.5 nM of 6-AA exhibited a range of microtubule spindle abnormalities, including bipolar spindles with

misaligned chromosomes, monopolar and multipolar spindles, while vehicle control (DMSO) showed cells with normal metaphase plates and bipolar anaphases (Fig. 4A). Phenotypic scoring of the 6-AA-induced spindle abnormalities revealed that, at 2.5 nM of 6-AA, 23% of cells displayed bipolar spindles with misaligned chromosomes and 35.8% and 41.3% of cells had monopolar and multipolar spindles, respectively. The latter defect visibly increased in a dose-dependent manner (Fig. 4A), which was consistent with our findings in HeLa cells (as shown above). A similar frequency of these spindle defects were observed in vinblastine-treated LNCaP cells (Fig. 4A). Measurement of the distance between spindle poles in cells with bipolar spindles revealed that 6-AA produced a dose-dependent reduction of the spindle length, similar to vinblastine (Fig. 4A). These defects were also observed in PC-3 cells (Supp. Fig. S5). Seven structural analogs of 6-AA induced a similar phenotype (Supp. Figs. S6A and S6B).

During normal mitosis, each of the two spindle poles contains one centrosome, formed by a pair of centrioles fixed in pericentriolar material (PCM), containing pericentrin (42). Immunofluorescence microscopy of vehicle-treated LNCaP cells in mitosis showed two similar-sized areas of pericentrin staining at both spindle poles, while 6-AA (5 nM) or vinblastine (20 nM) caused a smaller, more punctuated pericentrin staining and frequently two discrete spots per pole (Fig. 4B) and in some cases pericentrin staining was absent from the spindle pole (data not shown). These results demonstrate that 6-AA disrupts normal spindle organization in mitosis.

6-AA directly targets tubulin by inhibiting tubulin polymerization and microtubule dynamics

Inhibition of mitotic kinases Aurora kinase A (Aurora A) and Polo-like kinase-1 (Plk1) have been shown to cause defective spindle organization (43, 44). Immunofluorescence microscopy revealed that 6-AA-treated cells displayed strong Aurora A staining at the spindle

poles, which was absent at this localization with the Aurora A inhibitor MNL8237 (Supp. Fig. S7A). The Plk1 inhibitor BI2536 exclusively induced the formation of monopolar spindles (43) often with dispersed chromosomes at the cell periphery (Supp. Fig. S7B). Together, these distinct phenotypes strongly suggest that 6-AA does not target the mitotic kinases Aurora A or Plk1.

To determine whether 6-AA inhibits mitotic spindle organization by directly targeting microtubule formation, we performed a microtubule assembly assay with purified components in a cell-free system (45). 6-AA inhibited *in vitro* tubulin polymerization in a concentration-dependent manner, similar to the microtubule-depolymerizing compounds vinblastine and nocodazole, whereas the microtubule-stabilizing molecule paclitaxel increased polymerization (Fig. 5A). Anopterine, a 6-AA analog, also inhibited tubulin polymerization (Supp. Fig. S6C).

In LNCaP cells, high doses of 6-AA ($5\text{--}80\times\text{IC}_{50}$) significantly reduced the cellular tubulin polymer mass (46) in a dose-dependent manner and visibly disrupted cellular microtubule networks in LNCaP cells, similar to the effect of vinblastine, while paclitaxel caused an increase in tubulin polymer mass (Fig. 5B), which was consistent with previous studies (47). Inhibitor washout experiments revealed that disruption of the cellular microtubule network by 6-AA and vinblastine was reversible in LNCaP cells (Supp. Fig. S8) (48), whereas with colchicine, the destabilizing effect was irreversible (49).

We studied the effect of 6-AA on microtubule dynamics in HeLa cells expressing the end-binding protein (EB1-GFP) using real-time spinning disk microscopy. EB1 remains attached to the plus end of the growing microtubules but dissociates during the depolymerization phase (50) and can be used to track polymerizing microtubules and measure changes to microtubule dynamics (51). 6-AA visibly disrupted directional microtubule growth, as exemplified by maximum intensity projections of the EB1 comet time-lapse sequences (Fig.

5C and Videos S3–S5). Analysis of the time-lapse videos showed that 6-AA significantly shortened the displacement length of the EB1 tracks in a concentration-dependent manner when compared to the vehicle control (Supp. Fig. S9).

Taken together, 6-AA directly and reversibly inhibits microtubule dynamics (low concentration) and induces tubulin depolymerization (high concentration), leading to a decrease of cellular tubulin polymer mass and disrupted microtubule networks.

Microtubule alterations that confer resistance to *vinca* alkaloids or 2-methoxyestradiol did not affect 6-AA activity

To address if the activity of 6-AA is affected by resistance mechanisms that human T-cell acute lymphoblastic leukemia cell lines developed in response to chronic treatment with the microtubule inhibitors 2-methoxyestradiol (CEM/2ME2-14.4R and CEM/2ME2-28.8R cells) or vincristine (CEM/VCR-R cells), we measured the resistance factor of 6-AA in these cell lines. The 2ME2-resistant cell lines have acquired β I-tubulin mutations in the colchicine binding pocket, while the vincristine-resistant CEM/VCR-R cells have acquired multiple microtubule alterations (e.g. mutations in β I-tubulin, altered expression of β -tubulin isotypes) (18) and overexpression of P-glycoprotein (P-gp), thereby rendering cells multidrug-resistant to non-microtubule-targeting inhibitors such as doxorubicin (52), daunorubicin, actinomycin D (17), and tetracycline (53).

Consistent with previous work, CEM/2ME2-14.4R and CEM/2ME2-28.8R cells displayed resistance factors (RF) for 2ME2 of 18.7 and 30.6, respectively when compared to the parental CEM line. By contrast, the potency of 6-AA remained largely unaffected (RF: CEM/2ME2-14.4R=2.3, CEM/2ME2-28.8R=1.7) (Fig. 6A, Supp. Table S4).

Previous studies have shown that CEM/VCR-R cells are highly resistant to vincristine and vinblastine (17). Consistent with this, the RF for vincristine was 12,336 in CEM/VCR-R cells. Yet, 6-AA was 52-times more potent than vincristine, and CEM/VCR-R displayed an

RF of only 45.3 (Fig. 6A, Supp. Table S4). Similarly, and as previously shown (52), the RF for doxorubicin, a non-microtubule-targeting drug, was 24.9 in these cells, suggesting that the multidrug transporter P-gp confers resistance to doxorubicin (Fig. 6A, Supp. Table S4). Analysis of cells positive for caspase-3/7 activity by quantitative fluorescence microscopy demonstrated that 6-AA induced apoptosis in both CEM and CEM/VCR-R cells in a concentration-dependent manner (Fig. 6B). A 50 to 100-fold higher dose of 6-AA was required to induce a similar level of apoptosis (~30%) in CEM/VCR-R cells, while a 200-fold higher dose of vincristine was required.

To determine to what extent P-gp activity was responsible for the observed resistance of CEM/VCR-R cells to vincristine, 6-AA, and doxorubicin, the multidrug efflux pump was inhibited with verapamil (54). Co-treatment with verapamil partially restored sensitivity of CEM/VCR-R cells to vincristine (RF=63.6, Fig. 6C, Supp. Table S5), suggesting that previously described mechanisms (e.g. microtubule alterations (18)) caused the remaining resistance. In comparison, verapamil almost completely re-sensitized CEM/VCR-R cells to 6-AA and doxorubicin as demonstrated by RFs of 2.1 and 1.1, respectively. This strongly suggested that P-gp activity was solely responsible for resistance to 6-AA and doxorubicin and, more importantly, that 6-AA potency was not affected by the reported microtubule alterations (18).

In summary, the absence of resistance that is mediated by microtubule alterations provides evidence that 6-AA interacts with tubulin with a mechanism that is distinct to *vinca* alkaloids and 2ME2.

Discussion

In this work we investigated the mechanism of action of 6-AA and discovered that this compound is a novel potent mitotic inhibitor in prostate and cervical cancer cells. The natural

product 6-AA and seven analogs were previously identified by our group from the Australian endemic tree *Anopterus macleayanus* and are C₂₀ diterpenoid alkaloids featuring the unique anopterine scaffold (15). This is the first report that the anopterine diterpenoid scaffold is associated with inhibition of microtubule dynamics, representing a novel structure class of microtubule-targeting agents.

Depending on their effect on microtubules at high, micromolar concentrations, microtubule-targeting agents are categorized into microtubule-stabilizing compounds (e.g. taxanes, epothilones) and microtubule-destabilizing compounds (e.g. *vinca* alkaloids, nocodazole, colchicine) (4). Our data show that 6-AA is a reversible microtubule-destabilizing molecule that directly interacts with tubulin (Fig. 5). Using wash out experiments to investigate the reversibility of inhibition and relationship of treatment period with cell fate, we showed that LNCaP cells treated with 6-AA for a short period of time (8 h) could exit the mitotic block and resume proliferation. Longer treatment (24 h) with 6-AA or vinblastine showed that LNCaP and HeLa cells could partially recover and start to proliferate again. Gajate *et al.* (49) reported that cells treated with the bicyclic colchicine analog MCT for a short period of time (before induction of the apoptotic response) resumed proliferating, however, once the treatment period exceeded the tolerable threshold (cell line specific) and apoptosis was triggered, the cell proliferation capacity could not be restored by the removal of the inhibitor. Furthermore, Towle *et al.* showed that, after 12 h of treatment of U-937 cells (histiocytic lymphoma) with microtubule-targeting agents at concentrations that induced complete mitotic block, and 10 h of washout, the mitotic arrest was reversible or irreversible depending on the compound (38). Small changes in chemical structures between analogs (e.g., eribulin versus ER-076349, vincristine versus vinblastine, colchicine versus colcemid) led to profound differences in mitotic block reversibility.

Most microtubule-targeting agents of this class bind to the *vinca* or colchicine sites on

microtubules (4). The *vinca* site is located on the β -subunit of tubulin dimers (11). Studies have shown that vinblastine can also bind at the inter-dimer interfaces to both α - and β -tubulin (55, 56). The relatively large colchicine site is positioned between the α - and β -tubulin subunits within the same dimer (12). Point mutations located within these bindings pockets or sites that alter the tubulin conformation have been frequently observed and confer resistance through changed interaction with microtubule inhibitors of the same pharmacophore class (cross-resistance) (18, 19). The minimal resistance of 6-AA in the 2ME2-resistant leukemia cell lines (CEM/2ME2-14.4R and CEM/2ME2-28.8R, Fig. 6), suggests a tubulin binding mechanism of 6-AA that is different to 2ME2. Notably, CEM/2ME2-28.8R cells express class I β -tubulin with several mutations (S25N, D197N, A248T, and K350N), with A248T and K350N residing in the colchicine pocket, while CEM/2ME2-14.4R cells contain only the S25N and D197N mutations (19). On the basis that both cell lines lack significant cross-resistance to colchicine, we cannot dismiss the possibility that 6-AA might bind reversibly to the colchicine site.

A large body of different experiments presented here show that, at low nanomolar concentration, 6-AA acts phenotypically and functionally indistinguishable from the *vinca* alkaloid vinblastine (7, 8). The antiproliferative effect of both compounds was due to inhibition of microtubule dynamics leading to mitotic catastrophe.

Vincristine/vinblastine-resistant leukemia CEM/VCR-R cells possessed noticeable resistance to 6-AA (Fig. 6). In contrast to parental CEM cells or the 2ME2-resistant sublines, CEM/VCR-R cells display the classic multidrug-resistance phenotype mediated through high expression of P-gp that render cells resistant to inhibitors which do not target microtubules but are known substrates of P-gp (e.g., anthracyclines) (17). Inhibition of P-gp with verapamil almost completely restored 6-AA sensitivity of CEM/VCR-R cells to levels seen in parental CEM cells (Fig. 6), demonstrating that 6-AA was not affected by the remaining

resistance mechanisms like the reported microtubule alterations that render CEM/VCR-R cells resistant to *vinca* alkaloids. In light of our discovery that 6-AA is a P-gp substrate, future studies with our panel of anopterine derivatives will address if modifications of the chemical structure could reduce its affinity for P-gp.

Altogether, our results strongly suggest that 6-AA interacts with tubulin in a way that is distinct to both *vinca* alkaloids and 2ME2. However, we cannot rule out that 6-AA is binding in the colchicine or *vinca* alkaloid binding pockets, with a distinct orientation in the site and interacting with different amino-acids. Further experiments are warranted to investigate the exact binding of 6-AA with tubulin by molecular docking, detailed analysis of the crystal structure of the complex between tubulin and 6-AA, and site-directed mutagenesis of key amino acids combined with binding studies.

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Figures legends

Figure 1. 6-AA inhibits growth and induces a cell cycle arrest in G₂-M and apoptosis.

A, chemical structure of 6 α -acetoxyanopterin (6-AA).

B, dose-response curves of cell viability after treatment with 6-AA.

C, analysis of real-time cell proliferation of 6-AA treated LNCaP cells. DMSO (vehicle) and vinblastine (Vinb) were used as controls. Representative images for 6-AA (5 nM) and Vinb (20 nM) treatments are shown that track individual cells (bottom panel). Scale bar=50 μ m.

D, cell cycle was analyzed by flow cytometry. 6-AA arrests LNCaP cells in the G₂-M phase in a concentration-dependent manner after 24 h. DMSO and vinblastine were used as controls (left panel, n=3, mean $\pm$ SD, statistics in Supp. Table S6). Representative histograms for DMSO and 6-AA are shown (right panel).

E, F, 6-AA treatment of LNCaP cells (24 h) leads to cell death (**E**, FACS analysis of sub G₀-G₁ cell population, n=3, mean $\pm$ SD) by apoptosis (**F**, Western blot analysis of cleaved PARP protein). DMSO, vinblastine, paclitaxel (Paclit) and nocodazole (Noc) were used as controls. β -actin was used as a loading control. Please note that the β -actin control in Fig. 1F and 3A are the same data and that cropped lanes in Fig. 1F and 3A were from the same gel.

Figure 2. 6-AA and vinblastine transcriptionally deregulate identical mitotic networks.

A, pathway analysis of microarray data (Metacore) identified the top ten pathways affected by 6-AA (10 nM) and vinblastine (3.25 nM) in LNCaP cells after 24 h.

B, validation of differential expression of critical cell cycle genes identified in **A** by qRT-PCR (n=3, mean $\pm$ SD).

Figure 3. 6-AA arrests cells in mitosis, leading to asymmetric cell division and apoptosis.

A, 6-AA-treated LNCaP cells (24 h) displayed increased levels of histone H3 phosphorylation (PHH3, Western blot). DMSO, paclitaxel (Paclit) and nocodazole (Noc) were used as controls. β -actin was used as a loading control. Please note that the β -actin control in Fig. 1F and 3A are the same data and that cropped lanes in Fig. 1F and 3A were from the same gel.

B, quantitative immunofluorescence microscopy revealed that 6-AA and vinblastine caused a concentration-dependent increase of PHH3-positive LNCaP cells after 24 h (n=3, mean $\pm$ SD).

C, quantification of time-lapse microscopy showed that 6-AA and vinblastine increased the

time HeLa-H2B-GFP cells spend in mitosis (left panel). The cell fate after 24 h (bipolar division, asymmetric division or apoptosis) was quantitatively assessed (right panel, $n=2$, mean $\pm$ SD).

D, representative images of asymmetric cell division leading an interphase-like state (left panel) or cell death during the next cell cycle (right panel) of HeLa-H2B-GFP cells treated with 6-AA (2.5 nM) were captured by real-time live-cell imaging (PC: phase contrast). Arrows indicate cells that have undergone fusion. Scale bar=50 μ m.

Figure 4. 6-AA disrupts mitotic spindle organization.

A, 6-AA and vinblastine-treated LNCaP cells (24 h) were subjected to immunofluorescence microscopy of α -tubulin (green) and PHH3 (red). Representative images (top panel) of abnormal metaphase alignments of chromosomes and spindle pole organization are shown (scale bar=10 μ m). Quantification by scoring phenotypic differences (bottom left panel) and measuring the distance between spindle poles (bottom right panel) in bipolar cells based on the α -tubulin staining ($n=3$, mean $\pm$ SD).

B, 6-AA and vinblastine-treated LNCaP cells (24 h) caused abnormal centrosomal organization, as shown by immunofluorescence microscopy (pericentrin (red), DNA (blue) and α -tubulin (green), scale bar=10 μ m).

Figure 5. 6-AA destabilizes microtubules and inhibits microtubule dynamics.

A, 6-AA, like vinblastine (Vinb), inhibited tubulin polymerization in a cell-free system, while paclitaxel (Paclit) stimulated polymerization. Representative experiment ($n=3$) is shown.

B, high-dose treatment of LNCaP cells (24 h) with 6-AA and vinblastine reduced α -tubulin polymer mass, while paclitaxel increased α -tubulin polymer mass (top right panel), as shown by quantitative immunofluorescence microscopy of the cellular α -tubulin mean intensity (expressed as fold-change relative to DMSO control, $n=3$, mean $\pm$ SD). Representative images are shown (lower panel), illustrating disrupted cellular microtubule networks. Scale bar=5 μ m.

C, HeLa-EB1-GFP cells were treated with 6-AA and vinblastine (2h) and imaged by spinning disk microscopy. 6-AA and vinblastine affects the growing end of microtubules in HeLa cells, as shown in maximum intensity projections of EB1 comets (scale bar=10 μ m).

Figure 6. Vincristine and 2ME2-induced tubulin alterations do not mediate resistance to 6-AA.

A, dose-response curves (upper panel) of cell viability in indicated cell lines after treatment with 6-AA, vincristine (Vinc) or 2-methoxyestradiol (2ME2). For IC₅₀ values, see Supp. Table S4. The resistance factor (RF) expresses the quotient IC₅₀(drug-resistant cell line)/IC₅₀(CEM); (doxorubicin=Dox).

B, Assessment of caspase-3/7 activity revealed that 6-AA and vincristine induced apoptosis in both CEM and CEM/VCR-R cells after 24 h (left panels, n=3, mean±SD). Representative images of CEM/VCR-R cells (right panel, Casp: CellEvent Caspase-3/7 substrate). Scale bar=20 µm.

C, dose-response curves of cell viability (alamarBlue) after treatment with indicated compounds in combination with verapamil (Verap) for 72 h. For IC₅₀ values, see Supp. Table S5.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6