In vitro anticancer properties and biological evaluation of novel natural alkaloid Jerantinine B.

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Abstract

Natural products play a pivotal role in medicine especially in the cancer arena. Many drugs that are currently used in cancer chemotherapy originated from or were inspired by nature. Jerantinine B (JB) is one of seven novel *Aspidosperma* indole alkaloids isolated from the leaf extract of *Tabernaemontana corymbosa*. Preliminary antiproliferative assays revealed that JB and JB acetate significantly inhibited growth and colony formation, accompanied by time- and dose-dependent apoptosis induction in human cancer cell lines. JB significantly arrested cells at G2/M cell cycle phase, potently inhibiting tubulin polymerisation. Polo-like kinase 1 (PLK1; an early trigger for the G2/M transition) was also dose-dependently inhibited by JB (IC_{50} 1.5μM). Furthermore, JB provoked significant increases in reactive oxygen species (ROS). Annexin V+ cell populations, dose-dependent accumulation of cleaved-PARP and caspase 3/7 activation, and reduced Bcl-2 and Mcl-1 expression confirm apoptosis induction. Preclinical *in silico* biopharmaceutical assessment of JB calculated rapid absorption and bioavailability >70%. Doses of 8-16 mg/kg JB were predicted to maintain unbound plasma concentrations >GI_{50} values in mice during efficacy studies. These findings advocate continued development of JB as a potential chemotherapeutic agent.

Keywords: Natural products. Cell cycle. Tubulin. PLK1. Reactive oxygen species. Apoptosis.

Abbreviations: EB 1, end-binding protein 1; JB, jerantinine B; JBA, jerantinine B acetate; MTA, microtubule targeting agent; NP, natural product; ROS, reactive oxygen species; PLK1, Polo-like kinase 1.

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1. Introduction

Natural products (NP) have historically been used in the treatment of many diseases and continue to provide pharmacological agents used in pharmaceutical, biological and medical fields [1]. NPs are known for their structural diversity and have played inspirational roles in drug discovery [2]; ~ 50% of pharmaceuticals are derived from natural sources [3]. Thus it is important to pursue NP drug discovery to identify novel molecules from fragile rainforest habitats which may possess anticancer activity. Success in this field has led to the use of microtubule targeting agents (MTAs) in cancer chemotherapy. Vincristine and vinblastine, isolated from *Catharanthus roseus*, and taxol, isolated from the Pacific Yew, *Taxus brevifolia*, remain integral to the treatment of haematological and intractable solid cancers [4]. MTAs can act as stabilising agents (e.g. paclitaxel), promoting microtubule assembly, or destabilising agents (e.g. vincristine) to promote microtubule disassembly, altering microtubule dynamicity by binding to tubulin dimers [5]. Efficacy in the treatment of cancer arises from their ability to bind to tubulin and specifically inhibit mitosis [6, 7]. The cell cycle is governed by a rigorous system of checkpoints that consists of cyclin-dependent kinase (CDK)-cyclin complexes that allow progression from one cell cycle phase to the next [8, 9, 10]. Taxanes and vinca alkaloids arrest cell cycle at G2/M, affecting levels of cyclin B1 [8, 11, 12]. In 2008, seven novel *Aspidosperma* indole alkaloids were isolated, jerantinines A–G, from a leaf extract of Malayan plant *Tabernaemontana corymbosa* [13]. Jerantinine A (JA) evoked potent inhibitory activity against human-derived cancer cells causing profound G2/M block and clear inhibition of tubulin polymerisation [8]. Similarly, jerantinine E (JE) was shown to disrupt microtubules in PtK2 kidney cells [14]. Herein, we report *in vitro* biological evaluation of JB, a structural analogue of JA. *In vitro* activities of JB and its acetate derivative have been examined in human-derived colorectal (HCT-116), breast (MCF-7), lung (A549), pancreatic (MIA PaCa-2), vincristine resistant (VR) HCT-116 carcinoma cell lines and MRC5 fibroblasts by MTT assays. Cell counts, clonogenic assays, cell cycle analyses, confocal microscopy, annexin-V/PI apoptosis, caspase 3/7 activity assays and Western blots have been undertaken. Tubulin polymerisation, PLK1 activity and generation of reactive oxygen species (ROS) were ~~also~~ assessed in efforts to elucidate mechanisms of action of JB. In addition, the physicochemical and pharmacokinetic properties of JB were assessed *in silico*.

2. Materials and methods

2.1 Isolation and characterisation of JB and JBA

JB (Fig. 1) was isolated from *Tabernaemontana corymbosa* leaf extract. JBA (Fig. 1) was prepared by dissolving 20mg (0.050mM) in 1mL of pyridine and 1mL acetic anhydride. The mixture was stirred for 20 min. Water (10mL) was added, and pH adjusted to 9 using 10% Na_2CO_3 solution [13].

2.2 Agent stocks

JB and JBA were provided as solids and reconstituted with DMSO to yield concentrations of 10mM. Stocks were stored as 10μL aliquots at -80ºC protected from light.

2.3 Cell culture

Cells were passaged twice weekly upon reaching 70-80% confluency. Cells were sub-cultured in RPMI 1640 medium containing sodium bicarbonate supplemented (2g/L), L-glutamine (0.3g/L) and 10% heat-inactivated foetal bovine serum.

2.4 Generation of vincristine resistance

A variant HCT 116 cell line was established with acquired resistance to vincristine following continued exposure (>6 months) of cells to escalating concentrations of vincristine. Resistant subclones (VR) are those surviving and proliferating in the presence of 2µM vincristine.

2.5 MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, adapted from Mosmann [15], was used to assess the ability of test agents to inhibit cell growth and/or evoke cytotoxicity [16]. MTT assays were performed at the time of agent addition (T zero) and following 72h exposure of cells to test agents, as previously described [8].

2.6 Cell counting assay

Cell counts were performed to substantiate MTT assay results. Cells (2×10^4 cells/well) were seeded in 6 well plates are incubated overnight before treatment with JB at 0.1, 0.2, 0.5 and 1µM (72h). Cells were harvested and counted by haemocytometer.

2.7 Clonogenic assay

The clonogenic cell survival test measures the ability of a single cell to survive brief exposure to test agent and maintain proliferative potential to form progeny colonies [17.18]. The assay was performed as previously described [8].

2.8 Cell cycle analysis

Cell cycle analysis was carried according to Nicoletti et al (1991) [19]. Cells were seeded in cell culture dishes at densities of $3\text{-}5\times10^5$ cells/dish in 10mL medium. Following treatment, cells were harvested and pelleted by centrifugation then re-suspended in 0.5-1mL fluorochrome solution (50µg/mL propidium iodide (PI), 0.1mg/mL ribonuclease A, 0.1% v/v Triton X-100, and 0.1% w/v sodium citrate in dH_2O). Cells were stored overnight in the dark at 4ºC. Cell cycle analyses were performed on a Beckman Coulter FC500 flow cytometer. EX- PO32 software was used to analyse data.

2.9 Tubulin polymerisation assay

The tubulin polymerisation assay [20, 21, 22] was conducted in 96-well plates according to manufacturer's (Cytoskeleton, Inc) instructions. Incubations included 1mM EGTA, 1mM $MgCl_2$, tubulin buffer: 80mM Na-PIPES (pH 6.9), test compound (JB, 1 and 5µM; paclitaxel and nocodazole, 5µM) and HTS-tubulin (4mg/mL). Paclitaxel (microtubule stabiliser) and nocodazole (tubulin polymerisation inhibitor) were included as positive and negative controls respectively: Tubulin polymerisation was triggered by addition of 1mM GTP and carried out at 37°C; fluorescence absorbance at 340nm was measured over 1.15h. Scattered light is directly proportional to microtubule polymer concentration.

2.10 Annexin V – FITC and propidium iodide apoptosis assay
Reagents used were Annexin V-FITC Ab, 1× annexin binding buffer (1:10 dilution 10× annexin binding buffer in dH_2O), and PI solution (50µg/mL in PBS). Cells (1×10^5 cells/well) were seeded in 6 well plates are incubated overnight before treatment with JB at 1× and $2\times GI_{50}$ (24, 48 and 72h). Cells were trypsinised and collected in FACS tubes then kept on ice for 10 min. Annexin-V-FITC (5µL) plus 100µL 1× annexin-V buffer were added to cells after centrifugation. After 15 min incubation in the dark at room temperature, PI (10µL; 50µg/mL in PBS) plus 400µL annexin-V buffer were added. Samples were protected from light and kept on ice for 10 min before analysis on a Beckman Coulter FC500 flow cytometer. EX- PO32 software was used to analyse data.

2.11 Caspase-3/7 activity assay
The Apo-ONE® Homogeneous caspase 3/7 assay (Promega) was used to determine caspase activity. Cells (5×10^3 per well) were seeded in 96 well opaque white or black cell culture plates and incubated overnight at 37°C. JB or vincristine ($1\times GI_{50}$; 48h exposure) was introduced. Apo-ONE® caspase-3/7 reagent was added to each well with gentle mixing (300–500rpm) for at least 30 seconds. Plates were incubated for 30 min at room temperature. Well fluorescence was measured using an EnVision multilabel plate reader (PerkinElmer) at wavelengths between 499nm and 521nm.

2.12 Western blots
Western blotting was carried out as described [23]. Cells were seeded in dishes (100×20 mm) at a density of 1-2 x 10^6 per dish, allowed 24 hours to attach, and exposed to $1\times GI_{50}$ and $2\times GI_{50}$ JB. Following exposure, cell lysates were prepared and protein concentrations evaluated by Bradford assay [24]; 50µg protein per sample were separated by PAGE. Whole PARP, cleaved PARP, Mcl-1, Bcl-2, PLK1 and P glycoprotein (Pgp) 1° Abs were purchased from Cell Signaling Technologies. β-Tubulin 1° Ab (TUBB) was bought from Source BioScience. Anti-rabbit and anti-mouse immunoglobulin G (IgG) horseradish peroxidase-conjugated 2° Abs were obtained from Dako. Densitometric analyses of Western blots were performed using Image J.

2.13 Confocal microscopy
Confocal imaging was performed as previously described [25]. Procedures were performed at room temperature. Cells were fixed in formaldehyde (3.7 % in PBS; 10–15 min) then permeabilised by PBT (PBS + 0.1% Triton X-100; 2–3 min). Blocking agent (PBT + 1% BSA; 1h) was used to prevent binding non-specific protein binding. Cells were incubated with 1° Ab (monoclonal anti α-tubulin Ab, VWR International Ltd.; 2h), washed with PBT before incubation in the dark with 2° Ab for 1h. Cells were incubated with DNA binding dye (DRAQ5) for 5 min in the dark; a Zeiss LSM510 Meta confocal microscope was used to capture images.

2.14 Detection of reactive oxygen species
The ROS-Glo™ H_2O_2 luminescent assay is a sensitive, rapid, homogeneous assay that measures H_2O_2 levels directly in cell culture. Cells ($5x10^3$) were seeded in 96-well white

opaque plates in 80µL medium, incubated overnight and then treated with JB or vincristine for 24h. H_2O_2 substrate was added to incubates for 6h. The ROS-Glo™ detection solution was introduced (100µL) and plates incubated for 20 min at room temperature. Relative luminescence was measured using an EnVision multilabel plate reader (PerkinElmer).

2.15 PLK1 assay

PLK1 (5-20mU diluted in 50mM Tris pH 7.5, 0.1mM EGTA, 0.1% b-mercaptoethanol, 1mg/mL BSA, 100µM vanadate) was assayed against a substrate peptide (ISDELMDATFADQEAKKK) in a final volume of 25.5µL containing 50mM Tris pH 7.5, 300µM substrate peptide, 10mM magnesium acetate, 0.05% b-mercaptoethanol, 10µM vanadate and 0.005mM [^{33}P-g-ATP] (50-1000 cpm/pmole) and incubated for 30 min at room temperature. Assays were stopped by addition of orthophosphoric acid (5µL 0.5M; 3%) and then harvested onto P81 unifilter plates with a wash buffer of 50mM orthophosphoric acid. Concentrations of JB tested were 0.5, 1, 5, 10, 50µM.

2.16 *In silico* pharmacokinetic analyses

All predictions for both JB and JBA were conducted using GastroPlus™ version 9.0.0007 with built-in ADMET Predictor version 7.2.0.0 module. The input parameters for the simulations are shown in supplementary information (Suppl Table 1). *In silico* predictions of physicochemical and pharmacokinetic properties were performed based on the chemical structures of the two compounds by GastroPlus™. Built-in physiologically based pharmacokinetic (PBPK) models of relevant species were used for simulation of the pharmacokinetic profiles. Plasma concentration-time profiles following intravenous bolus injection and oral administration were predicted in mice and humans. For oral administration, PBPK models of fed state were applied for the predictions.

2.17 Statistical analyses

Experiments were conducted >3 times; with representative experiments demonstrated. One-way and two-way analysis of variance (ANOVA) were used to determine statistical significance. The minimal level of significance (p<0.05) was determined using Dunnett's multiple comparison tests.

3. Results

3.1 MTT assay

Both JB and JBA revealed potent growth inhibitory activity against five cell lines (Fig. 2; Table 1). GI_{50} values for JB were between 0.7 - 0.9µM for HCT-116, A549 and MCF-7. However, JBA exhibited more potent growth inhibitory activity against HCT-116, A549 and MCF-7 than JB with GI_{50} values ranging between 0.36 – 0.6µM. MIA PaCa-2 cells showed greatest sensitivity to JB and JBA with GI_{50} values ~ 0.25µM. 2-Way ANOVA between control and treatment groups revealed significant (p<0.01) growth inhibition by JB concentrations >0.1µM in HCT-116, A549 and MCF-7 and > 0.05µM in MIA PaCa-2 cells. JBA >0.1 µM significantly inhibited (p<0.01) growth in all cell lines. DMSO had no effect on absorbance readings for all cell lines (data not shown).

3.2 Cell counts

Cell counts were performed following 72h exposure of HCT-116 and MIA PaCa-2 cells to JB to validate MTT assay results. Dose-dependent reductions in cell numbers were particularly evident between 200nM and 500nM JB. At 1µM, JB caused cytotoxicity as significantly fewer cell numbers were recorded than were initially seeded (Figure 2 E and F; $p<0.01$).

3.3 Effect of JB and JBA on HCT-116 Colony Formation

Both JB and JBA significantly inhibited colony formation after 24h treatment of cells at concentrations equivalent to $1\times GI_{50}$ and $2\times GI_{50}$ (Fig. 3A). JB inhibited colony formation in A549 cells at $1\times GI_{50}$ and $2\times GI_{50}$ values by 64% and 80% respectively. Although MCF-7 and MIA PaCa-2 showed different sensitivity to JB (GI_{50} values 0.91 µM and 0.25 µM respectively), colony formation was similarly inhibited (by 95% and 99%) at $1\times GI_{50}$ and $2\times GI_{50}$ values respectively. Complete (100%) inhibition of colony formation was observed in HCT-116 cells exposed to 1× and $2\times GI_{50}$ JB, reflecting potency and cytotoxicity of JB (Fig. 3B). JBA showed significant and dose-dependent inhibition; interestingly, inhibition of colony formation by JBA in HCT-116 and MCF-7 was less potent than JB. At $1\times GI_{50}$ and $2\times GI_{50}$ values, JBA inhibited colony formation in HCT-116 by 40% and 96% respectively and in MCF-7 by 66% and 80% respectively. JBA revealed inhibitory effects similar to JB in MIA PaCa-2 cells (> 95% and 99% at $1\times GI_{50}$ and $2\times GI_{50}$ respectively).

3.4 Cell cycle analysis

MTT and clonogenic assays suggest that JB compromises cancer cell growth and viability. Guided by these observations, we investigated the effect of JB on cell cycle perturbation by flow cytometry. Cells, treated with JB at $1\times GI_{50}$ and $2\times GI_{50}$ for either 24, 48 or 72h. Stark G2/M cell cycle arrest was observed following 24 h exposure to JB in HCT-116, MCF-7 and A549 at $1\times GI_{50}$ and $2\times GI_{50}$ (Fig. 4). Profound G2/M accumulation was sustained after 48h and 72h in HCT-116, MCF-7 and A549 cells at $1\times GI_{50}$ and $2\times GI_{50}$ with time-dependent accumulation of HCT-116 and A549 events in pre-G1 at 1× and $2\times GI_{50}$. The most sensitive cell line indicated by MTT assay, MIA PaCa-2, revealed significant cell cycle arrest in G2/M phase at $2\times GI_{50}$ at 24h and 48h (Fig. 4). Interestingly, a significant pre-G1 was observed, earlier in MIA PaCa-2, following 24h exposure and sustained up to 72h at $2\times GI_{50}$. Cell cycle profiles are shown in supplementary Fig. 1.

3.5 JB inhibits tubulin polymerisation

Profound G2/M cell cycle arrest led us to investigate the effect of JB on tubulin polymerisation and compare effects to tubulin-stabilising and destabilising agents paclitaxel and nocodazole respectively (Fig. 5). Paclitaxel (5µM) induced rapid polymerisation of tubulin. In contrast, 5µM nocodazole arrested tubulin polymerisation. The effect of JB on tubulin polymerisation was unequivocal and similar to nocodazole: JB comprehensively inhibited tubulin polymerisation at 1 and 5µM.

3.6 JB induces apoptosis in cancer cells

Annexin-V/PI apoptosis and caspase-3 activation assays were performed to investigate apoptosis-inducing properties of JB in HCT-116, A549 and MIA PaCa-2 cell lines exposed to JB (1 and 2 × GI_{50}; 24, 48 and 72h). Apoptotic populations were confirmed by dual annexin V-FITC/PI staining (supplementary Fig. 2). JB revealed profound time-dependent incidence of apoptosis. JB induced significant (p<0.01) early (A^+/PI^-) and late (A^+/PI^+) HCT-116 apoptosis at 1× and 2×GI_{50} following 24, 48 and 72 h exposure (Fig. 6A). A549 revealed significant (p<0.01) apoptosis in the presence of JB at 1× and 2×GI_{50} after 48 and 72h treatment (Fig. 6B). On the other hand, the most sensitive cell line to JB (MIA PaCa-2), indicated by MTT, appeared more resistant to apoptosis at 1×GI_{50}. However, MIA PaCa-2 showed significant (p<0.01) early and late apoptosis at 2×GI_{50} concentration (Fig. 6C). The highest percentage apoptosis at 2×GI_{50} was observed in HCT 116 cells after 72h exposure to JB (50±4.71 %), compared to MIA PaCa-2 (45±4.74 %) and A549 (34±1.62 %). Induction of caspase-3/7 activity was measured in HCT-116, VR HCT-116 and MIA PaCa-2 cells after exposure to either JB or vincristine at 1×GI_{50} for 48h (Fig. 6D). JB evoked significantly (p<0.01) increased caspase activity in all three cell lines (HCT-116 230%, VR HCT-116 150% and MIA PaCa-2 155%) compared to control (100%). Vincristine clearly increased caspase activity in HCT-116 (200%) and MIA PaCa-2 (180%), in contrast, no enhanced activity was detected in VR HCT-116 cells.

3.7 JB alters protein expression

Western blot was used to investigate expression of proteins possessing roles in mitosis, apoptosis and cell survival. Lysates of HCT-116 and MIA PaCa-2 cells following 72h exposure of cells to 1× and 2× GI_{50} JB were prepared. Down-regulation of PLK1 and β-tubulin was observed after exposure to JB for 72h. Dose-dependent elevation in cleaved PARP accompanied reduction in expression of anti-apoptotic/pro-survival proteins Mcl-1 and Bcl-2 (Fig. 7A and B).

3.8 JB causes severe disruption in cytoskeletal architecture

Based on the ability of JB to inhibit microtubule assembly, prior to induction of apoptosis confocal microscopy was conducted to determine cellular changes in cytoskeletal architecture. Images were captured after 24 h exposure of HCT-116 cells to 1×GI_{50} JB or vincristine or vehicle alone. Representative images (Fig. 8) reveal the effect of JB on cell morphology. JB caused multinucleation (1) nuclear fragmentation (2) and membrane blebbing (3). Multipolar spindles were also evident in cells treated with JB and vincristine (4).

3.9 Generation of vincristine resistant (VR HCT-116) cells

A variant cell line possessing >300-fold resistance to vincristine was generated from HCT-116 cells. Cells were initially exposed to 5nM vincristine. Drug concentrations were escalated stepwise over 6 months until VR HCT-116 cells, able to survive subculture in the continued presence of 2μM were established. Vincristine GI_{50} values were as follows: HCT-116, 5nM; VR HCT-116, 1.64 μM. Western blot determined expression of the ATP binding cassette protein ABCB1, P-glycoprotein (Pgp), product of the multi-drug resistance (*MDR1*) gene in VR HCT-116 (Fig. 7). In contrast to

vincristine, JB and JA retained activity in VR HCT-116 cells, demonstrating ability to overcome Pgp efflux; GI_{50} values <700 nM JB were obtained in HCT-116 and VR HCT-116 (Table 2).

3.10 JB induces ROS production in cancer cells

Production of ROS was measured in HCT-116, VR HCT-116 and MIA PaCa-2 cells following treatment with either JB or vincristine at $1\times GI_{50}$ following exposure for 24h (Fig. 9). JB evoked significantly increased ROS production in HCT-116 (348%), VR HCT-116 (170%) and MIA PaCa-2 (281%) following 24h. Vincristine showed clear increase in ROS production in HCT-116 (239%) and MIA PaCa-2 (277%). Consistent with caspase-3 activity assay, vincristine failed to induce ROS in VR HCT-116 cells.

3.11 JB potently inhibits the activity of PLK1

JB significantly ($p<0.05$) and dose-dependently inhibited PLK1 activity (0.5, 1, 5, 10 and 50µM; Fig. 10; IC_{50} ~1.5 µM). In addition, in cells exposed to JB ($1\times$ and $2\times GI_{50}$) down-regulation of PLK1 expression was detected (Fig. 7A and B).

3.12 Predicted pharmacokinetic profiles of JB and JBA

In silico simulations by GastroPlus^{TM} predicted pharmacokinetic profiles for JB and JBA. The plasma concentration-time profiles for both compounds in humans and mice (the species commonly used for preclinical efficacy studies) are shown in Fig. 11. While both JB and JBA were predicted to have relatively high oral bioavailability (>70%) without major species differences, higher oral bioavailability was revealed for JB than JBA (Table 3). The elimination rate of JBA was higher than that of JB, which may be a consequence of JBA`s rapid conversion to JB.

Predictions to predetermine the therapeutic doses needed for future preclinical efficacy studies were performed in mice with unbound plasma concentration of the compounds, because it will be the unbound fraction of the compounds that exert pharmacodynamic activities *in vivo*. Also, fed state was applied because the mice are likely to be in fed state during preclinical efficacy studies where multiple dosing will be applied. Unbound plasma concentration-time profiles were plotted together with the GI_{50} values obtained from *in vitro* experiments to provide preliminary prediction of efficacious doses. From Fig. 12, it can be seen that dose-linear pharmacokinetics are expected from the dose range simulated (2-64 mg/kg). As a result, doses of 8-16 mg/kg are predicted to be able to maintain the unbound plasma concentrations of both compounds above the GI_{50} values for most cell lines in mice during efficacy studies.

4. Discussion

JB a novel *aspidosperma* indole alkaloid and its acetate derivative revealed profound growth inhibitory and cytotoxic activity against human-derived HCT-116, VR HCT-116, MCF-7, A549 and MIA PaCa-2 cancer cell lines. Generally, JBA exhibited greater potency against HCT-116, A549 and MCF-7 than JB in the MTT assay. JBA's enhanced potency may be attributed to its stability when compared to JB, which may undergo hydrolysis and degradation thereby impacting activity [8]. Additionally, the presence of an acetate group reduces overall polarity possibly facilitating compound diffusion across hydrophobic cell membranes [26]. Therefore enhanced intracellular concentrations of JBA may be achieved, leading to slightly higher potency. Interestingly, JB possessed greater activity in most cell lines tested when compared to JA notably against A549; GI_{50} JB ~ 0.7 μM; GI_{50} JA 3.7 μM [8]. JB possesses an epoxide moiety which may confer enhanced activity over JA [8]. Subsequent cell counts, performed following 72 h exposure of cells to JB, corroborated potent growth inhibitory properties detected by MTT assay. Estimated JB and JBA GI_{50} values, calculated from MTT assays were used in subsequent assays. JB and JBA (24h exposure) significantly inhibited HCT 116, MCF-7, MIA PaCa-2 and A549 colony formation (Fig. 3A). These outcomes indicate that cells have either been killed or have lost their ability to proliferate and form progeny colonies (Fig. 3B). In contrast, the effect of JA on MCF-7 colony formation at $1 \times GI_{50}$ was not significant, thus exposure of MCF-7 to $1 \times GI_{50}$ JA for 24 h was not sufficient to cause cytotoxicity or inhibit proliferation [8]. Although, JBA showed potent growth inhibitory activity in MTT assays compared to JB after 72h continued treatment, reduced potency against HCT-116, MCF-7 and MIA PaCa-2 in clonogenic assays was observed after exposure for 24h (Fig. 3A). JBA may act as a prodrug requiring the presence of cellular esterases for bio-activation [26]. Because of the low cell densities seeded in clonogenic assays, there may be insufficient esterase activity to catalyse enzymatic hydrolysis. Indeed, incomplete or slow bioconversion of prodrugs can lead to lower than predicted bioavailability [26]. JB evoked significant G2/M arrest (Fig. 4); at $2 \times GI_{50}$ JB was able to sustain significant G2/M arrest throughout the 72h exposure. It was hypothesised that JB like analogue JA may perturb microtubule dynamics underpinning G2/M halt. Many drugs show similar inhibitory activity on the cell cycle, e.g. taxanes, vinca alkaloids, nocodazole and colchicine. These drugs act as microtubule-disrupting agents and block the cell cycle at G2/M through interference with tubulin (de)polymerisation and microtubule (dis)assembly [27]. Tubulin polymerisation was carried out in the presence of paclitaxel, nocodazole, JB (1 and 5μM) or vehicle alone. Paclitaxel was used as a positive control (microtubule stabiliser), while nocodazole, a negative control, inhibits tubulin polymerisation and destabilises microtubules. JB unambiguously and comprehensively inhibited tubulin polymerisation (Fig. 5). In addition, significant reduction in expression of β-tubulin protein in JB-treated cells further inferred JB may induce microtubule disarray (Fig. 7B). Confocal microscopy following treatment of cells with JB and vincristine was undertaken; DNA and tubulin were stained to accentuate changes in cytoskeletal architecture and cell morphology triggered by these agents. Treatment of tumour cells with MTAs is responsible for features characteristic of failed polymerisation including multipolar spindles and irregular chromosome segregation in addition to apoptotic signs such as DNA fragmentation and blebbing [28, 29]. Confocal microscopy images of JB-treated cells revealed such features. Multipolar spindles and

misaligned chromosomes were evident (Fig. 8); multinucleation (aneuploidy), nuclear fragmentation and membrane blebbing were also detected. It has emerged that like the vinca alkaloids; JB caused G2/M cell cycle arrest, overwhelmingly inhibited tubulin polymerisation and caused microtubule disarray leading to formation of multipolar spindles. In addition, jerantinine analogues share structural similarity with vincristine; cross-resistance between these alkaloids was therefore investigated. We endeavoured to generate variant HCT-116 cell lines displaying acquired resistance to vincristine or jerantinine. After 6 months in the presence of escalating vincristine concentrations, VR HCT-116 cells evolved which survived and proliferated in medium spiked with 2μM vincristine. VR HCT-116 cells were >300-fold more resistant to vincristine than HCT-116 cells. In contrast, VR HCT-116 cells were slightly more sensitive than parent HCT-116 cells to JB and JA (Table 2). Western blot analysis determined expression of P-glycoprotein (Pgp) in VR HCT-116 (Fig. 7A). Cell membrane Pgp is an efflux pump of the ATP-binding cassette (ABC) class, it exports drugs to the extracellular environment and is associated with multidrug resistance [30]. Expression confers resistance not only to vincristine, but to multiple chemotherapeutic agents possessing structural diversity and distinct modes of action. Clinically, Pgp expression is an immense problem limiting treatment success and cancer survival. Thus discovery of a novel alkaloid which appears not to be a Pgp substrate, able to evade multiple drug resistance is encouraging; however, it should be cautioned that agents able to evade ATP binding cassette pumps may by cyto- and neurotoxic; thus future research endeavours could include investigation of targeted delivery systems for such molecules. Intriguingly, following 2 years of continuous efforts, we have been unable to generate a variant HCT-116 cell line with acquired resistance to jerantinine. The effect of JB on activity of additional proteins which possess critical roles in mitoses were examined. Human PLK1, a member of the serine/threonine family of kinases is an important enzyme in control of progression mitosis, and maintenance of genomic integrity [31, 32]. PLK1 regulates multiple cell cycle-related events, including cdc2 activation, centrosome maturation, chromosome segregation, formation of bipolar spindle and execution of cytokinesis [33]. Substantially elevated expression of PLK1 has been reported in solid malignancies compared to healthy tissue [31, 32], leading to its classification as an oncogenic kinase. PLK1 has been proposed as a novel diagnostic and poor prognostic marker, and is a validated therapeutic target for cancer [33]. In preclinical studies, small interfering RNA molecules that inhibit PLK1 inhibited cancer cell proliferation and tumour growth, causing mitotic arrest and apoptosis [34]. Small molecule PLK1 inhibitor B12536 inhibited the self-renewal of cancer cells with high PLK1 expression [35, 36]. JB caused dose-dependent reduction in PLK1 activity (IC_{50} 1.5μm; Fig. 10) and down-regulated PLK1 expression in treated cells (Fig. 7B). This provides supportive evidence that, although comparable to vincristine in ability to disrupt microtubules, JB may interact upstream of tubulin *via* PLK1, which may be one of multiple possible targets that contribute to G2/M arrest.

Like vincristine, JB significantly increased ROS production in HCT-116 and MIA PaCa-2 cells (Fig. 9). The source of ROS may be enzymatic (NADPH oxidase, cytochrome P450s for example) or non-enzymatic (mitochondrial respiratory chain). ROS may be formed as by-products of metabolism of oxygen-containing compounds; enzyme-catalysed reactions that generate ROS include those metabolising vinca alkaloids (and possibly jerantinines). Vinca alkaloids also promote release of cytochrome C from

mitochondria inducing cell death and interfere with the electron transport chain resulting in production of superoxide radicals [37, 38, 39]. Beyond the scope of this study, we hypothesise that jerantinines may generate ROS through both these routes. Natural product anticancer agents such as vincristine and taxanes can induce apoptosis *via* ROS production [40]. The greater sensitivity of some tumour cells (over normal cells) to oxidative stress is considered a therapeutic option for new anticancer drugs [40]. Arsenic derivatives are examples of compounds that elicited anticancer activity and apoptotic effects by production of high levels of ROS [40]. ROS may be responsible for initiating caspase activation [41]. Through oxidation of mitochondrial pores, ROS may cause release of cytochrome c which plays an important role in caspase activation and induction of apoptosis [41]. Furthermore, through ROS production, MTAs may modulate microtubule dynamics through EB1 phosphorylation and lead to anti-proliferative and anti-migratory effects [42]. In VR HCT-116 cells, only JB caused a significant rise in ROS, again demonstrating that JB overcomes Pgp-mediated vincristine (and multi-drug) resistance. These data, consistent with MTT results (Table 2), support the thesis that the mechanism of antitumour action of JB involves generation of ROS. Inhibition of microtubule assembly and failed mitoses may lead to a number of consequences, including aneuploidy and apoptosis [43, 44]. PLK1 inhibition and ROS induction, as discussed, may also precede apoptosis. At 72 h HCT-116, MIA PaCa-2, and A549 cells exhibited a significant ($p<0.01$) increase in pre-G1 events following treatment with JB (Fig. 4). Interestingly, MCF-7 did not show significant elevation in pre-G1 events, because MCF-7 cells do not express caspase 3 [45]. Pre-G1 events are an indication of DNA cleavage by DNAase enzymes, an event integral to apoptosis [8]. Annexin-V/PI apoptosis and caspase-3 activation assays were used to confirm these observations. After 72h JB elicited time- and dose-dependent HCT-116, MIA PaCa-2, and A549 apoptosis (Fig. 6). Clear induction of caspase 3 activity was revealed in HCT-116, VR HCT-116 and MIA PaCa-2 cells at $1\times GI_{50}$ following 48h exposure to JB, while vincristine precipitated significantly increased caspase activity in HCT-116 and MIA PaCa-2 but not VR HCT-116 cells (Fig. 6D). Concomitant with caspase activation, cleaved PARP was observed in A549 (data not shown), HCT-116 and MIA PaCa-2 cells exposed to JB. Corresponding down-regulation of anti-apoptotic/pro-survival proteins Mcl-1and Bcl-2 (Fig. 7A and B) were also detected. Cancers commonly express elevated levels of anti-apoptotic oncogenic Bcl-2 family members. Their down-regulation diminishes cancer cell survival. Taken together, morphological features characteristic of apoptosis (e.g. membrane blebbing), exposure of phosphatidylserine on the cell membrane exterior, reduced Mcl-1 and Bcl-2 expression, caspase activation and cleaved PARP strongly suggest a cellular apoptotic destiny. In summary, JB elicited potent anti-proliferative effects and significantly inhibited colony formation in human-derived carcinoma cell lines. Profound G2/M cell cycle arrest and inhibition of tubulin polymerisation was demonstrated. JB inhibited PLK1 activity and significantly increased ROS production in treated cancer cells, ultimately inducing apoptosis. Intriguingly, JB retained sensitivity in VR HCT-116 cells expressing Pgp. The ability to generate a corresponding HCT-116 variant cell line resistant to jerantinine was however elusive; we postulate that JB`s multiple targets, which possess key roles in tumourigenesis, preclude (to date) evolution of jerantinine resistance compatible with cell viability, and conclude that further preclinical evaluation of these novel alkaloids is justified.

Thus, *in silico* preclinical biopharmaceutical evaluation of JB and JBA were performed using GastroPlus™, a powerful tool used in drug discovery programmes for lead optimisation and compound selection [46, 47]. The built-in generic PBPK models have provided predictions for a wide range of drugs [48, 49, 50] and there are numerous reports where GastroPlus™ has successfully predicted pharmacokinetic profiles in preclinical species and humans [51]. Predictions can be made based on chemical structure and/or physicochemical properties [52, 53, 54, 55]. In the present study, calculations by GastroPlus™ were based on the chemical structure of JB and its acetate derivative; rapid absorption and bioavailability values >70% were predicted for both compounds. Predictions to predetermine the doses needed for preclinical efficacy studies were performed in mice: doses of 8-16 mg/kg would be expected to maintain the unbound plasma concentrations of both compounds above the GI_{50} values for most cell lines. These data provide useful guidelines for future preclinical and clinical evaluation.
This new natural indole alkaloid, which targets mechanisms pertinent to tumourigenesis and cancer cell survival is worthy of continued development for treatment of malignant disease.

Acknowledgements
Authors would like to thank Dr. Cornelia de Moor, Dr. Hilary Collins and International Centre for Kinase Profiling in University of Dundee. The VR HCT-116 cell line was generated by V.J. Raja. T.S. Kam and K.H. Lim thank MOHE, Malaysia (HIR-005) for financial support. M.E. Qazzaz thanks the Higher Committee for Education Development in Iraq (HCED). Data represented in Figs. 11 and 12 were generated by GastroPlus™ software provided by Simulations Plus, Inc, Lancaster, California, USA.

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Figure legends.

Fig.1 The structures of jerantinine B and jerantinine B acetate

Fig. 2 Growth inhibitory effects of JB and JBA on HCT-116 (A), MCF-7 (B), A539 (C) and MIA PaCa-2 cell lines (D). Cells were seeded at a density of 3×10^3 cells/well in 96-well plates and incubated for 72h (n=8, ≥ 3 trials). Effects of JB on HCT-116 (E) and MIA PaCa-2 (F) cell numbers. Cells (2×10^4 cells/well) were seeded in 6 well plates are incubated overnight before treatment with JB at 0.1, 0.2, 0.5 and 1µM (72h). Cells were harvested and counted by haemocytometer.

Fig. 3A Mean survival fraction (%) of treated cells as a percentage of the control population for A549, HCT-116, MCF-7 and MIA PaCa-2 with SEM. JB exhibited significant reduction in colony formation ($p<0.01$, n=2 for each of the 2 trials). B Representative photograph showing effect of JB on HCT-116 colony formation at $1\times GI_{50}$ and $2\times GI_{50}$

Fig. 4 Effect of $1xGI_{50}$ and $2xGI_{50}$ JB following 24, 48 and 72h exposure on HCT-116 (A), A549 (B), MCF-7 (C) and MIA PaCa-2 (D) cell cycle. JB evoked significant arrest in G2/M phase ($p<0.05$, n=2; experiments were repeated ≥3 times)

Fig. 5 Effect of JB (1µM and 5µM) on tubulin polymerisation. Paclitaxel and nocodazole (5µM) were used as positive and negative controls respectively. JB strongly inhibited tubulin polymerisation.

Fig. 6A, B and C Effects of JB on HCT-116, A549 and MIA PaCa-2 apoptosis. Cells were treated with 1x and $2xGI_{50}$ JB for 24, 48 and 72h. Annexin-V/PI apoptosis assay was adopted to determine the percentage apoptotic cells. Total apoptosis comprises early apoptotic (annexin V-positive) and late apoptotic (annexin V-positive and PI positive) populations. Fig. 6D Effect of JB on caspase 3/7 activity in HCT-116,VR HCT-116 and MIA PaCa-2cells after 48h exposure to $1\times GI_{50}$. Mean± SEM ≥3 independent trials (n=2 per trial).

Fig. 7A Protein expression in HCT-116 and MIA PaCa-2 lysates following 72h exposure of cells to JB. Lysates containing 50µg protein were loaded into each well and proteins separated by SDS-PAGE. Western blots were performed using antibodies to detect whole and cleaved PARP, Mcl-1, Bcl-2, PLK1, TUBB, Pgp and housekeeping gene GAPDH. HCT-116 and MIA PaCa-2 cells were treated at $1\times GI_{50}$ and $2 \times GI_{50}$.

Fig. 7B Collated densitometric measurement of protein expression levels PARP cleavage was observed at $1\times GI_{50}$ and was accompanied by a dose-dependent decrease in Mcl-1 and Bcl-2. PLK1 and TUBB down-regulation was observed in HCT-116 and MIA PaCa-2 cells. GAPDH was used as an internal loading control. Expression of Pgp was observed in VR HCT-116. Mean± SD ≥3 independent trials.

Fig. 8 Effects of JB and vincristine (24h exposure) on HCT-116 cell morphology. 1st row (A,B,C,D): controls with vehicle only; 2nd row (E,F,G,H): JB ($1xGI_{50}$ = 0.7µM); 3rd row (I,J,K,L): vincristine ($1xGI_{50}$ = 5nM). JB caused multinucleation (1) nuclear fragmentation (2) and blebbing (3). Multipolar spindles were also evident in samples treated with JB and vincristine (4). Experiments were repeated 3 times.

Fig. 9 JB (24h) potently increased ROS production in HCT-116, VR HCT-116 and MIA PaCa-2 cells compared to vincristine which caused ROS production in HCT-116 and MIA PaCa-2 cells only. Cells were treated with JB ($1xGI_{50}$ = 0.7µM) or vincristine ($1xGI_{50}$ = 5nM). Mean± SEM ≥3 independent trials (n=2 per trial)

Fig. 10 Dose-dependent inhibition of PLK1 activity by JB. Mean±SD was calculated of two independent trials (n=2 per trial).

Fig. 11 Predicted plasma concentration-time profiles. (A) Prediction after administration of 4 mg/kg of JB to mice. (B) Prediction after administration of 100 mg capsule of JB to 70 kg humans. (C) Prediction after administration of 4 mg/kg of JBA to mice. (D) Prediction after administration of 100 mg capsule of JBA to 70 kg humans.

Fig. 12 Predicted unbound plasma concentration-time profiles plotted with GI_{50} values obtained from MTT assays. (A) Prediction after oral administration of JB in mice and GI_{50} values of JB on various cancer cell lines. Curves in grayscale from top to bottom represent unbound plasma concentration-time profiles after oral administration at doses of 64, 32, 16, 8, 4 and 2 mg/kg, respectively. Straight lines in colour scheme from top to bottom represent GI_{50} values from MRC-5, MCF-7, A549, HCT-116, VR HCT-116 and MIA PaCa-2 cell lines, respectively. (B) Prediction after oral administration of JBA in mice and GI_{50} values of JBA on various cancer cell lines. Curves in grayscale from top to bottom represent unbound plasma concentration-time profiles after oral administration at doses of 64, 32, 16, 8, 4 and 2 mg/kg, respectively. Straight lines in colour scheme from top to bottom represent GI_{50} values from A549, MCF-7, HCT-116 and MIA PaCa-2 cell lines, respectively.

Table 1. Effect of JB and JBA on growth of human-derived cancer cells

	Mean GI_{50} values (μM)				
	Breast carcinoma MCF-7	Lung carcinoma A549	Pancreas carcinoma MIA PaCa-2	Colon carcinoma HCT-116	Human foetal lung fibroblast MRC-5
JB	0.917±0.004	0.701±0.010	0.245±0.033	0.682±0.026	1.915±0.036
JBA	0.482±0.009	0.547±0.092	0.253±0.010	0.362±0.006	

MTT assay, following 72 h exposure of cells to test agents. Mean ± SD GI_{50} values (μM) values from ≥ 3 independent trials where n = 8

Table 2. Effect of JB, JA and vincristine on growth of wild type and VR HCT-116 cells

	Mean GI_{50} values (μM)			
	Designation	JB	JA	Vincristine
Wild colon carcinoma	HCT-116	0.682±0.026	0.762±0.13	0.005±0.001
Vincristine resistant colon carcinoma	VR HCT-116	0.490±0.015	0.438±0.1	1.64±0.45

MTT assay, following 72 h exposure of cells to test agents. Mean ± SD GI_{50} values (μM) values from ≥ 3 independent trials where n = 8

Table 3. Species differences in input values and predicted outcomes for JB and JBA

	JB		JBA	
	Mouse	Human	Mouse	Human
Body weight (kg)	0.025	70	0.025	70
Dose (mg)	1	100	1	100
Dose volume (mL)	0.25	250	0.25	250
Formulation	Solution	Capsule	Solution	Capsule
CL_h (L/h/kg)	0.44	0.12	1.48	0.42
CL_r (L/h/kg)	0.03	0.01	0.04	0.01
$t_{1/2}$ (hr)	1.25	5.88	0.297	1.827
F (%)	92.0	90.9	77.6	74.4

CL_h, hepatic clearance; CL_r, renal clearance; $t_{1/2}$, elimination half-life; F, oral bioavailability.