# Cardiac glycoside Cerberin exerts anticancer activity by PI3K/AKT/mTOR signal transduction inhibition.

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## Abstract

Natural products have played a significant role in anticancer drug discovery and many anticancer drugs used currently are of natural origin. Cerberin (CR), which is a cardenolide isolated from the fruit kernel of *Cerbera odollam*, was found to potently inhibit cancer cell growth ($GI_{50}$ values <90 nM), colony formation and migration. Significant G2/M cell cycle arrest preceded time- and dose-dependent apoptosis-induction in human cancer cell lines corroborated by dose-and time-dependent PARP cleavage and caspase 3/7 activation, in addition to reduced Bcl-2 and Mcl-1 expression. CR potently inhibited PI3K/AKT/mTOR signalling depleting polo-like kinase 1 (PLK-1), c-Myc and STAT-3 expression. Additionally, CR significantly increased the generation of reactive oxygen species (ROS) producing DNA double strand breaks. Preliminary *in silico* biopharmaceutical assessment of CR predicted >60% bioavailability and rapid absorption; doses of 1–10 mg/kg CR were predicted to maintain efficacious unbound plasma concentrations (>$GI_{50}$ value).

CR's potent and selective anti-tumour activity, and its targeting of key signalling mechanisms pertinent to tumourigenesis supports further preclinical evaluation of this cardiac glycoside.

***Key words***:

**Cardenolide; DNA Damage; Apoptosis; Reactive oxygen species; *Cerbera odollam*.**

## 1. Introduction

Cancer has caused great mortality and morbidity worldwide, with an estimated 18.1 million new cases and 9.6 million deaths by 2018 [1]. The pathogenesis of cancer is highly complicated

but is the basis of various molecular targets used in anticancer chemotherapy. To date, cytotoxic chemotherapy remains the mainstay treatment option for many types of metastatic cancers [2,3], although the issues of side effects and drug resistance have driven the urge to search for newer and safer cytotoxic drugs. Natural products (NPs) play a crucial role in drug discovery, with >50% of clinically approved drugs sourced from, or inspired by NPs [4]. Taking into account that many effective anticancer drugs like taxanes, vincristine and etoposide are plant NPs, our research aimed to elucidate the potent, yet selective, cytotoxic mechanism of a plant-derived cardiac glycoside, cerberin (CR), and to explore its potential as a new cytotoxic agent.

CR was originally isolated and characterised from the seed kernels of a Southeast Asian tree, *Cerbera odollam* (syn. *Cerbera manghas*) [5]. *C. odollam* is well-known within Southeast Asia to be highly poisonous, with restricted ethnobotanical uses as a purgative and rat poison [6]. Many instances of human fatalities have been attributed to the plant, with CR named as the principle toxin [7]. CR and *C. odollam* extracts have been shown to be potently cytotoxic against different *in vitro* cancer cell lines [8–12], but the exact mechanism(s) of action has remained unexamined. All cardiac glycosides are comprised of a 17 carbon steroidal backbone linked to a sugar moiety via a glycosidic bond and differ through alterations to the steroidal and glycosidic moieties. CR has a five-membered lactone ring and a distinctive sugar moiety identified as 2′-*O*-acetyl-L-thevetose [5,8,11]. Cardiac glycosides like digoxin and ouabain are well known for their inhibitory actions on the $Na^+/K^+$-ATPase transmembrane ion pump, leading to a positive inotropic effect on the heart. Digoxin (Lanoxin®) is widely indicated for the treatment of congestive heart failure despite having a narrow therapeutic window [13]. During the last two decades, the antiproliferative and apoptotic effects of various cardiac glycosides have become apparent. Many plant derived cardiac glycosides like convallatoxin, digoxin, peruvoside, oleandrin, ouabain and strophantidin have all been shown to exert *in vitro*

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anticancer effects on various cancer cell lines [10,14,15]. Due to their pleiotropic anticancer activity at low nanomolar concentrations against many cancer cell types, cardiac glycosides have been proposed as potential anticancer agents [16,17]. However, CR`s potential as an anticancer agent has yet to be explored and no data reporting anticancer mechanisms have been reported. In this study, the effects of CR were investigated against a panel of cancer cell lines; guided by preliminary screening, the focus of the work then concentrated on pancreatic (PANC-1), triple negative breast (MDA-MB-468) and non-small cell lung (A549) carcinoma cell lines for further biological evaluation and elucidation of the mechanism(s) of action.

## 2. Materials and methods

### *2.1 Plant collection and extraction*

The matured fruits of *Cerbera odollam* (20 kg) were collected in the autumn of 2015 from Penang, Malaysia. All plant specimens were positively identified as *C. odollam* and a voucher specimen (SH_UNMC-069B) was deposited in the herbarium of the School of Pharmacy, University of Nottingham Malaysia Campus. The fruits of *C. odollam* were cut open to retrieve the seeds from which their kernels were exposed and dried in an oven with aeration at 45 °C for 1 week. The dried seed kernels (500 g) were ground and extracted with 9:1 dichloromethane: methanol to yield a dark brown crude extract (15 g).

### *2.2 Isolation of cerberin*

The crude kernel extract of *C. odollam* was dissolved in methanol (200 mL) and partitioned against petroleum ether (3 × 100 mL) to remove fatty constituents. The methanol-soluble fraction was recovered and dried *in vacuo* to yield a brown amorphous solid extract (9.5 g), which was separated using preparative layer chromatography (PLC, Silica Gel 60 Merck 7759,

230-400 mesh) with ethyl acetate-hexane-methanol (9:1:0.2) as the eluent and visualised under UV light at 254 nm. A distinctively separated band ($R_f$ 0.25) on the PLC plate was scraped off and extracted with 200 mL dichloromethane-methanol (9:1) to yield a white amorphous solid (30 mg). The isolated compound was analysed with a Waters ACQUITY®ARC UHPLC system equipped with Waters Quarternary Solvent Manager-R, Sample Manager FTN-R and 2998 PDA Detector. The separation was conducted on a Waters CORTECS® C18 column (4.6 × 50 mm, 2.7 μm) with a mobile phase of A ($H_2O$, 0.1% TFA) and B (ACN) under gradient elution of 10–90% B over 30 min at a flow rate of 1 mL/min. Total injection volume was 5 μL (5 mg/mL CR stock solution in ACN). Two different chromatograms with PDA detection at 254 nm and MaxPlot (200-400 nm) were generated. $^1$H NMR spectrum of the pure sample was obtained in $CD_3OD$ on a Bruker 600 MHz NMR spectrometer, using TMS as internal standard.

### *2.3 Cell culture*

CR activity was tested against a panel of cancer cells derived from breast (MCF-7, MDA-MB-231, MDA-MB-468 and SKBR3), colon (HT-29, HCT-116 and vincristine-resistant- (VR)-HCT-116), pancreatic (PANC-1 and MIA PaCa-2), lung (A549), liver (HepG2) and nasopharyngeal (HK1) carcinomas. All cell lines were obtained from the American Type Culture Collection (ATCC) except for the HK1 and NP-69 cell lines which were a kind gift from Prof. GSW Tsao, Faculty of Medicine, The University of Hong Kong [18]. The VR-HCT-116 cell line was developed at the University of Nottingham following continued exposure (>6 months) of the HCT-116 cell line to escalating concentrations of vincristine [19]. Cell cultures were maintained in RPMI 1640 (Gibco) supplemented with sodium bicarbonate (2 g/L), 10% foetal bovine serum (FBS) (Gibco) and 2 mL-glutamine (Gibco) under a humidified atmosphere containing 5% $CO_2$ in air at 37 °C. The VR-HCT-116 cell line was subcultured

additionally in the presence of 2 µM vincristine. Cells were passaged twice weekly upon reaching 70–80% confluency.

### *2.4 MTT assay*

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was adapted from a previously-established protocol [20]. Briefly, cells were seeded at a density of $3 \times 10^3$ cells per well into 96-well plates (Nunc, Roskilde, Denmark) 24 h prior to treatment with test agents at serially-diluted concentrations. MTT assays were performed at the time of agent addition (T zero) and followed by 72 h exposure of the cells to test agents [21]. GraphPad Prism 7.04 (Graphpad Software Inc, USA) and Microsoft Office 365 were used for generating curves and obtaining 50 % Growth inhibition ($GI_{50}$) values. Results are expressed as the mean of total ≥ 4 independent experiments (n = 6).

To authenticate MTT assay results, cell counts were performed. Briefly, into 6-well plates, cells were seeded at a density of $2 \times 10^4$ per well and incubated overnight before treatment with CR at 4-5 different concentrations proximate to $GI_{50}$ values (obtained by MTT assay following 72 h exposure). The cells were incubated with the test agent for 72 h and counted after harvesting using a haemocytometer [19].

### *2.5 Clonogenic assay*

The clonogenic assay is an *in vitro* cell survival assay that measures the ability of single cells to survive and retain proliferative capacity to form progeny colonies after brief exposure to test compounds [22]. The assay was performed according to our previously described procedure [19,21].

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### *2.6 Migration assay*

Migration assays were performed to measure the ability of test agents to inhibit the migration of cancer cells. Cells were seeded in 6-well plates at a density of 1 × $10^6$ in 2 mL medium supplemented with 10 % FBS and allowed to grow until 90 % confluent. A scratch or "wound" was formed using a sterile 200 µL yellow pipette tip. Subsequently, cells were washed with phosphate-buffered saline (PBS) and incubated with the test compound at 2 ×$GI_{50}$ concentration in the presence of FBS (10 %) for 48 h. Acquisition of microscopy images was carried out using an inverted microscope (Nikon ECLIPSE TS100) equipped with a Nikon COOLPIX 4500 camera at 0, 24 and 48 h. Images were analysed using ImageJ software (NIH, Maryland, USA). Results were expressed as a percentage of the original wound area [23].

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### *2.7 Cell cycle analysis*

Cell cycle analyses were carried out as described [23]. Briefly, cells were seeded in 6-well plates at densities of 0.5–1 × $10^6$ cells/well. Following treatment, cells were harvested, centrifuged and then resuspended in 0.3–0.5 mL fluorochrome solution containing 50 µg/mL propidium iodide (PI), 0.1 mg/mL Ribonuclease A, 0.1 % v/v Triton X-100, and 0.1 % w/v sodium citrate in deionised water ($dH_2O$). Cells were stored overnight in the dark at 4 °C. Cell cycle analyses were performed on a Beckman Coulter FC500 flow cytometer (Indianapolis, IN, USA). Weasel flow cytometry analysis software (v 3.5) (WEHI, Melbourne, VIC, Australia) was used to analyse the data.

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### *2.8 Detection of DNA Double Strand Breaks*

γH2A.X foci appear at the sites of DNA double-strand breaks (DSBs) and therefore represent a biomarker of DNA DSB damage [24]. Detection of γH2A.X foci was carried out as reported previously [23,25]. Cells were seeded at a density of 1–1.5 × $10^6$ in 10 $cm^2$ dishes and allowed

to adhere for 24 h at 37 °C. Cells were treated with 1 × and 2 × $GI_{50}$ of CR for 24 and 48 h. Cells were then harvested and fixed with 1 % methanol-free formaldehyde in PBS. Cells were then incubated for 5 min at room temperature and permeabilised using 0.4 % Triton-X-100 in PBS (500 µL). Cells were rinsed with PBS, centrifuged and resuspended in 200 µL γH2A.X 1º Ab (EMD-Millipore, 1:3333 dilution) and incubated for 1.5 h at room temperature. Goat anti-mouse Alexa Fluor 488 2º Ab (1:1750 dilution) was later added and cells incubated at room temperature for 1 h in the dark. Cells were washed with PBS and then resuspended in 300 µL solution of 50 µg/mL PI/0.1 mg/mL RNase A in PBS followed by incubation for ≥10 min at room temperature. A Beckman Coulter Cytomics FC500 MCL flow cytometer (Indianapolis, IN, USA) was used to take the measurements; data were analysed *via* Weasel flow cytometry analysis software (v 3.5) (WEHI, Melbourne, VIC, Australia).

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***2.9 Annexin V-FITC and propidium iodide apoptosis assay***

Cells were seeded in 12-well plates at densities of 1.5 × $10^5$ cells/well (24 h), 1 × $10^5$ cells/well (48 h) and 5 × $10^4$ (72 h). Cells were incubated overnight prior to exposure to CR at 1 × and 2 × $GI_{50}$ concentration for 24 h -72 h. Following treatment, cells were trypsinised, centrifuged, collected in FACS tubes and kept on ice for 10 min in 2 mL cold medium. Following centrifugation, cells were washed with ice-cold PBS, then pelleted by centrifugation. Annexin-V-FITC (5 µL) plus 100 µL 1 × annexin-V buffer was added to cells; after 15 min incubation in the dark at room temperature, PI (10 µL; 50 µg/mL in PBS) plus 400 µL annexin-V buffer was added. Cells were placed on ice and kept in the dark for 10 min prior to analyses using Beckman Coulter FC500 flow cytometer (Indianapolis, IN, USA). Weasel flow cytometry analysis software was used to evaluate data (v 3.5) (WEHI, Melbourne, VIC, Australia).

### *2.10 Caspase-3/7 activity assay*

Caspase 3/7 assay kit (Promega) was used to determine caspase activity as previously reported [23]. Briefly, cells ($5 \times 10^3$ per well) were seeded in white opaque 96-well plates and incubated overnight at 37 °C. CR or vincristine was introduced at 1 × $GI_{50}$. Caspase-Glo 3/7 reagent was added to the cells in a 1:1 ratio of reagent to cell culture medium. An orbital shaker was used to mix the reagent. Plates were incubated for 60 min at room temperature. The resulting luminescence was read using the Envision 2104 multi-label plate reader (PerkinElmer, Waltham, MA, USA).

### *2.11 Western blots*

Western blotting was carried out as previously described [23]. Briefly, cells were seeded in 10 $cm^2$ dishes at a density of 1–2 × $10^6$ per dish and allowed to attach for 24 h prior to exposure to 1 × $GI_{50}$ and 2 × $GI_{50}$ CR. Following the desired treatment period cell lysates were prepared and protein concentrations were calculated by Bradford assay [26]. Protein (50 µg per sample) was separated using the SDS PAGE and transferred to the nitrocellulose membrane. Whole PARP, cleaved PARP, Mcl-1, Bcl-2, PLK1, p- mTOR, mTOR, p-4EBP1, 4EBP1, p-Akt, Akt, p-p53, p53, p-eIF4e, eIF4e, c-MYC, MAPK ERK1/2, p-ERK1/2, p-STAT3, STAT-3 and GAPDH Primary antibodies (Abs) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit and anti-mouse immunoglobulin G (IgG) horseradish peroxidase-conjugated Secondary Abs were obtained from Dako (. Proteins were detected by immunoblotting, as previously described [27]. ImageJ software (NIH, Maryland, USA) was used for densitometric analyses.

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### *2.12 Confocal microscopy*

Confocal imaging was conducted as described [28]. All procedures were performed at room temperature unless stated otherwise. Cells at a density of $1 \times 10^4$ were seeded in 8-well µ-slides (Ibidi, Planegg, Germany) in 200 µL medium and incubated overnight to adhere. Following 24 h treatment, cells were fixed with formaldehyde (3.7% in PBS; 10–15 min). Cells were then permeabilised by PBT (PBS + 0.1% Triton X-100; 2–3 min). To prevent non-specific protein binding cells were blocked for 1 h using PBT and 1% bovine serum albumin (BSA). Cells were incubated for 2 h with 1° monoclonal anti-α-tubulin Ab (Thermo Scientific), washed with PBT and incubated in the dark for 1 h with fluorescent 2° Ab (anti-mouse IgG Alexa Fluor@ 488 F, 1:500 dilution). DNA binding dye DRAQ5 (1:3000) was included with cells and incubated for 5 min in the dark. Cells were visualised and images captured using a Zeiss LSM510 Meta confocal microscope conjugated with Zeiss LSM image browser software (version 4.2.0.121).

### *2.13 Detection of reactive oxygen species*

To measure the $H_2O_2$ levels in cells, the rapid ROS-Glo™ $H_2O_2$ luminometric-based assay (Promega, UK) was adopted. Briefly, $5 \times 10^3$ cells were seeded in white opaque 96-well plates in 80 µL medium, incubated overnight and then treated with CR or vincristine for 24 h. $H_2O_2$ substrate (25 µM) was added to the cells 30 min before completion of the 6 h incubation period; 100 µL ROS-Glo™ detection solution was then added, and samples were incubated for 20 min at room temperature. Relative luminescence was measured using an Envision 2104 multilabel plate reader (PerkinElmer, Waltham, MA, USA).

### *2.14 In silico pharmacokinetic analyses*

*In silico* prediction of CR pharmacokinetics was performed using GastroPlus™ v9.5.0004 (SimulationsPlus, Lancaster, CA, USA) with built-in ADMET Predictor™ v8.1.0.0. All

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physicochemical and biopharmaceutical properties were predicted from the chemical structure, specific input parameters for the simulations can be found in the supplementary information (Supplementary Table S1). Advanced compartmental and transit (ACAT) and physiologically-based pharmacokinetic (PBPK) models were used with physiological parameters of a 0.025 kg mouse for the simulations. Paracellular permeability and enterohepatic circulation were turned on, and log D was estimated by structure-based model v6.1. Hepatic clearance ($CL_h$) was simulated with recombinant CYP predictions from the ADMET Predictor™. Renal clearance ($CL_r$) was predicted by the product of fraction unbound in plasma and glomerular filtration rate. Simulations were performed for i.v., p.o. and s.c. administration routes.

### *2.15 Statistical analyses*

Experiments were conducted ≥3 times and representative experiments are shown in the Figures. One-way and two-way analyses of variance (ANOVAs) were used to determine statistical significance. Levels of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$ compared to untreated control) were determined using Dunnett's multiple comparison test.

## 3. Results

### *3.1 Characterisation of Cerberin from Cerbera odollam*

The CR sample isolated in section 2.2 was positively characterised by comparison of its $^1$H NMR spectral data with available literature [8,9,29–31] (Supplementary Fig 1; Supplementary Table S2). The $^1$H NMR spectrum of CR showed a distinctive methyl singlet resonance at $\delta_H$ 2.05 ppm, corresponding to its 2′-*O*-acetyl substituent (H-2′) (Fig 1). A methoxy methyl singlet resonance (H-3′) detected at $\delta_H$ 3.57 ppm has indicated the glycosidic moiety of CR to be 2′-

*O*-acetyl-L-thevetose as reported in the literature [9,29,32]. An α,β-unsaturated-γ-lactone moiety of the cardenolide skeleton in CR was readily determined based on an olefinic proton resonance detected at $\delta_H$ 5.89 ppm (H-22), which was coupled to two downfield methylene proton resonances at $\delta_H$ 4.82 (H-21′) and $\delta_H$ 4.98 ppm (H21′′) respectively. The stereochemistry of H-17 was established as 17β due to its chemical shift at $\delta_H$ 2.83 ppm, consistent with published data on 17β cardenolide isomers such as 17β neriifolin [11,30] (Supplementary Table S2). The CR sample was tested and found to be pure via UHPLC analysis, which showed a single well resolved peak at $R_t$13.04 min (PDA 254 nm, PDA MaxPlot 200–400 nm) (Supplementary Fig. S6).

### *3.2 Growth inhibitory effect of Cerbera odollam extract*

The growth inhibitory activity of the defatted dichloromethane: methanol (9:1) extract of *C. odollam* fruits was initially tested against six cancer types (nine cancer cell lines) using the MTT assay. Treatment with the extract revealed various growth inhibitory activities against all the cell lines tested as reported in Table 1. A statistical analysis between control and treatment groups revealed significant ($p$ <0.001) growth inhibition following treatment with extract concentrations ≤0.01 µg/mL in all the cancer cell lines tested except the MDA-MB-231 cell line which showed significant growth inhibition at concentrations of ≤0.5 µg/mL. The extract exhibited 50% *in vitro* growth inhibitory ($GI_{50}$) activities between 0.03 and 0.17 µg/mL in all tested carcinoma cell lines. When $GI_{50}$ values of *C. oddolam* extract in cancer cell line were compared to that of non-tumourigenic MRC-fibroblasts ($GI_{50}$ = 146 µg/mL), large selectivity indices (SI) were encountered (858- and 4866-fold SI). Subsequently, the pure compound CR was isolated from this extract; the anticancer activity of CR was then rigorously examined, and elucidation of anticancer mechanisms of action undertaken.

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### *3.3 MTT assay results: Evaluation of the Cytotoxic Potential of CR in a Panel of Cancerous vs Non-transformed Human Cells*

The *in vitro* antitumour activity and selectivity of CR was tested against a panel of cell lines derived from breast, colon, pancreatic, lung, liver and nasopharyngeal cancers, MRC-5 fibroblasts and NP-69 nasopharyngeal epithelial cells by MTT assay. CR elicited a concentration-dependent decrease in the number of viable cells (exemplified for PANC-1 and A549 in Fig 2A) and exhibited potency against all the cancer cell lines tested. Similar to the *C. odollam* extract, significant growth inhibition (determined by a two-way ANOVA between control and treatment groups) was evoked by nanomolar concentrations of CR in all cancer cell lines tested. The vehicle control DMSO did not affect absorbance readings in any of the cell lines (data not shown). The $GI_{50}$ values for CR in 12 cancer cell lines in addition to non-transformed fibroblasts are summarised in Table 2 which ranged between 22.23 nM (HK-1) and 130.2 nM (SKBR-3). Interestingly, CR demonstrated 315.64 – 1838.92-fold and 26 – 155.5-fold selectivity towards cancer cells compared to the MRC-5 ($GI_{50}$ = 41.10 µM) and NP-69 ($GI_{50}$ > 3.46 µM) cells respectively (Table 2).

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To validate the MTT assay results, cell counts were performed following 72 h exposure of PANC-1 and A549 cells to CR. Dose-dependent reductions in cell numbers were particularly evident between 17 nM - 200 nM in PANC-1 and 10 nM – 70 nM in A549 cells. Nanomolar CR concentrations caused cytotoxicity in both cell lines as significantly fewer cell numbers were counted than originally seeded (Fig. 2 B and C; $p < 0.0001$).

### *3.4 CR inhibited cancer colony formation*

CR significantly inhibited colony formation after 24 h exposure of cells to concentrations equivalent to 1 × $GI_{50}$ and 2 × $GI_{50}$ (Fig. 3 A and B). At 1 × and 2 × $GI_{50}$ respectively, CR inhibited colony formation in: HT-29 cells by 35% and 74%; HCT-116 cells by 85% and

99.3%; MIA PaCa-2 cells by 79% and 100%; HK-1 cells by 70% and 100%; A549 cells by 95% and 100%; MDA-MB-468 cells by 71% and 84%; MDA-MB-231 by 51% and 89%; MCF-7 cells by 76% and 93%; PANC-1 cells by 70% and 97.3% (Fig. 3A and C). The clonogenic assay corroborated the anticancer potency of CR and also demonstrated the cytotoxicity of CR. Although there were differences in sensitivity to CR between the cell lines, colony formation was significantly inhibited.

### *3.5 CR inhibited cancer cell migration*

In metastasis, migration plays a crucial role as primary stroma cancer cells migrate *via* the circulation and lymphatic systems to distant sites, then invade and colonise new niches in distant organs forming secondary metastatic tumours [33]. A wound healing assay was implemented to investigate the effect of CR on the migration of PANC-1, MDA-MB-468 and A549 cells (Fig. 4). Compared to the control, CR exhibited significant dose-dependent migration inhibition activity in PANC-1, MDA-MB-468 and A549 cells. The untreated controls of PANC-1 and A549 showed 100% closure of the wound indicating cell migration there was no evidence of the wound after 48 h. In MDA-MB-468 control cells, migration progressed slower than the other two cell lines (wound healing estimated at 93% after 48 h).. CR exhibited 4- to 26-fold inhibition of wound healing in all the three cell lines at 2 × $GI_{50}$ concentration after 48 h (Fig. 4).

### *3.6 Generation of DNA double strand breaks*

γ-H2A.X is considered a DNA double-strand break (DSB) marker, which can be quantified to assess DNA damage [24]. The induction of DNA damage by CR was assessed by detecting the number of cells expressing γ-H2A.X by flow cytometry. As demonstrated in Fig. 5, CR significantly increased γ-H2A.X foci in PANC-1, MBA-MB-468 and A549 cells by 9-, 2.6-

and 12-fold respectively, relative to the control. The positive control etoposide caused similar γ- H2A.X elevation in PANC-1 (6.18-fold), MDA-MB-468 (2.7-fold) and A549 (7.5-fold).

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### *3.7 Cell cycle analysis*

The MTT, cell count and clonogenic assays suggest that CR inhibits cancer cell growth and viability. Based on these observations, we investigated the effect of CR on cell cycle perturbation by flow cytometry. Cells were exposed to CR (1 × $GI_{50}$ and 2 × $GI_{50}$) for 24, 48 and 72 h. Time- and dose-dependent accumulation of events in G2/M phases were sustained in PANC-1, MDA-MB-468 and A549 cell lines, with significant G2/M cell cycle arrest observed following 24, 48 and 72 h exposure at 1 × $GI_{50}$ and 2 × $GI_{50}$, as shown in Fig. 6. CR induced the highest accumulation of events in G2/M phases after 72 h exposure: 56.8%, 43% and 33.5% in MDA-MB-468, PANC-1 and A549 populations respectively. In Supplementary Fig. S3, the stark dose-dependent accumulation of HCT 116 events in the G2/M cell cycle phase was clear. Accumulation of A549 events in G1 at 1 × $GI_{50}$ and 2 × $GI_{50}$ was observed after 48 h exposure accompanied by significant elevation of cells in the sub-G0/G1 phase. One of the most sensitive cell lines (inferred by MTT assays), PANC-1 revealed that G2/M cell cycle arrest was accompanied by a significant pre-G1 population at 2 × $GI_{50}$ following 72 h exposure. Cell cycle profiles are shown in Supplementary Fig. S2.

### *3.8 CR induces apoptosis in cancer cells*

Annexin-V/PI evaluation of apoptosis in conjunction with caspase 3/7 activation assays were performed to investigate the apoptosis-inducing properties of CR in PANC-1, MDA-MB-468 and A549 cell lines exposed to CR (1 and 2 × $GI_{50}$; 24, 48 and 72 h). Apoptotic populations were confirmed by dual annexin V-FITC/PI staining (corresponding scatter plots are shown in Supplementary Fig. S4). CR evoked profound time-dependent apoptosis. PANC-1 cells

showed only significant early apoptosis at 1 x $GI_{50}$ (48 and 72 h) and 2 × $GI_{50}$ at 24, 48 and 72 h exposure ($p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively); cells appeared more resistant to late apoptosis at both 1 × $GI_{50}$ and 2 × $GI_{50}$ (Fig. 7A), possibly indicating slower apoptosis onset. However, CR induced significant early (A+/PI−) MDA-MB-468 apoptosis at 1 × $GI_{50}$ and 2 × $GI_{50}$ following 48 and 72 h exposure ($p < 0.0001$); late (A+/PI+) MDA-MB-468 apoptosis was observed at 1 × $GI_{50}$ (72 h) and 2 × $GI_{50}$ at both 48 and 72 h exposure (Fig. 7B). Similarly, A549 revealed significant early apoptosis following CR treatment at 1 × and 2 × $GI_{50}$ after 48 and 72 h treatment ($p < 0.001$ and $p < 0.0001$, respectively), and late apoptosis at 2 x $GI_{50}$ only after 48 and 72 h exposure (Fig. 7 C). The highest percentage apoptosis at 2 × $GI_{50}$ was observed in A549 populations after 72 h exposure to CR (36.99 ± 4.50%), compared to MDA-MB-468 (28.13 ± 4.35%) and PANC-1 (23.45 ± 5.07%).

Induction of caspase-3/7 activity was measured in PANC-1, MDA-MB-468 and A549 cells after 24 h exposure to either CR (1 × $GI_{50}$ and 2 × $GI_{50}$) or vincristine (10 nM; Fig. 7D). CR significantly increased caspase 3/7 activity in all three cell lines compared to the control (100% increase) ($p < 0.0001$). In PANC-1, MDA-MB-468 and A549 populations, 1 × $GI_{50}$ and 2 × $GI_{50}$ CR concentrations enhanced caspase activity by 170% and 322%; 190% and 439%; 133% and 194% respectively. Vincristine also increased caspase activity in PANC-1 (115%, not significant), MDA-MB-468 (197%, $p < 0.0001$) and A549 (190%, $p < 0.0001$) cells.

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### *3.9 CR induces ROS production in cancer cells*

ROS production was measured in PANC-1, MDA-MB-468 and A549 cells following 24 h treatment with CR (1 × $GI_{50}$ and 2 × $GI_{50}$) or vincristine (10 nM) (Fig. 8). At 1 × $GI_{50}$ CR significantly increased ROS production to 147%, 156% and 155% compared to the control in PANC-1, MDA-MB-468 and A549 cells, respectively; at 2 × $GI_{50}$ CR, ROS generation

increased to 165%, 305% and 190% of the control in PANC-1, MDA-MB-468 and A549 cells, respectively.

### *3.10 CR treatment leads to alterations in protein expression*

Western blot was used to investigate protein expression and the activation of signal transduction cascades known to possess roles in apoptosis, mitosis and cell survival. Lysates of PANC-1, MDA-MB-468 and A549 cells following 24 and 72 h exposure of cells to 2 × $GI_{50}$ CR were prepared. Significant time-dependent elevation in cleaved PARP accompanied a reduction in expression of anti-apoptotic/pro-survival proteins Mcl-1 and Bcl-2 compared to the untreated control (Fig. 9 A-E). Significant down-regulation of PLK1 was observed after exposure of cells to CR (24 and 72 h; $p < 0.0001$) (Fig. 9A and F).

### *3.11 CR Modulates Signalling through PI3K/AKT/mTOR Pathways*

The PI3K-AKT-mTOR signalling pathway plays an essential role in tumourigenesis including cancer cell survival, proliferation and protein translation [34]. Therefore, we examined whether CR impacts activation of this pathway in PANC-1, MDA-MB-468 and A549 cancer cells. Cultures of all three cancer cell lines were treated with CR at 2 × $GI_{50}$ for 24 and 72 h. Time-dependent attenuation of PI3K, AKT, and 4EBP1 phosphorylation was observed, whereas the total protein expression of PI3K, AKT and 4EBP1 remained unchanged (Fig. 10 A-D). The oncogenic transcription factor STAT3 is commonly constitutively activated in tumours and tumour-derived cell lines [35]. Our results also revealed that CR downregulates STAT3 phosphorylation with no changes in total STAT3 expression (Fig. 10 A and E). The mammalian target of rapamycin (mTOR) is also commonly activated in cancer and regulates cell proliferation, metabolism, survival and metastasis. Therefore, we investigated the effect of CR on mTOR phosphorylation. Interestingly, CR downregulated phosphorylation of mTOR (p-

mTOR) (Fig. 10 F-G), correlating with PI3K/AKT/mTOR inhibition. Again, no significant changes in total mTOR protein were observed.

### *3.12 CR causes severe disruption in cytoskeletal architecture*

G2/M arrest revealed during cell cycle analyses may be indicative of tubulin/microtubule disruption, therefore, following 24 h exposure to vehicle alone (control), CR (1 × $GI_{50}$) or vincristine (10 nM), MDA-MB-468 cells were immunostained and prepared for inspection by confocal microscopy. To investigate the morphological changes in cytoskeletal, chromosomal and cellular characteristics, tubulin and DNA were stained, and CR-treated cells were compared to those exposed to the vehicle alone or vincristine. Fig. 11 A-D represent MDA-MB-468 control cells. Cells treated with the microtubule depolymerising agent vincristine (10 nM; Fig. 11 E-H) revealed irregular chromosomal separation (Fig. 11 E and F), nuclear membrane disruption, microtubule network disruption and uneven cell division, consistent with its mechanism of action; additionally, chromatin condensation, heralding apoptosis, was detected (Fig. 11 G and H). Similar to vincristine, CR caused tubulin network disruption leading to improper chromosome segregation, accompanied by aneuploidy and multinucleation (Fig. 11 J-K). Uneven cell division was also detected in CR-treated MDA-MB-468 cells (Fig. 11 L-P). CR treatment also evoked nuclear fragmentation, chromatin lunate morphology and condensation accompanied by severe membrane blebbing (Fig. 11 M-P), characteristics most commonly associated with apoptosis.

### *3.13 In Silico Pharmacokinetic properties of CR*

The pharmacokinetic (PK) properties of CR were simulated in mice, as rodents are most likely to be the first species tested in preclinical evaluation (Fig. 12, Table 3). GastroPlus™ is a powerful *in silico* prediction tool in the area of biopharmaceutics and pharmacokinetics with

the ability to provide useful information and guidelines prior to expensive and time-consuming *in vivo* experiments [19,36–38]. Plasma concentration-time profiles of CR following various routes of administration were simulated to predict the levels of systemic exposure for each route (Fig. 12 A). CR was predicted to be rapidly eliminated with an elimination half-life of 1.6 h following intravenous administration. Following oral administration, CR was predicted to be rapidly absorbed with maximum concentration achieved at 0.4 h with relatively high bioavailability (61.2%). Subcutaneous absorption was predicted to show slower but almost complete absorption with a bioavailability of 96.6% (Table 3).

Simulations were performed to predict the dose range needed in preclinical development of CR. As it is an unbound drug that exerts its pharmacological effect [39], the unbound concentration profiles of CR in plasma following oral administration at a range of doses were simulated (Fig. 13 B). The lowest (HK-1) and the highest (SKBR-3) $GI_{50}$ values obtained for cancer cell lines were also plotted to visualise effective dose range. The modelling suggested that doses between 1 – 10 mg/kg CR would exert anti-tumour activity in preclinical murine models. A dose of 100 mg/kg was predicted to cause possible issues with absorption of the drug, showing irregular profiles. As GastroPlus™ employs the ACAT model for oral absorption, the amount (%) of CR predicted to be absorbed in each compartment of the GI tract was also shown (Fig. 13 C). CR was predicted to be absorbed mostly at the proximal region of the GI tract. Simulated PK results provide a useful guideline in further preclinical development of CR as a promising anticancer therapy.

## 4. Discussion

Throughout history, it has been recognised that Nature provides a rich source of potential medicinal agents – molecules possessing structural complexity and diversity able to perturb molecular mechanisms critical to disease pathogenesis. Pursuit of natural product drug

discovery is critical to identify novel compounds able to combat intractable and drug-resistant diseases before habitat loss leads to extinction.

Recent research has focussed on CGs such as ouabain, oleandrin, digoxin, digitalis, as putative anticancer agents [40]. These CGs exert potent *in vitro* anticancer activity and are non-toxic towards normal cells [41]. Epidemiological studies have shown that patients receiving CG treatment for heart failure appear protected from certain cancer types [42, 43]; three CGs have progressed to clinical evaluation as anticancer therapies [46].

In this study we have isolated cerberin (CR), a cardenolide cardiac glycoside (CG) and shown that it evokes significant, potent (nanomolar), broad-spectrum and selective (>300-fold) anticancer activity. Herein, we report our efforts to clarify the nature of *in vitro* antitumour activity and elucidate molecular mechanisms of action of CR.

MTT and cell count assays revealed the potent growth inhibitory activity of CR; inhibition of carcinoma colony formation indicated that cells were either killed, or lost the proliferative ability to form progeny colonies. Estimated $GI_{50}$ values (calculated from MTT assays) were adopted in subsequent assays adopted to interrogate molecular targets of CR.

Flow cytometric analyses of CR-treated PANC-1, MDA-MB-468 and A549 cells demonstrated significant G2/M cell cycle arrest, which may indicate interrupted mitoses and cytoskeletal protein targets. Indeed, confocal microscopy revealed tubulin network disorder, multipolar spindles, misaligned chromosomes and multinucleation – clear evidence of cytoskeletal architecture disruption. Microtubule disruption is likely to impact cell migration, a key component of invasion and metastasis – a fundamental cancer hallmark [ref Hanahan and Weinberg 2011] and important target for intervention in anticancer drug discovery [47]. It was shown that CR exhibited significant anti-migratory activity in the same cancer cell lines (PANC-1, MDA-MB-468, A549). Polo-like kinase 1 (PLK1) possesses key roles leading to

promotion of mitosis including modulation of cdc2 activation, chromosome segregation and formation of bipolar spindles. Enhanced levels of PLK1, a recognised oncogene and validated anticancer drug target, are evident in malignant compared to normal tissue [19,55]. CR caused significant time-dependent down-regulation of PLK1. Similarly, the CG bufalin supressed PLK1 protein expression, delaying entry of cells into prophase, arresting cell cycle, ultimately leading to apoptosis [55].

CR is known to inhibit $NA^+/K^+$-ATPase (NKA), reducing $NA^+$ and elevating $Ca^{2+}$ intracellular levels, impacting signal transduction cascades and modulating key cellular functions including proliferation and apoptosis [43]. NKA also interacts with signalling proteins (PKC, PKA, PI3K, MAPK, EGFR; known as the signalosome), inspiring further interest in CGs as anticancer agents [44, 45].

With this knowledge in mind, the effects of CR on transcription factor c-Myc expression, PI3K-AKT-mTOR and signal transducer and activator of transcription 3 (STAT 3) activation were interrogated by western blot. Down-regulation of c-Myc was evident after treatment of cells with CR; c-Myc depletion is known to negatively impact cancer cell survival [56]. PI3K pathway activation similarly has important consequences on cancer cell survival, and is pivotal for protein translation and angiogenesis. Mutations leading to PI3K pathway deregulation promote tumourigenesis [57]. The eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) is regulated by PI3K, AKT and mTOR signalling; interestingly, Bcl-2 is regulated by 4E-BP1. In MDA-MB-468 and A549 cells, CR significantly inhibited mTOR phosphorylation. For sustainable, long-term benefit, dual mTOR and AKT/PI3K inhibitors may need to be employed to thwart cancer recurrence or emergence of drug-resistance [57]; herein, we demonstrate down-regulation of PI3K, AKT and mTOR phosphorylation by CR. 4EBP1, a repressor of protein translation, is the first downstream substrate of mTOR; phosphorylated 4EBP1 is associated with tumour progression and poor prognosis [59]. CR also

downregulated phosphorylation of 4EBP1. Finally, CR significantly supressed STAT 3 phosphorylation in the 3 cell lines examined (PANC-1, MDA-MB-468 and A549) following treatment with CR (24 h; 48 h); STAT 3 has regulatory roles in diverse cellular functions including metastasis, angiogenesis, and apoptosis and its constitutive activation leads to poor cancer prognosis [58].

This study therefore demonstrates that CR possesses anti-oncogenic properties concurrently perturbing multiple molecular targets pertinent to tumourigenesis. That the ultimate consequence of CR treatment is cancer cell apoptosis has been shown by flow cytometry, caspase activation, confocal microscopy and western blot.

During early apoptosis, cellular membrane integrity is lost exposing phosphatidylserine (PS) on the outer membrane leaflet. Annexin V-FITC binds with high affinity to PS, a property that has been extensively exploited to quantify early apoptosis [52]. As apoptosis progresses, membrane integrity is lost allowing cells to stain positive for PI. Activation of caspases is a hallmark of apoptosis, specifically executioner caspase 3 [23, 53]. Therefore, annexin V-FITC/PI flow cytometry detection and caspase 3/7 activation assays were adopted, and confirmed emergence of PANC-1, MDA-MB-468 and A549 apoptotic populations. Consistent with cell cycle arrest and the appearance of DNA damage, time- and dose-dependent annexin V-positive (early apoptotic) populations was evident in all 3 cell lines; whereas, significant late apoptosis was observed in MDA-MB-468 and A549 cells (48 h; 72 h exposure CR).Clear induction of caspase 3/7 activity was demonstrated in all 3 cell lines. Confocal microscopy revealed evidence of apoptotic characteristics such as chromatin condensation, DNA fragmentation and membrane blebbing [9. 49]. Concomitantly, cleaved PARP, and down-regulation of anti-apoptotic proteins Bcl-2 and Mcl-1 were detected. Cancers commonly express elevated levels of oncogenic Bcl-2 protein family members and their down-regulation modulates cancer cell survival.

In order for promising experimental anticancer agents to progress through *in vivo* and clinical evaluation, drug metabolism and pharmacokinetic studies are necessary to determine agent bioavailability. Gastroplus™ is a powerful preclinical tool used in drug discovery: results of *in silico* biopharmaceutical evaluation inferred that CR concentrations able to evoke antitumour activity and perturb molecular targets and signal cascades driving tumourigenesis were achievable following oral administration of CR doses between 1 and 10 mg/kg. Oral (self) administration is known to improve patient adherence and reduces costs associated with in-patient treatment [60].

In summary, we have shown that CR elicits potent and selective anticancer activity, significantly inhibiting cancer cell proliferation, migration and colony survival in human-derived pancreatic, triple negative breast and non-small cell lung cancer cell lines. Profound G2/M cell cycle arrest and disruption of cytoskeletal architecture were demonstrated. Intriguingly, CR inhibited molecular targets portending potential to perturb multiple cancer hallmarks: CR suppressed PI3K/AKT/mTOR and STAT 3 signal transduction and down-regulated PLK 1, c-Myc, Bcl-2 and Mcl-1 expression. CR significantly increased ROS production and caused DNA DSBs, ultimately inducing apoptosis. Finally, we predict that CR may be administered at doses that provide efficacious anticancer plasma concentrations, therefore, further preclinical evaluation of CR is justified.

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## Conflicts of interest

The authors have no conflicts of interest.

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Ref 5, 30, 31 : Capital letters in authors’ names
Ref 7, 8, 9, 11, 12, 29, 30, 31: Italicise plant names
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