Novel semi-synthetic Cu (II)-cardamonin complex exerts potent anticancer activity against triple negative breast and pancreatic cancer cells via inhibition of the Akt signaling pathway

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Abstract

Triple negative breast cancer (TNBC) and pancreatic cancer are two of the most aggressive types of cancer that lack effective treatments. We have previously reported the semi-synthesis of a novel Cu (II)-cardamonin complex (**19**) that demonstrated potent anti-tumour activity. In this study, we further investigated the bioactivity and mode of action of **19** against MDA-MB-468 and PANC-1 cancer cells in an attempt to discover an effective anticancer agent for TNBC and pancreatic cancer, respectively. Results revealed that **19** abolished formation of MDA-MB-468 and PANC-1 colonies, exerted growth inhibitory activity and inhibited the migration of cancer cells. Further mechanistic studies showed that it induced DNA damage resulting in G2/M-phase arrest and microtubule network disruption. Moreover, there was an increase in ROS production which may have contributed to the observed induction of apoptosis, corroborated by activation of caspase-3/7, cleavage of PARP and downregulation of Mcl-1. Complex **19** also decreased the expression levels of p-Akt and p-4EBP1 in both cell lines, which indicates that the compound exerted its activity, at least in part, *via* inhibition of the Akt signaling pathway. Furthermore, it decreased the expression of c-Myc in PANC-1 cells only which suggests that it may exert its bioactivity *via* multiple mechanisms of action pertinent to tumourigenesis. These results demonstrate the potential of **19** as a promising therapeutic agent for TNBC and pancreatic cancer.

Introduction

Cancer is a major health problem worldwide with ~ 17 million new cases and 9.6 million deaths in 2018. It is predicted that the number of new cases will increase to 27.5 million cases by the year 2040. Breast cancer is one of the most common types of cancer globally, with ~ 2 million cases reported in 2018. A subgroup of breast cancer known as triple negative breast cancer (TNBC) represents 15–20% of all breast cancers and is characterised by the lack of oestrogen receptor (ER) and progesterone receptor (PR) expression, in addition to the lack of human epidermal growth factor receptor (HER2) amplification. TNBC is of particular concern as it displays poor prognosis due to its inherently aggressive nature and lack of recognised molecular targets for therapy. Pancreatic cancer is another cancer phenotype that is highly lethal and is a major cause of cancer-related mortality with an overall 5-year survival of < 7%. The appalling prognosis that is associated with pancreatic cancer is partially due to its aggressive nature and difficult early diagnosis. There is currently a lack of efficient treatments for TNBC and pancreatic cancer, therefore, further research is required in order to develop more effective treatments for these types of cancer [1–5].

Natural products have historically provided an important source of bioactive compounds and have been heavily studied for the purpose of developing effective anticancer agents. Cardamonin is a natural product that belongs to a larger family of compounds known as chalcones and was found to exhibit pronounced cytotoxic activity against a variety of cancer cell lines such as A549, M14, U266, MDA-MB-231, HK1 and MCF-7 [6–7]. Moreover, there were many studies that attempted to investigate cardamonin's mechanism of action where it was found to exert its cytotoxic activity *via* targeting a variety of signaling pathways such as JNK-FOXO3a, Wnt/ β -catenin and mTOR cascades [8–10].

As part of our efforts to discover and develop novel anticancer agents, we synthesised several analogues of cardamonin, and discovered a highly active analogue [11]. This active analogue was the Cu (II)-cardamonin complex (**19**) (Fig. 1a) which displayed cytotoxic activity against lung cancer and nasopharyngeal carcinoma cell lines. However, to confirm the potential of **19** as a promising anticancer agent, we still have to investigate its cytotoxic activity against other types of cancer and elucidate in detail its mode(s) of action.

Therefore, the main aim of the present study was to examine the cytotoxic activity of **19** against intractable MDA-MB-468 (TNBC) and PANC-1 (pancreatic) cancer cell lines to further explore the potential of **19** as an effective anticancer agent and investigate its mode(s) of action. The study employed a wide range of biological assays to fully assess the anti-tumour activity of **19**; ranging from cell viability, apoptosis and migration assays to cell-cycle and Western blot analysis. Herein, we describe the potential of the novel semi-synthetic compound, **19**, as a promising anticancer agent against TNBC and pancreatic cancer, in addition to reporting a detailed description of its mechanisms of action.

Materials And Methods

Synthesis of **19**

Compound **19** was synthesised and its structure fully characterised as reported in our previous study [11].

Cell culture

MDA-MB-468 (TNBC) and PANC-1 (pancreatic) cancer cell lines were first obtained from the American Type Tissue Culture Collection (ATCC). Cell cultures were maintained using RPMI 1640 (Sigma-Aldrich UK) supplemented with sodium bicarbonate (2 g/l, Sigma-Aldrich UK), 1% 200 mM L-glutamine (Sigma-Aldrich UK) and heat inactivated 10% foetal bovine serum (FBS) (Sigma-Aldrich UK) under humidified atmosphere containing 5% CO₂ in air at 37°C.

Cell viability assay

MTT assay was used to measure cell viability. Cells were seeded in 96 well plates at a density of 3×10^3 cells per well and incubated for 24 h at 37°C prior to treatment. Cells were then treated with **19** at different concentrations and incubated for 72 h. Finally, MTT solution was added to each well and the resulting absorbance was read.

Colony formation assay

The assay was performed as reported [12].

Migration assay

Cells were seeded in 6-well plates at a density of 1×10^6 cells per well and allowed to grow until confluency. This was followed by forming a scratch or “wound” in the layer of cells using a sterile 200 µl

pipette tip. The cells were washed with PBS and incubated with **19** in the presence of 10% FBS for 48 h. Images of the cells were then analysed *via* ImageJ.

Cell-cycle analysis

Cell-cycle analysis was performed as reported [11].

γ -H2AX detection for DNA damage assessment

The assay was performed as reported [11].

Confocal microscopy

Cells were seeded in 8-well μ -slides at a density of 1×10^4 cells per well and incubated overnight. Cells were then treated with **19** for 24 h, fixed with formaldehyde (3.7% in PBS; 10–15 min) and permeabilised using PBT (PBS + 0.1% TritonX-100; 2–3 min). Cells were incubated/treated for 1 h with PBT and 1% bovine serum albumin (BSA) in order to prevent non-specific protein binding. They were later incubated for 2 h with monoclonal anti- α -tubulin antibody (Thermo Scientific), washed with PBT and incubated for 1 h with fluorescent secondary antibody (anti-mouse IgG Alexa Fluor 488 F, 1:500 dilution) in the dark. DNA binding dye DRAQ5 (1:3000) was added and cells were incubated for 5 min in the dark. Finally, visualisation of the cells was performed using a Zeiss LSM510 Meta confocal microscope [13].

Annexin V-FITC/propidium iodide (PI) apoptosis assay

Cells were seeded in 12-well plates at a density of 1.5×10^5 cells per well and incubated overnight. Later, cells were treated with **19** for 24 h and then trypsinised, collected in FACS tubes and kept on ice for 10 min, washed with PBS and centrifuged to form a pellet. Annexin V-FITC (5 μ l) and 100 μ l $1 \times$ annexin-V buffer were added to the cells followed by incubation for 15 min at room temperature. PI (10 μ l; 50 μ g/ml in PBS) and 400 μ l annexin-V buffer were later added and the cells were finally placed on ice for 10 min prior to analysis using a Beckman Coulter FC500 flow cytometer (Indianapolis, USA).

Caspase-Glo 3/7 assay

The Caspase-Glo 3/7 assay kit (Promega) was used. Cancer cells were seeded at a density of 5×10^3 cells per well and incubated overnight. The cells were then treated with **19** followed by addition of Caspase-Glo 3/7 reagent and incubation for 60 min prior to reading the results *via* a multi-label plate reader.

Western blotting analysis

Cells were seeded in dishes at a density of $1-2 \times 10^6$ per dish. After 24 h, cells were treated with **19** for 24 h and 72 h. Following treatment, cell lysates were prepared, and protein concentrations calculated using the Bradford assay. Protein was subjected to SDS-PAGE for separation and transferred to the nitrocellulose membrane. Whole PARP, cleaved PARP, Mcl-1, Akt, p-Akt, 4EBP1, p-4EBP1, c-Myc and GAPDH primary antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-

rabbit and anti-mouse immunoglobulin G (IgG) horseradish peroxidase-conjugated secondary antibodies were obtained from Dako (Santa Clara, USA).

Reactive oxygen species (ROS) detection

The ROS-Glo H₂O₂ luminometric-based assay (Promega, UK) was used. Cancer cells were seeded at a density of 5×10^3 cells per well and incubated overnight. The cells were then treated with **19** for 24 h. H₂O₂ substrate (25 μ M) was then added to the cells followed by the addition of 100 μ l ROS-Glo detection solution, and samples were finally incubated for 20 min at room temperature. Resulting luminescence was detected using a multi-label plate.

Statistical analysis

Experiments were generally repeated $\geq$ three times and data were expressed as mean $\pm$ SD. Experimental data were analysed *via* Graphpad Prism. Statistical differences among means were analysed by one-way and two-way ANOVA followed by Dunnett's multiple comparisons tests. Data represent significant values as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Results And Discussion

Compound 19 inhibited the growth and migration of MDA-MB-468 and PANC-1 cancer cells

We have previously been able to synthesise a highly active Cu (II) complex of cardamonin (**19**) which demonstrated potent cytotoxic activity [11]. In order to further assess the cytotoxic activity of **19**, we examined its effect against MDA-MB-468 (TNBC) and PANC-1 (pancreatic) cancer cell lines. The effect of **19** on the cancer cell lines was initially investigated *via* an MTT cell viability assay and results are summarised in Table 1. Compound **19** demonstrated potent growth inhibitory activity against both MDA-MB-468 and PANC-1 cells with GI₅₀ values of 6.14 μ M and 12.48 μ M, respectively, and it decreased their viability in a dose-dependent manner (Fig. 1b). Moreover, **19** showed an almost 6-fold enhancement in activity against MDA-MB-468 cells relative to its parent compound, cardamonin, while a slight enhancement was observed against PANC-1 cells. This indicates that complexation of cardamonin to Cu²⁺ was a successful technique to enhance the natural product's bioactivity. Compound **19** was also screened against human normal lung fibroblasts (MRC-5) in order to investigate the compound's selectivity towards cancer cells. Results of the screening showed that **19** exhibited 5.3- and 2.6-fold selectivity towards MDA-MB-468 and PANC-1 cancer cells relative to non-transformed MRC-5 cells, respectively. Overall, results showed that **19** was more active towards MDA-MB-468 cells than PANC-1 cells, exhibited superior cytotoxic activity relative to its parent compound, cardamonin, and demonstrated cancer-selectivity.

Table 1 GI₅₀ values of **19** against MDA-MB-468, PANC-1 and MRC-5 cells

Compound	GI ₅₀ (μM) ^a			Selectivity index (SI) ^b	
	MDA-MB-468	PANC-1	MRC-5	MDA-MB-468	PANC-1
19	6.14 ± 0.06	12.48 ± 0.70	32.60 ± 5.13	5.3	2.6
Cardamonin	34.33 ± 0.55	19.21 ± 2.37	–	–	–
Vincristine sulfate	0.03 ± 0.0005	0.48 ± 0.24	5.58 ± 0.38	186	11.6

^a GI₅₀ values are reported as the mean (GI₅₀ ± SD) of at least three independent experiments.

^b SI = (GI₅₀ of MRC-5)/(GI₅₀ of MDA-MB-468 or PANC-1).

To further examine the effect of **19** on the growth of cancer cells, a colony formation assay was conducted. Cultured cancer cells are capable of anchorage-independent growth, possessing the ability to grow in the absence of extracellular matrix and adjacent cells. This property of cancer cells has been linked to tumour cell aggressiveness, *in vivo* [14]. Anchorage-independent growth may be assessed *via* a colony formation assay. Colony formation/clonogenic assays also detect the ability of cancer cells to survive a brief challenge with test agent and form progeny colonies, therefore, clonogenic assays were performed to investigate the effect of **19** on the ability of MDA-MB-468 and PANC-1 cells to form colonies (Fig. 1c). Results show that **19** was able to completely prevent the formation of colonies by the cancer cells after 24 h of treatment. These data indicate that **19** has the ability to kill MDA-MB-468 TNBC and PANC-1 pancreatic tumour-initiating cells and prevent tumour re-colonisation, highlighting its cytotoxic potential.

We subsequently investigated the effect of **19** on cancer cell migration. Cancer cells exhibit the ability to metastasise or migrate from their site of origin to other organs within the body which leads to the spread of cancer. Therefore, wound healing migration assays were performed to examine the effect of **19** on the migration of MDA-MB-468 and PANC-1 cells (Fig. 1d and e). Migration of MDA-MB-468 cells was reduced by ~ 3-fold and 13-fold, while that of PANC-1 cells was reduced by around 2-fold and 4-fold relative to the control after 48 h of treatment with **19** at concentrations of 1 × GI₅₀ and 2 × GI₅₀, respectively. Therefore, these results indicate that **19** exhibits significant inhibitory effects on the migration of MDA-MB-468 and PANC-1 cells with a more pronounced effect on the former. The migration ability of cancer cells is a crucial factor in invasion and metastasis [15], therefore, these results and those of the clonogenic assay, reflect the potential of **19** to reduce or inhibit cancer cell survival, invasion and metastasis in patients.

Fig. 1 Compound **19** inhibited the growth and migration of cancer cells. **a** The chemical structure of compound **19**. **b** Representative dose-response profiles demonstrating growth-inhibitory effects of **19** against MDA-MB-468 and PANC-1 cells. **c** Compound **19** completely inhibited the formation of colonies

by MDA-MB-468 and PANC-1 cells after 24 h of treatment at concentrations of $1 \times \text{GI}_{50}$ and $2 \times \text{GI}_{50}$. **d-e** Compound **19** significantly inhibited the migration of MDA-MB-468 and PANC-1 cells after 24 h and 48 h of treatment at concentrations of $1 \times \text{GI}_{50}$ and $2 \times \text{GI}_{50}$. All assays were repeated at least three times, while bars/points and error bars refer to mean $\pm$ S.D. **** $p < 0.0001$ vs. control

Compound 19 induced G2/M-phase cell-cycle arrest by triggering DNA damage in MDA-MB-468 and PANC-1 cancer cells

To further characterise the cytotoxic and growth inhibitory effect of **19** against cancer cells, cell-cycle analysis was conducted in order to investigate the effect of our compound on cell-cycle progression. MDA-MB-468 and PANC-1 treated with **19** at concentrations of $1 \times \text{GI}_{50}$ and $2 \times \text{GI}_{50}$ for 24 h showed a significant increase in the fraction of cells in the G2/M-phase (Fig. 2a). Compound **19** induced the highest accumulation of cells in the G2/M-phase at a concentration of $2 \times \text{GI}_{50}$ for MDA-MB-468 cells (64.3% compared to 29.1% in the untreated cells). However, in PANC-1 cells, the highest accumulation of cells at the G2/M-phase was induced at a concentration of $1 \times \text{GI}_{50}$ (40.3% compared to 15.5% in the untreated cells), while increasing the treatment concentration to $2 \times \text{GI}_{50}$ resulted in significantly increased numbers of events in G2/M and also pre-G1 phases. These observations indicate that **19**, at $2 \times \text{GI}_{50}$, causes apoptosis in PANC-1 cells.

The induction of G2/M-phase cell-cycle arrest by certain agents often indicates that they also cause DNA damage [16]. Therefore, since compound **19** was able to induce G2/M-phase cell-cycle arrest, we investigated its ability to cause DNA damage. The formation of DNA double-strand breaks (DSB) as a result of DNA damage is accompanied by phosphorylation of histone H2AX to γ -H2AX [17], thus DNA damage may be assessed *via* quantification of γ -H2AX. The induction of DNA damage by compound **19** was assessed by quantifying γ -H2AX-expressing cells post-treatment *via* flow cytometry. Results showed that **19** ($2 \times \text{GI}_{50}$) was able to significantly increase the formation of γ -H2AX by ~ 8 -fold in both MDA-MB-468 and PANC-1 cells relative to the control (Fig. 2b). Moreover, it is interesting to note that **19** also resulted in the formation of γ -H2AX at higher levels than the positive control, etoposide ($2 \mu\text{M}$). This clearly indicates that the observed cytotoxic activity of **19** involves the induction of DNA damage in the cancer cells.

Fig. 2 Compound **19** induced G2/M-phase cell-cycle arrest by triggering DNA damage in MDA-MB-468 and PANC-1 cancer cells. **a** Compound **19** induced G2/M-phase cell-cycle arrest in MDA-MB-468 and PANC-1 cells after 24 h of treatment at concentrations of $1 \times \text{GI}_{50}$ and $2 \times \text{GI}_{50}$. The figure also shows representative cell-cycle histograms from one of the experiments showing the effect of **19** on cell-cycle progression **b** Representative dot-plots illustrating the formation of γ -H2AX in MDA-MB-468 and PANC-1 cells after 24 h of treatment with etoposide and **19** at concentrations of $2 \mu\text{M}$ and $2 \times \text{GI}_{50}$, respectively. All assays were repeated at least three times, while bars and error bars refer to mean $\pm$ S.D. * $p < 0.05$ vs. control, *** $p < 0.001$ vs. control, **** $p < 0.0001$ vs. control

Compound 19 causes severe disruption in cytoskeletal structure

Cell-cycle analysis revealed that **19** caused G2/M-phase arrest in MDA-MB-468 and PANC-1 cancer cells, and this could be indicative of microtubule disruption [18]. Therefore, in order to determine whether **19** affects the microtubule network; MDA-MB-468 and PANC-1 cells were treated for 24 h with **19** at GI_{50} concentration, stained with DRAQ5 and monoclonal anti α -tubulin antibody, and morphology investigated using confocal microscopy. Confocal microscopy images (Fig. 3) revealed that **19** was able to induce characteristic features in cancer cells that are typical of a microtubule disrupting agent (MDA), such as multinucleation, nuclear fragmentation and microtubule network disruption [18, 19]. Moreover, apoptosis-related morphological changes were also observed following treatment with **19**, such as membrane blebbing, chromatin condensation and nuclear fragmentation. Therefore, these data suggest that **19** interferes with the cancer cells' microtubule network leading to G2/M-phase cell-cycle arrest which later results in cell death and growth inhibition.

Fig. 3 Compound **19** disrupts the cytoskeletal structure of MDA-MB-468 and PANC-1 cells. **a** Effect of **19** on MDA-MB-468 cells. **b** Effect of **19** on PANC-1 cells. Cells were treated with **19** for 24 h at GI_{50} concentration. Compound **19** causes nuclear fragmentation (1), multinucleation (2), membrane blebbing (3), microtubule network disruption (4), membrane disruption of nuclei (5), lunate morphology of chromatin and chromatin condensation (6). The experiments were repeated 3 times. Cells were immunostained with an antibody specific for α -Tubulin (green) and counterstained with DRAQ5 (purple).

Compound 19 induced caspase-dependent apoptosis and ROS generation in MDA-MB-468 and PANC-1 cancer cells

Cell-cycle analysis and confocal microscopy suggest that **19** causes apoptosis in MDA-MB-468 and PANC-1 cells. Therefore, to further confirm the induction of apoptosis by **19** in cancer cells, an annexin V/propidium iodide (PI) apoptosis assay was conducted. MDA-MB-468 and PANC-1 cells were treated with **19** for 24 h at concentrations of $1 \times GI_{50}$ and $2 \times GI_{50}$, and apoptotic populations were confirmed by annexin V-FITC/PI dual staining. Results have shown that **19** caused significant dose-dependent early apoptosis (A+/PI-) along with an insignificant number of necrotic cells in both cancer cell lines (Fig. 4a). A small increase in late apoptotic events (A+/PI+) was observed in PANC-1 cells, however, MDA-MB-468 cells demonstrated a significant increase in late apoptotic cells after treatment with **19** at $2 \times GI_{50}$, and this may be a consequence of more rapid apoptosis onset at this concentration. The highest percentage of apoptotic cells at $2 \times GI_{50}$ was ~ 36% and 44% for MDA-MB-468 and PANC-1 cells, respectively (consistent with cell cycle observations).

Apoptosis can be further confirmed by investigating the expression levels of apoptosis-related proteins such as PARP and Mcl-1. PARP is a substrate of caspase-3 which plays an active role in a variety of key biological processes, and its cleavage is indicative of apoptosis [20]. Mcl-1 is an antiapoptotic Bcl-2 family protein. Therefore, MDA-MB-468 and PANC-1 cells were treated with **19** and the expression levels of PARP, cleaved PARP and Mcl-1 were analysed *via* Western blot. Results of Western blot analyses showed that **19** cleaved PARP and downregulated Mcl-1 in a time-dependent manner which indicates that it induced apoptosis in the cancer cells (Fig. 4b).

We next explored whether caspases were involved in the induction of apoptosis by **19**, especially crucial executioner caspase 3, whose activation is considered to be a hallmark of apoptosis [21]. Therefore, MDA-MB-468 and PANC-1 cells were treated with **19** and Caspase-Glo 3/7 assays were conducted to investigate the compound's effect on caspase activation. Results showed that **19** was able to induce caspase-3/7 activation in a dose-dependent manner in both MDA-MB-468 and PANC-1 cells, with more pronounced activity observed in the former (Fig. 4c). This suggested that **19** exerted its cytotoxic activity, at least in part, *via* the induction of caspase-dependent apoptosis. It is also crucial to note that cleavage of PARP that was observed in the Western blot assay is also indicative of caspase activation which corroborates the caspase assay results. In conclusion; annexin V-FITC/PI assay, caspase assay and Western blot analysis indicate that **19** induces apoptosis in MDA-MB-468 and PANC-1 cells.

After confirming the induction of apoptosis by **19**, we wanted to investigate the possibility that it was triggered *via* reactive oxygen species (ROS), as ROS are generated by many natural product-derived anti-tumour agents [22] and may damage DNA and play a role in apoptosis-induction. Therefore, cells were treated with **19**, and ROS production was measured using a ROS-Glo H₂O₂ luminescence assay. Compound **19** significantly increased ROS production in MDA-MB-468 and PANC-1 cells by ~ 7- and 10-fold, relative to the untreated control, respectively (Fig. 4d). Increasing the treatment concentration from 1 × GI₅₀ to 2 × GI₅₀ did not result in further significant increases in ROS production in either cell line. So, it can be deduced that the induction of apoptosis by **19** in MDA-MB-468 and PANC-1 cells might be mediated, at least in part, *via* the production of ROS. However, further experiments are required to confirm the relationship between ROS production and apoptosis induction by **19** in the cancer cells.

Fig. 4 Compound **19** induced caspase-dependent apoptosis and ROS production in MDA-MB-468 and PANC-1 cancer cells. **a** Results of the annexin V/PI apoptosis assay showed that **19** induced apoptosis in MDA-MB-468 and PANC-1 cells after 24 h of treatment at concentrations of 1 × GI₅₀ and 2 × GI₅₀. Total apoptosis comprises both early and late apoptotic populations. **b** Western blot analysis of PARP, cleaved PARP and Mcl-1 in MDA-MB-468 and PANC-1 cells. Cells were treated with **19** for 24 h and 72 h at a concentration of 2 × GI₅₀; GAPDH was used as an internal control. **c** Caspase-3/7 activity of MDA-MB-468 and PANC-1 cells after 24 h of treatment with **19** at concentrations of 1 × GI₅₀ and 2 × GI₅₀. **d** ROS production in MDA-MB-468 and PANC-1 cells after 24 h of treatment with **19** at concentrations of 1 × GI₅₀ and 2 × GI₅₀. All assays were repeated at least three times, while bars and error bars refer to mean ± S.D. ***p*<0.01 vs. control, ****p*<0.001 vs. control, *****p*<0.0001 vs. control

Compound 19 exerted its cytotoxic activity in MDA-MB-468 and PANC-1 cells via inhibition of Akt/4EBP1 signaling and downregulation of c-Myc

Several studies have reported that cardamonin exerted its cytotoxic activity *via* inhibition of mTOR signaling [23-25]. A further study has shown that cardamonin reduced the expression level of phosphorylated Akt, which is an upstream regulator of mTOR [26]. Akt signaling pathway is normally involved, *via* the PI3K/Akt/mTOR pathway, in cell proliferation and survival. However, this pathway is abnormally activated in several types of breast cancer, including TNBC, which contributes to the disease's

aggressiveness [27]. Akt activation was also often observed in pancreatic cancer, whereby it is considered as a “master regulator” of cancer cell metastasis [28].

Therefore, we reasoned that the observed cytotoxic activity of **19**, an analogue of cardamonin, against MDA-MB-468 and PANC-1 cells might have occurred *via* a similar mechanism to that of cardamonin. Western blot analysis showed that **19** decreased the expression of phosphorylated Akt (p-Akt) in a time-dependent manner in both MDA-MB-468 and PANC-1 cells (Fig. 5a). Moreover, results have shown that **19** also downregulated the expression levels of phosphorylated 4EBP1 (p-4EBP1), and this protein is the first downstream substrate of mTOR. Therefore, these results suggest that **19** exerted its cytotoxic activity in MDA-MB-468 and PANC-1 cells, at least in part, *via* inhibition of the Akt signaling pathway. It can be further deduced that **19** is involved in the modulation of the mTOR signaling pathway, as our data clearly show that it downregulated p-Akt and p-4EBP1 which are upstream and downstream components of mTOR, respectively.

TNBC and pancreatic cancer are also characterised by an overexpression of the oncoprotein c-Myc which contributes to the growth and proliferation of cancer cells [29-30]. Therefore, we wanted to investigate if the cytotoxic activity of **19** involved suppression of c-Myc expression. Western blot analysis showed that **19** downregulated the expression level of c-Myc in PANC-1 cells (Fig. 5b), however, no change was observed in MDA-MB-468 cells (result not shown). These results are consistent with a c-Myc's role in promotion of pancreatic malignancy and pancreatic cancer cell proliferation and migration and concur with findings of previous studies which reported that inhibition of c-Myc affects the progression of pancreatic cancer cells [31-33]. This suggests that **19** may perturb multiple signaling nodes pertinent to tumourigenesis and can exert its cytotoxic activity in PANC-1 cells *via* a different/additional mechanism than that of MDA-MB-468.

Conclusion

In summary, the present study involves detailed assessment of the cytotoxic activity of novel semi-synthetic compound **19**, investigating its activity and molecular mechanisms of action in MDA-MB-468 and PANC-1 cell lines. Compound **19** demonstrated significant cytotoxic activity against the cancer cell lines and showed higher bioactivity than cardamonin which reflects the positive effect of complexation on bioactivity in this case. Furthermore, **19** inhibited cancer cell colony formation and migration ability. After establishing the efficacy of **19**, we next explored its mode(s) of action, whereby it was found that our compound increased ROS production which may have resulted in the observed induction of DNA damage and subsequent G2/M-phase cell-cycle arrest. It was also found that **19** induced caspase-dependent apoptosis in the cancer cells and it could be due to the production of ROS, while further mechanistic studies showed that it inhibited Akt signaling. Fig. 6 summarises the proposed signaling pathways triggered by compound **19** in the cancer cells. Taken together, this study further highlighted the anticancer potential of **19**, corroborating the findings of our previous study [11], and has provided a better understanding of the mode(s) of action of our compound. To the best of our knowledge, compound **19** is the first synthetic cardamonin analogue that has demonstrated promising anti-tumour activity, and the

only analogue whose mechanism of action has been studied in such detail, as presented in this study. It is expected that such groundwork will guide future *in vivo* studies on **19** in order to further develop the compound as an effective anticancer drug for TNBC and pancreatic cancer.

Declarations

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Conflicts of interest

The authors declare that they have no competing interests.

Availability of data and material

The data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable

Author contributions

Md Shahadat Hossan was involved in the conceptualisation of the study and writing of the manuscript in addition to conducting all the biological assays/experiments and analysing the resulting data.

Mohammed Khaled Bin Break synthesised the Cu(II)-cardamomin complex used in the study, analysed the biological data and wrote the manuscript. Tracey D. Bradshaw supervised the study and was involved in its conceptualisation, in addition to reviewing and editing the manuscript. Hilary M. Collins provided assistance with regards to the use of confocal microscopy in addition to reviewing and editing the manuscript. Christophe Wiart and Teng-Jin Khoo reviewed the manuscript and provided feedback. All authors have approved the final article.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

Figure 1

Compound 19 inhibited the growth and migration of cancer cells. a The chemical structure of compound 19. b Representative dose-response profiles demonstrating growth-inhibitory effects of 19 against MDA-MB-468 and PANC-1 cells. c Compound 19 completely inhibited the formation of colonies by MDA-MB-468 and PANC-1 cells after 24 h of treatment at concentrations of 1 × GI₅₀ and 2 × GI₅₀. d-e Compound 19 significantly inhibited the migration of MDA-MB-468 and PANC-1 cells after 24 h and 48 h of treatment

at concentrations of $1 \times \text{GI}_{50}$ and $2 \times \text{GI}_{50}$. All assays were repeated at least three times, while bars/points and error bars refer to mean $\pm$ S.D. **** $p < 0.0001$ vs. control

Figure 2

Compound 19 induced G2/M-phase cell-cycle arrest by triggering DNA damage in MDA-MB-468 and PANC-1 cancer cells. a Compound 19 induced G2/M-phase cell-cycle arrest in MDA-MB-468 and PANC-1 cells after 24 h of treatment at concentrations of $1 \times \text{GI}_{50}$ and $2 \times \text{GI}_{50}$. The figure also shows

representative cell-cycle histograms from one of the experiments showing the effect of 19 on cell-cycle progression b Representative dot-plots illustrating the formation of γ -H2AX in MDA-MB-468 and PANC-1 cells after 24 h of treatment with etoposide and 19 at concentrations of 2 μ M and 2 $\times$ GI50, respectively. All assays were repeated at least three times, while bars and error bars refer to mean $\pm$ S.D. *p<0.05 vs. control, ***p<0.001 vs. control, ****p<0.0001 vs. control

Figure 3

Compound 19 disrupts the cytoskeletal structure of MDA-MB-468 and PANC-1 cells. a Effect of 19 on MDA-MB-468 cells. b Effect of 19 on PANC-1 cells. Cells were treated with 19 for 24 h at GI50 concentration. Compound 19 causes nuclear fragmentation (1), multinucleation (2), membrane blebbing (3), microtubule network disruption (4), membrane disruption of nuclei (5), lunate morphology of chromatin and chromatin condensation (6). The experiments were repeated 3 times. Cells were immunostained with an antibody specific for α -Tubulin (green) and counterstained with DRAQ5 (purple).

Figure 4

Compound 19 induced caspase-dependent apoptosis and ROS production in MDA-MB-468 and PANC-1 cancer cells. a Results of the annexin V/PI apoptosis assay showed that 19 induced apoptosis in MDA-MB-468 and PANC-1 cells after 24 h of treatment at concentrations of 1 × GI50 and 2 × GI50. Total apoptosis comprises both early and late apoptotic populations. b Western blot analysis of PARP, cleaved PARP and Mcl-1 in MDA-MB-468 and PANC-1 cells. Cells were treated with 19 for 24 h and 72 h at a concentration of 2 × GI50; GAPDH was used as an internal control. c Caspase-3/7 activity of MDA-MB-468 and PANC-1 cells after 24 h of treatment with 19 at concentrations of 1 × GI50 and 2 × GI50. d ROS production in MDA-MB-468 and PANC-1 cells after 24 h of treatment with 19 at concentrations of 1 × GI50 and 2 × GI50. All assays were repeated at least three times, while bars and error bars refer to mean ± S.D. **p<0.01 vs. control, ***p<0.001 vs. control, ****p<0.0001 vs. control

Figure 5

Compound 19 inhibited Akt signaling and decreased the expression of c-Myc in PANC-1 cells. a Western blot analysis of p-Akt, Akt, p-4EBP1, 4EBP1 in MDA-MB-468 and PANC-1 cells. b Western blot analysis of c-Myc in PANC-1 cells only. Cells were treated with 19 for 24 h and 72 h at a concentration of 2 × GI50. GAPDH was used as an internal control. All assays were repeated at least three times.

Figure 6

Schematic representation of the proposed signaling pathways perturbed by 19 in MDA-MB-468 and PANC-1 cells.