Diterpenoid natural compound C4 (Crassin) exerts cytostatic effects on triple-negative breast cancer cells via a pathway involving reactive oxygen species.

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Diterpenoid natural compound C4 (Crassin) exerts cytostatic effects on triple-negative breast cancer cells via a pathway involving reactive oxygen species

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Abstract

Purpose Triple-negative breast cancers (TNBC) lack expression of three common cell surface receptors, i.e., estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2). Accordingly, TNBCs are associated with fewer treatment options and a relatively poor prognosis. Having screened a National Cancer Institute natural compound library, the purpose of this study was to investigate the bioactivity of compound C4 (Crassin) in TNBC cells.

Methods Cell viability assays were performed in two TNBC cell lines, MDA-MB-231 and 4T1, following C4 treatment in the presence or absence of the antioxidant N-acetyl-L-cysteine (NAC). Phosphorylation of Akt and ERK was assessed by Western blotting. Apoptosis, necrosis, autophagy, necroptosis, ferroptosis and cytostasis assays were performed to explain viability deficits resulting from C4 exposure.

Results We found that the viability of the TNBC cells tested decreased in a concentration- and time-dependent fashion following C4 treatment. This decrease coincided with an unexpected increase in the expression of the cell survival effectors pAkt and pERK. In addition, we found that both the decreased cell viability and the increased pAkt/pERK levels could be rescued by the antioxidant NAC, suggesting a central role for reactive oxygen species (ROS) in the mechanism of action of C4. Necrosis, apoptosis, necroptosis and ferroptosis could be ruled out as cell death mechanisms. Instead, we found that C4 induced cytostasis downstream of ROS activation. Finally, we observed a synergistic effect between C4 and the chemotherapeutic drug doxorubicin in TNBC cells.

Conclusions From our in vitro data we conclude that C4 exerts cytostatic effects on triple-negative breast cancer cells via a pathway involving reactive oxygen species. Its potential value in combination with cytotoxic therapies merits deeper investigation in pre-clinical models.
1 Introduction

As the second most common cancer in the world, breast cancer is responsible for approximately 25% of all cancer-related deaths. Triple-negative breast cancers (TNBC) account for 12-20% of all breast cancer cases and are typically associated with a poor prognosis and a high risk of early recurrence [1]. TNBCs are tumours that fail to express three common cell-surface receptors: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) [1, 2]. Accordingly, TNBCs do not benefit from most of the currently available targeted therapies and are restricted to more conventional therapeutic regimens [2].

Chemotherapeutic drugs elicit many undesirable off-target effects, and it has been argued that compounds derived from natural sources may exhibit better toxicity profiles [3, 4]. In fact natural compound screening libraries have been used as a cancer drug discovery tool for decades, and have led to the discovery of the microtubule inhibitor Taxol [5, 6] and others. Taxol, a diterpenoid compound derived from the bark of the Pacific yew tree, has become a key component of front-line combination therapy in several cancers including breast and prostate cancer [7], in which its ability to halt cell division through G2/M arrest powerfully synergizes with cytotoxic drugs. Other diterpenoids have recently been shown to induce cytotoxicity in leukemic and lung cancer cell models [8]. In this study, a natural compound library obtained from the Developmental Therapeutics Program of the US National Cancer Institute (https://dtp.cancer.gov/) was screened against TNBC cells using in vitro viability assays. Based on this screen, diterpenoid compound C4 (Crassin) was selected for further analysis. We present evidence for a ROS-dependent cytostatic effect of C4 in TNBC cells and suggest potential clinical benefits in combination with other cytotoxic chemotherapeutic drugs.

2 Materials and methods

2.1 Cell Culture

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MDA-MB-231 and 4T1 cells were obtained from the American Type Culture Collection. Human primary breast cancer cells (198T) were isolated from a triple-negative breast cancer patient as reported before [9] with full ethical approval from the Beaumont Hospital Medical Ethics (Research) Committee. MDA-MB-231 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, Poole, UK) supplemented with 10% Fetal Bovine Serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamine. 4T1 cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin. Primary cells were cultured in MEGM medium (DMEM/HAMS F12 (Sigma) supplemented with EGF (10 ng/ml), Hydrocortisone (0.5 µg/ml), Insulin (5 µg/ml), BPE (70 µg/ml), Transferrin (5 µg/ml), Ethanolamine (1x10^{-4} M) and O-phosphoethanolamine (1x10^{-4} M). All cell cultures were performed using aseptic techniques.

2.2 Pharmacological treatment

C4 (NSC-210236; PubChem CID 5357909; Crassin / (4E,8E)-2,10-dihydroxy-4,8,12-trimethyl-16-methylidene-14-oxabicyclo[11.3.1]heptadeca-4,8-dien-15-one) was donated by the US National Cancer Institute (NCI) Developmental Therapeutic Program (DTP) and dissolved in DMSO at a stock concentration of 20 mM. Cells were seeded and treated according to their specific doubling periods. C4 was applied at a range of concentrations (20 µM, 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.6 µM, 0.3 µM) to establish the IC_{50} in MDA-MB-231 cells. For each drug concentration, the corresponding matched concentration of DMSO was used as a vehicle control (yielding final concentrations ranging from 0.1 to 0.00156% v/v). In all cases, C4 and DMSO were added to complete culture media. The antioxidant N-acetyl-L-cysteine (NAC; Sigma, # A7250) solution was freshly prepared before each treatment (10 mg/ml in sterile PBS pH 7.4, sterile-filtered). At the desired concentration (3 mM) NAC was added to fresh complete culture media and incubated for 1 hour pre-treatment before the addition of C4 (5µM). After pre-treatment, fresh NAC was re-added in parallel with C4. DMSO (0.025% v/v) was used as vehicle control. Necrostatin-1 (Sigma, # N9037) was dissolved in DMSO (10 mg/ml) and used at a concentration of 100 µM, whereas Ferrostatin-1 (Sigma, #SML0583) was dissolved in...
DMSO (10 mg/ml) and used at a concentration of 5 µM. These compounds were added to cell culture wells with or without C4 or NAC for 24 or 48 hours at 37°C. Similar to NAC treatment, cells were pre-treated with either Nectrosatin-1 or Ferrostatin-1 after which the compounds were re-added following 1 hour incubation with or without C4 or NAC. DMSO (0.025% v/v) was used as vehicle control. Doxorubicin (Dox) was obtained from the Beaumont Hospital pharmacy (2 mg/ml), dissolved in H₂O and applied at a concentration of 2.5 µM with or without C4 (5 µM). DMSO (0.025% v/v), H₂O (0.067% v/v) or a combination of both were used as vehicle controls. Staurosporine was dissolved in H₂O (1 mg/ml) and cells were treated at a concentration of 10 µM for 3 hours or 1 µM for 48 hours prior to Western blotting or flow cytometry, respectively.

2.3 Western blot analysis

Cell lysates were prepared as follows: cells were washed 3 times with ice-cold PBS, after which 200 µl RIPA buffer was added containing 1% Triton-X100, a protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3 (Sigma). Next, the cells were scraped off, trituated, and stored on ice for 10 minutes before incubation at -80°C for 24 hours. The resulting samples were thawed and centrifuged (15300 rcf, 20 min, 4°C) after which the supernatants stored at -20°C until use. Equivalent protein concentrations were electrophoresed (40 mA/gel, or 35 V/gel for caspase-3), after which the proteins were wet-transferred to nitrocellulose membranes (100 V for 75 min, or 200 mA for 2 hours for caspase-3). Next, the membranes were blocked for 1 hour with either 5% milk or 5% BSA in 1x TBS containing 0.1% Tween-20 (TBS-T) and incubated with primary antibodies overnight at 4°C. Following washes, the membranes were incubated with horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit secondary antibodies for 1 hour at room temperature (RT), after which they were immersed in enhanced chemiluminescence solution (ECL; Western Lightning Plus, Perkin Elmer Ireland) for 1 minute before being processed in a BioRad ChemiDoc MP imager. Subsequently, the membranes were washed 3 times for 5 min in TBS-T and stripped in a β-mercaptoethanol-based buffer at 65°C for 15 min, prior to being washed and incubated with additional antibodies as
described above. Actin was used as an internal reference for signal intensities between treatment groups.

2.4 Statistical analysis

Average mean (± standard error of the mean (SEM)) values from the Western blot experiments were calculated and graphed using Graphpad Prism 6 based on the band volume intensities using ImageLab software 5.2.1. The band volume intensity of the protein of interest was calculated relative to that of the actin control. The experimental data (n = 3) were analysed using one-tailed, unpaired t-tests to determine statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001) differences between conditions.

2.5 Cell viability assay

Cellular viabilities were measured using Alamar Blue - Resazurin assays (Invitrogen, # DAL1025). Briefly, varying cell numbers were seeded per well in 96-well plates and incubated with drug. Next, the media were aspirated and 100 µl Alamar blue solution (43.8 µM) and fresh media were added, after which the cells were incubated in the dark for 5 hours at 37°C and fluorescence was measured at 610 nm using a VICTOR™ X3 Multilabel Plate Reader. Statistical analyses were performed using Graphpad Prism 6 software and significance levels (*p < 0.05, **p < 0.01, ***p < 0.001) were calculated following recommended tests and post-tests included in the software.

2.6 Annexin-V apoptosis assay

Apoptotic cell death was assessed using a BD FITC, Annexin-V Apoptosis Detection Kit I (Becton Dickinson, Oxford, UK, # 556547). Cells were seeded at a density of 300,000 cells per well in 6-well-plates, incubated for 48 hours and treated with either vehicle control, C4 (5 µM) or Staur. (1 µM) as a positive control. After the required treatment period, the supernatant media were transferred to 15 ml tubes after which the cells were washed using chloride- and magnesium-free PBS (CMF-PBS), trypsinized and transferred to tubes. The tubes were centrifuged (670 rcf for 5 min) and washed 3x in CMF-PBS, after which the cell pellets were resuspended in 200 µl incubation buffer, 5 µl Annexin-V
and 5 µl propidium iodide (PI) and incubated at RT for 15 min. After this incubation, 20,000 cells were analysed on a Beckman Coulter FC500 flow cytometer and the % gated recorded in order to generate mean ± SEM of experimental data (n = 3) for statistical analysis of apoptotic cell death using Graphpad Prism 6 software (conditions relative to vehicle DMSO control).

2.7 Lactate dehydrogenase (LDH) necrosis assay
Necrosis was assessed using a Sigma LDH Assay Kit (# MAK066). MDA-MB-231 cells were seeded at a density of 5000 per well in 96-well plates and after 36 hours treated with C4 or vehicle DMSO control for 48 hours. Incubation with 1% (v/v) 1% 10x Triton-X100 for 45 minutes at 37°C was used as a positive control for maximum cell lysis. After treatment, the plates were centrifuged (670 rcf for 5 min) and the supernatants were transferred to new 96-well plates. LDH Assay buffer and substrate mix were added to these new plates as per the product manual and incubated for 2 minutes. Following this incubation, the plates were spectrophotometrically read at 450 nm on a VICTOR™ X3 Multilabel Plate Reader.

2.8 Bromodeoxyuridine (BrdU) proliferation assay
Cell proliferation was assessed using a Roche Cell Proliferation ELISA, BrdU (colorimetric) kit (#11647229001, Sigma-Aldrich). Following C4 treatment, cells were labelled with a BrdU labelling solution as per manufacturer’s instructions. Following a 2 hour incubation at 37°C, the labelling solution was aspirated and replaced by a FixDenant solution for 30 minutes at RT. This solution was subsequently removed and replaced by an anti-BrdU-POD antibody for 90 min at RT, after which the solution was removed and the cells were washed. Finally, a substrate solution was added for 10 min followed by a stop solution (1M H$_2$SO$_4$) before the plate was read at 450 nm (reference wavelength 690 nm) using a VICTOR™ X3 Multilabel Plate Reader.

3 Results
3.1 C4 reduces the viability of triple negative breast cancer cells

This study was conceived following reports of the anti-cancer properties of several compounds derived from natural sources [10-13]. Having identified C4 as a promising lead compound after screening a natural products chemical library using breast cancer cell viability assays (unpublished data), we established the IC₅₀ of C4 in triple negative breast cancer (TNBC) MDA-MB-231 cells as 9.16 µM (24 hours) and 4.65 µM (48 hours), respectively, using Alamar Blue - Resazurin assays (Fig. 1). In addition to this concentration sensitivity, we also established time sensitivities to C4 in two TNBC cell lines and in a primary TNBC cell culture (Fig. 2). We found that MDA-MB-231 cells exhibited C4-induced time-dependent reductions in viability of 38 ± 6.3% and 63.5 ± 4.1% at 24 hours and 48 hours, respectively (Fig. 2A). Similar time-dependent reductions in cell viability were observed following C4 treatment in TNBC 4T1 cells, namely 58.3 ± 9.9% and 86 ± 3.8% at 24 hours and 48 hours, respectively (Fig. 2B). In a single biological replicate of the primary TNBC cell culture 198T, also a significant reduction in viability (52 ± 0.02%) was observed across technical replicates of C4-treated cells compared to vehicle control (DMSO) treated cells (Fig. 2C).

3.2 C4 increases pAkt and pERK levels in an antioxidant-sensitive manner

Having established that C4 reduces viability in three (primary and established) TNBC cell models, we next set out to assess its effect on known cell survival signalling pathways. Unexpectedly, we found that the expression of the cell survival effector phosphorylated Akt (pAkt) [14-17] increased significantly after 24 and 48 hours of C4 treatment (Fig. 3C-D). Since Akt signalling has also been associated with cell death downstream of reactive oxygen species (ROS) release [18-20], we next examined the viability of cells pre-treated with the antioxidant N-acetyl-cysteine (NAC) (3 mM) prior to treatment with C4 (5 µM) for 24 or 48 hours. We found that NAC significantly counteracted the negative effects of C4 on the viability of both MDA-MB-231 and 4T1 cells (Fig. 3A and 3B, respectively) and, in addition, restored the pAkt levels to those of C4 untreated controls (Fig. 3C-G).

Interestingly, we also observed a reduction in total Akt in 4T1 cells, but not in MDA-MB-231 cells, following C4 treatment. This reduction may be attributed to cell density, potentially reflecting a
better access of C4 to the cell surfaces of loosely growing 4T1 cells than the more densely growing MDA-MB-231 cells, since the effect could be reproduced in MDA-MB-231 cells seeded at a lower density (Fig. 4). In light of the observed C4-dependent increases in pAkt levels, we next set out to explore whether ERK phosphorylation (also known to play a key role in cell survival [21]) was similarly affected by C4 treatment. By doing so, a small but statistically insignificant increase in pERK levels was observed after C4 treatment of MDA-MB-231 cells (Fig. 3H-J).

3.3 C4-induced cell viability decreases are not accounted for by common mechanisms of cell death

Having established that C4 reduces cell viability across three TNBC cell models, in conjunction with ROS-mediated Akt and ERK activation (phosphorylation), we next set out to examine potential downstream cell death pathways. To this end, we first examined Caspase-3 (Cas-3) cleavage to assess a possible contribution of apoptosis to cell death [22, 23]. Upon Western blotting, cleaved Cas-3 bands were only observed under positive control (staurosporine-treated) but not under C4-treated conditions (Fig. 5A). This apparent lack of apoptosis involvement was subsequently confirmed using flow cytometric Annexin-V assays. Specifically, we found that MDA-MB-231 cells treated with the positive control reagent staurosporine showed increased Annexin-V positivity compared to DMSO-treated cells ($p < 0.001$), whereas Annexin-V staining was not significantly increased above negative control levels in C4-treated cells, even after 48 hours (Fig. 5B and C).

The possibility that C4 may activate necrotic cell death was next explored using lactate dehydrogenase (LDH) assays in C4-treated cells (with Triton-X100-treated cells as a positive control). We found that LDH release was not different between control DMSO- or C4-treated cells, i.e., 10% (± 3) and 13% (± 2) at 24 hours, respectively, and 5% (± 1) and 8% (± 2) at 48 hours, respectively, compared to positive control cells (Fig. 6A). This suggests that membrane permeabilization is not a feature of C4-treated cells even after 48 hours. Interestingly, we noted a significant increase in LDH release by NAC-treated cells, but this may be due to a transient NAC-induced permeabilisation of the cell membranes, as this release was seen to tail off at 48 hours (31 ± 8% at 24 hours, reduced to 14 ± 2% at 48 hours).
Given the sensitivity of each assay to the ROS inhibitor NAC, we next set out to explore other modes of cell death putatively involving ROS induction. Both necroptosis and ferroptosis are apoptosis-independent mechanisms of cell death associated with increased ROS levels [24-27]. To test these possibilities, MDA-MB-231 cells were treated with C4 (5 µM) in the presence or absence of necroptosis or ferroptosis inhibitors (Necrostatin-1 and Ferrostatin-1, respectively). We found that neither Necrostatin-1 (100 µM) nor Ferrostatin-1 (5 µM) were able to block the C4-induced decreases in cell viability (Fig. 6B), whereas the C4-induced cell viability effects could still be rescued by NAC (3 mM). Erastin (10 µM) was used as a positive control inducer of ferroptosis. A positive control inducer of necroptosis in MDA-MB-231 cells could not be found (data not shown).

Next we explored autophagic cell death by examining LC3B II protein expression in C4-treated MDA-MB-231 cells. Significant increases in LC3B II conjugation were observed compared to controls at both 24 hours (Fig. 6C) and 48 hours (Fig. 6D) ($p = 0.0001$ and $p = 0.0494$, respectively). However we found that the morphology of cells following C4 treatment was not consistent with autophagy (Supplementary Fig. 1 and 2); which suggests that reductions in cell viability after C4 treatment may reflect cytostasis rather than overt autophagic toxicity.

To determine whether C4 may induce cell cycle arrest, flow cytometric cell cycle analyses were conducted in propidium iodide-stained MDA-MB-231 cells following C4 treatment. By doing so, we observed a significant shift in the C4-treated cells from G1 to G2/M (Fig. 7A). Specifically, we found that 37 ± 12% fewer C4-treated cells than DMSO-treated control cells were in the G1 phase of the cell cycle, whereas 30 ± 4% more C4-treated cells were in the G2/M phase. Subsequently, BrdU assays were carried out to examine the proliferative capabilities of the cells following treatment with C4 (Fig. 7B). Our data indicate diminished proliferative capabilities even after a 24 hour treatment with C4 (5 µM; reductions of 90.6 ± 4.7% and 95.9 ± 2.9% at 24 hours and 48 hours, respectively). Taken together, we conclude that our data are consistent with a cytostatic mode of action of C4, reflecting a reduced capability to metabolize resazurin to resorufin in Alamar Blue - Resazurin assays (see above).

3.4 C4 synergises with doxorubicin in targeting TNBC cell viability
Finally, the potential usefulness of a cytostatic compound like C4 in combination with an established chemotherapeutic drug (doxorubicin) was investigated in TNBC cells. We found that the combination of C4 and doxorubicin synergistically reduced cell viability over the responses to either compound alone (Fig. 8A-B, Dox. 2.5 µM; reductions of 30 ± 2% and 69 ± 2% at 24 hours and 48 hours, respectively; C4 5 µM; reductions of 37 ± 3% and 66 ± 2% at 24 hours and 48 hours, respectively; C4 and Dox combination reductions of 58 ± 3% and 82 ± 2% at 24 hours and 48 hours, respectively).

4 Discussion

Triple-negative breast cancers (TNBC) constitute 12-20% of all diagnosed breast cancers and are associated with a poor survival [2, 28-31]. Despite recent advances, few targeted therapies are available and, therefore, the treatment options are often limited to surgery, radiation or conventional chemotherapy [28]. Here, we identified a natural compound, C4 (Crassin), which effectively reduces the viability of two TNBC cell lines and a primary TNBC cell culture.

Based on this result, we assessed the expression levels of the survival effectors pAkt and pERK in TNBC cells following C4 treatment. Akt in its activated (phosphorylated) form has been shown to promote cell survival and to inhibit apoptosis [21, 32, 33]. With the expectation that the pAkt and pERK levels would decrease following C4 treatment, we surprisingly noted significant increases in pAkt and pERK expression levels. Based on this finding, we considered the involvement of reactive oxygen species (ROS) as a possible explanation to this conundrum. ROS are generated due to a partial reduction of oxygen, resulting in molecules with unpaired electrons [34]. ROS can oxidise members of the PI3K/Akt signaling pathway [34] and specifically activate ERK [35-37]. This phenomenon may occur naturally during mitochondrial oxidative metabolism, but can also be induced in response to external stimuli [38]. The unstable nature of ROS means they are highly reactive and can cause a further instability of cellular macromolecules, including lipids, proteins and DNA [38, 39]. If this process of ‘electron stealing’ is left unchecked and the stability of important cell structures
continues to deteriorate, a cell may enter the process of programmed cell death [39, 40]. Therefore, we used an anti-oxidant (NAC) in an attempt to prevent Akt and ERK activation (phosphorylation). Anti-oxidants have the ability to donate an electron to unstable free radicals, without compromising their own stability [38], and hence may prevent damage caused by ROS seeking or donating electrons. Strikingly, we found that NAC treatment prevented C4-induced increases in pAkt and pERK, indicating that this phenotype is mediated by a highly oxidised environment. Furthermore, NAC treatment prevented C4-induced reductions in cell viability in TNBC cells compared to C4 treatment alone. This is consistent with C4 inducing oxidative stress upstream of Akt activation, resulting in a reduced cell viability.

Since C4 treatment significantly reduced the viability of TNBC cells, we next attempted to identify the cell death mechanism known to involve ROS. Caspase-3 (Cas-3) is a key member of the apoptotic pathway [23], and its cleavage activates a downstream cascade of events leading to cytochrome c release and cell death [22]. However, no Cas-3 cleavage in C4-treated samples was found. Furthermore, no evidence of Annexin-V positivity in C4-treated cells was found, suggesting that the C4-induced reduction in cell viability was independent of traditional apoptotic pathways. To next test whether C4 could induce necrosis, we measured LDH release in TNBC cells and found that the C4-decreases in cell viability occurred independently of necrosis. Also, links between ROS activation and necroptotic or ferroptotic mechanisms of cell death have recently been reported [24-26], but we failed to obtain any evidence for necroptosis or ferroptosis in response to C4 treatment of TNBC cells. Interestingly, increases in LC3B cleavage (indicative of activation of an autophagic mode of cell death) were observed, but our morphologic observations suggest that these increases in expression may not reflect autophagy, or at most may indicate that C4-treated cells are in a primitive stage of cell death. Extended treatment times may uncover a shift from cellular stasis to induced autophagy, though significantly diminished cell numbers at later treatment time points may pose an inevitable concern for result validation.

While it is possible that the later (48 hour) C4 treatment time point represents a premature state of cell death, cell cycle analysis revealed a significant shift from G1 to G2/M in these cells compared to controls. Furthermore, BrdU assays revealed diminished proliferative capabilities of
TNBC cells following C4 treatment, supporting a role for C4 in cytostasis induction. The induction of cell cycle arrest by C4 may prove to be an important mechanistic effect of the non-cytotoxic natural compound. Our data showing synergism of C4 with the chemotherapeutic drug doxorubicin may indicate a potential future use in clinical settings. At this point in time, however, it is not known whether (and how) C4 may also synergise with anti-tumorigenic effects of other chemotherapeutic compounds (i.e., anti-metabolites, alkylating agents etc.). Nonetheless, supportive literature demonstrates benefits of anti-cancer cytostatic therapies when used in combination with cytotoxic chemotherapies [41], and this approach is now commonly employed in the design of chemotherapy protocol algorithms [42]. Such algorithms have shown, both in vivo and in silico, that cytostatic/cytotoxic combinations may improve the efficacy of treatment and the overall efficiency of chemotherapy [42]. It is also of interest to note that compound C4 belongs to the same broad chemical class as the widely used cytostatic chemotherapeutic drug, Taxol (the diterpenoid class). Whilst both compounds are of natural origin, they exhibit chemical differences that likely affect their bioactivities. C4 (Crassin) has the following formula and molecular weight: C_{20}H_{30}O_{4}, MW 334.456, while Taxol is a nitrogenous alkaloid with the following formula and molecular weight: C_{47}H_{51}NO_{14} / MW 853.918. Similar to C4, Taxol has been shown to induce G2/M cell cycle arrest, but under certain circumstances Taxol has also been found to act as a pro-apoptotic agent in a p53-independent manner [43, 44]. We found, however, that C4 exhibited no cytotoxicity at the tested concentrations. Taken together, we found that C4 may induce cell cycle arrest via the sensitisation of TNBC cells to ROS, thereby increasing Akt and ERK activation and decreasing cell proliferation. Our data warrant a further investigation of the therapeutic efficacy of C4 in combination with other cytotoxic agents.

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The authors declare no conflict of interest.
References

Figure legends

Figure 1. C4 dose-dependently reduces the viability of MDA-MB-231 cells via a pathway involving ROS. MDA-MB-231 cells were seeded at a density of 5000 cells per well in 96-well plates and treated 48 hours later with C4 at concentrations ranging from 0.325 to 20 µM. Cell viability was assessed spectrophotometrically using Alamar Blue metabolic assays, and expressed as % optical density relative to that in vehicle (DMSO) treated cells. Data (n = 3) are expressed as X=log(X) mean ± SEM. IC₅₀ values were calculated using the Graphpad Transform function and curve fitted using non-linear regression.

Figure 2. C4 time-dependently reduces the viability of TNBC cells. MDA-MB-231 cells (A) were seeded at a density of 5,000 cells per well, 4T1 cells (B) at 1,000 per well and primary breast cells at 5,000 per well (C) in 96-well plates and treated after one doubling time with C4 (5 µM) or vehicle control (DMSO). Viability was assessed at 24 hours and 48 hours for MDA-MB-231 and 4T1 cells and at 48 hours for 198T cells using an Alamar Blue assay, and expressed as % of the DMSO vehicle control. Data (n = 3) are expressed as mean ± SEM, with statistical significance determined using one-way ANOVA correcting for multiple comparisons using Dunnet’s test reporting adjusted p values (MDA-MB-231, 4T1 cells) or unpaired two-tailed t-test (primary cells) (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05).

Figure 3. C4 reduces cell viability and increases pAkt expression in a ROS-dependent fashion. MDA-MB-231 cells (A) were seeded at a density of 5,000 cells per well and treated 48 hours later with C4 (5 µM) or vehicle control (DMSO) +/- NAC (3 mM) for 24 or 48 hours. 4T1 cells (B) were seeded in 96-well plates at a density of 1000 cells per well and treated 24 hours later with C4 (5 µM) or vehicle control (DMSO) +/- NAC (3 mM) for 24 or 48 hours. Cell viabilities were assessed using Alamar Blue assays, and expressed as % of DMSO vehicle control. Data (n = 3) are expressed as mean ± SEM, with statistical significance determined using unpaired two-tailed t-tests. MDA-MB-
231 cells were seeded in 6-well plates at a density of 300,000 per well and treated 48 hours later with C4 (5 µM) or vehicle control (DMSO) +/- NAC (3 mM) for 24 or 48 hours (C). 4T1 cells were seeded at a density of 50,000 per well and treated 24 hours later with C4 (5 µM) or vehicle control (DMSO) +/- NAC (3 mM) for either 24 or 48 hours (D). The expression of pAkt (C, D) and pERK (H) was analysed using Western blotting, and densitometrically quantified using ImageLab software 5.2.1 in MDA-MB-231 (E, F, H) and 4T1 (G) cells (volume intensity of band relative to matched total Akt band and displayed relative to DMSO). Results are expressed as mean ± SEM (n = 3). Statistical significance was calculated by unpaired one-tailed t-tests (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05).

**Figure 4. Akt expression levels are sensitive to cell density.** MDA-MB-231 cells (A) were seeded in 6-well plates at a low density of 100,000 cells per well and treated 48 hours later with C4 (5 µM) or vehicle control (DMSO) +/- NAC (3 mM) for 24 or 48 hours. pAkt expression was analysed using Western blotting (A), and densitometrically quantitated using ImageLab software 5.2.1 (B) (volume intensity of band relative to matched total Akt band and displayed relative to DMSO). Results are expressed as mean ± SEM (n = 3). Statistical significance was calculated by unpaired one-tailed t-tests.

**Figure 5. C4 does not induce apoptotic cell death.** MDA-MB-231 cells were seeded in 6 well plates at a density of 300,000 per well and treated 48 hours later with C4 (5 µM) or vehicle control (DMSO) +/- NAC (3 mM) for either 24 or 48 hours [using Staurosporine/(staur.; 10 µM/3 hours or 1 µM/48 hours) as a positive inducer of apoptosis]. Caspase-3 expression was analysed using Western blotting (A). Cells were flow cytometrically analyzed after staining with a BD Annexin-V kit (B). The gated % of the treatment groups were analysed for Annexin-V positivity/apoptosis (quadrant D4) (C) using one-way ANOVA, correcting for multiple comparisons using Tukey’s test. Data (n = 3) are expressed as mean ± SEM and statistical significance relative to the matched vehicle control % ratio.
Figure 6. C4 does not induce cell death. MDA-MB-231 cells were seeded at a density of 5000 cells per well in 96-well plates and treated 48 hours later with C4 (5 μM) in the presence or absence of NAC (3 mM) for 24 and 48 hours (A), at which points positive control wells were treated with 1% Triton-X for 45 minutes. Necrosis was measured using a LDH Assay kit. Data (n = 3) are expressed as mean ± SEM. Statistical significance (**p < 0.001, *p < 0.05) was determined using one-way ANOVA correcting for multiple comparisons using Dunnet’s test reporting adjusted p values calculated using DMSO-treated controls for all groups. MDA-MB-231 cells were treated with C4 (5 μM) or vehicle control (DMSO) +/- NAC (3 mM) for 48 hours in the presence or absence of necrostatin-1 (nec-1; 100 μM), or ferrostatin-1 (fer-1; 5 μM) with Erastin (10 μM) as a positive control for ferroptosis, prior to performing Alamar Blue cell viability assays (B). Data (n = 3) are expressed as mean ± SEM. Statistical significance was determined using one-way ANOVA correcting for multiple comparisons using Dunnet’s test reporting adjusted p values. MDA-MB-231 cells were seeded at a density of 300,000 per well and treated 48 hours later with C4 (5 μM) or vehicle control (DMSO) +/- NAC (3 mM) for 24 or 48 hours. LC3B II expression was analysed using Western blotting at 24 hours (C) and 48 hours (D), and densitometrically quantitated using ImageLab software 5.2.1 (E) (volume intensity of band relative to matched Actin band). Results are expressed as mean ± SEM (n = 3). Statistical significance was calculated relative to DMSO by one-way ANOVA using Dunnet’s correction for multiple comparisons and reporting adjusted p-values.

Figure 7. C4 induces cell cycle arrest in a ROS-sensitive manner. MDA-MB-231 cells were seeded at a density of 2500 cells per well in 96-well plates and treated 48 hours later with C4 (5 μM) for 24 or 48 hours, +/- 1 hour pre-treatment with NAC (3 mM) (A, B). Next, a BrdU colorimetric assay was performed (C, D). Data (n = 3) are expressed as mean ± SEM. Statistical significance was assessed by comparing C4 to the pre-treated NAC group (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05; one-way ANOVA performed using Prism 6 software with multiple comparisons and corrections using Dunnet’s test, reporting adjusted p-values calculated using DMSO treated control for all groups).
Figure 8. C4- and doxorubicin-induced reductions in cell viability are synergistic. MDA-MB-231 cells were seeded at a density of 5000 cells per well in 96-well plates and treated with C4/Dox 48 hours after seeding (5 μM or 2.5 μM, respectively). Cell viabilities were assessed using Alamar Blue assays after 24 (A) or 48 (B) hours and expressed as % optical density of that in vehicle control (DMSO)-treated cells. Data (n = 3) are expressed as mean ± SEM. Statistical significance (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05) was determined using unpaired two-tailed t-tests.
Supplementary Figure Legends

**Supplementary Figure 1: C4 alters cellular morphology in MDA-MB-231 cells.** MDA-MB-231 cells were plated at either 300,000 or 100,000 per well and treated 48h later with C4 (5µM) or vehicle control (DMSO) for either 24h (A) or 48h (B) for 48h. Cells were then imaged at 40x magnification on an Olympus CKX41 microscope using Cell B imaging software.

**Supplementary Figure 2: C4 alters nuclear morphology in MDA-MB-231 cells and 4T1 cells.** MDA-MB-231 cells were plated at 75,000 per well and treated 48h later and 4T1 cells plated at 50,000 cells per well and treated 24h later with C4 (5µM) or vehicle control (DMSO) for either 24h (A) or 48h (B). Nuclei were then stained with DAPI and imaged at 40x magnification on an Olympus CKX41 microscope using Cell B imaging software.
Figure 1: Richards et al
Figure 2: Richards et al

A

% Cell Viability (Ratio to DMSO)

24h 48h

DMSO C4 DMSO C4

B

% Cell Viability (Ratio to DMSO)

24h 48h

DMSO C4 DMSO C4

C

Cell Viability

DMSO C4

Treatment (48 hours)
Figure 4: Richards et al

A

![Image of Western blot with protein bands at 24h and 48h, labeled pAkt, Total Akt, and Actin, with DMSO, NAC, and C4 treatment conditions]

B

![Bar graph showing protein expression relative to Total Akt, with DMSO, C4, C4 + NAC treatment conditions at 24h and 48h]
Figure 5: Richards et al

A

<table>
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Cas-3 Full Length
Cas-3 Cleaved
Actin

DMSO  NAC  C4  Staur.
      +   +   +   +
      -   +   +   +
      -   -   +   +
      -   -   -   +

B

DMSO
C4
C4 + NAC
Staur.

Annexin-V

C

Annexin-V Staining Ratio to DMSO (Gated %)

DMSO  C4  C4+NAC  Staur.

Treatment (48 hours)
Figure 6: Richards et al

A

LDH Release %
(Ratio % to 1% Triton-X)

Treatment (24 hours)

B

Cell Viability (%)
(Ratio to DMSO)

Treatment (48 hours)

C

kDa
19
17
42

DMSO + - - - NAC - + - + C4 - - + +

LC3B I

LC3B II

Actin

D

kDa
19
17
42

DMSO + - - - NAC - + - + C4 - - + +

LC3B I

LC3B II

Actin

E

Protein Expression
(Ratio to DMSO)

Treatment

24h

48h
Figure 7: Richards et al

A

PI Intensity (Relative to DMSO)

Cell Cycle Stage

DMSO
C4
C4+NAC

B

BrdU Staining (Ratio to DMSO)

Treatment

DMSO
NAC Alone
C4
C4 + PBS
C4 + NAC
C4 + PBS
C4 + NAC

24h
48h
Figure 8: Richards et al

A

% Cell Viability

DMSO  Dox  C4  C4 + Dox

Treatment (24h)

B

% Cell Viability

DMSO  Dox  C4  C4 + Dox

Treatment (48h)
Supplementary Figure 1: Richards et al

A

DMSO  
300,000
100,000

C4

24h 40x Magnification

B

DMSO  
300,000
100,000

C4

48h 40x Magnification
Supplementary Figure 2: Richards et al

MDA-MB-231  4T1

DMSO

C4

48h 40x Magnification