

A novel synthetic C-1 analogue of 7-deoxypancratistatin induces apoptosis in p53 positive and negative human colorectal cancer cells by targeting the mitochondria: enhancement of activity by tamoxifen

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Summary The natural compound pancratistatin (PST), isolated from the *Hymenocallis littoralis* plant, specifically induces apoptosis in many cancer cell lines. Unlike many other chemotherapeutics, PST is not genotoxic and has minimal adverse effects on non-cancerous cells. However, its availability for preclinical and clinical work is limited due to its low availability in its natural source and difficulties in its chemical synthesis. Several synthetic analogues of 7-deoxypancratistatin with different modifications at C-1 were synthesized and screened for apoptosis inducing activity in human colorectal cancer (CRC) cells. We found that a C-1 acetoxymethyl derivative of 7-deoxypancratistatin, JC-TH-acetate-4 (JCTH-4), was effective in inducing apoptosis in

both p53 positive (HCT 116) and p53 negative (HT-29) human CRC cell lines, demonstrating similar efficacy to that of natural PST. JCTH-4 was able to decrease mitochondrial membrane potential (MMP), increase levels of reactive oxygen species in isolated mitochondria, cause release of the apoptogenic factor cytochrome c (Cyto c) from isolated mitochondria, and induce autophagy in HCT 116 and HT-29 cells. Interestingly, when JCTH-4 was administered with tamoxifen (TAM), there was an enhanced effect in apoptosis induction, reactive oxygen species (ROS) production and Cyto c release by isolated mitochondria, and autophagic induction by CRC cells. Minimal toxicity was exhibited by a normal human fetal fibroblast (NFF) and a normal colon fibroblast (CCD-18Co) cell line. Hence, JCTH-4 is a novel compound capable of selectively inducing apoptosis and autophagy in CRC cells alone and in combination with TAM and may serve as a safer and more effective alternative to current cancer therapies.

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Abbreviations

ANT	adenine nucleotide translocase
CK	creatine kinase
CRC	colorectal cancer
CypD	cyclophilin D
Cyto c	cytochrome c
ER	estrogen receptor
IC ₅₀	half-maximal inhibitory concentration
JCTH-1	JC-TH-acid-1
JCTH-4	JC-TH-acetate-4

HK	hexokinase
LC3	microtubule-associated protein 1 light chain 3
MDC	monodansylcadaverine
MMP	mitochondrial membrane potential
MRC	mitochondrial respiratory chain
mtDNA	mitochondrial DNA
NFF	normal human fetal fibroblast
PBR	peripheral benzodiazepine receptor
PQ	paraquat
PST	pancratistatin
PTP	permeability transition pore
RFU	relative fluorescence units
ROS	reactive oxygen species
SDHA	succinate dehydrogenase subunit A
TAM	tamoxifen
TMRM	tetramethylrhodamine methyl ester
VDAC	voltage-dependent anion channel

Introduction

Globally, colorectal cancer (CRC) accounts for 9.4% of all newly diagnosed cancers [1]. In 2009, CRC was the third most common cancer responsible for 49,920 deaths in the USA [2]. Current treatments include surgery, radiotherapy, and chemotherapy. Most chemotherapeutics however, are genotoxic in nature and cause adverse effects in non-cancerous cells. More recent efforts have achieved improved rates of survival with metastatic CRC, using combination therapy of various drugs, such as administration of oxaliplatin or irinotecan with fluorouracil [3–6]. Despite these efforts, CRC remains as one of the most prominent and aggressive forms of cancer.

Many cancers develop as a result of defects in the apoptosis or programmed cell death pathway [7]. In the intrinsic pathway of apoptosis, various pro-apoptotic proteins act to permeabilize the mitochondria, dissipate mitochondrial membrane potential (MMP), and cause release of apoptogenic factors, such as cytochrome c (Cyt c), from the mitochondrial intermembrane space into the cytosol [8]. Once in the cytosol, apoptogenic factors can directly or indirectly execute apoptosis. As most cancers possess malfunctions in this pathway, much effort has been put into restoring or selectively activating this pathway for cancer therapy.

Pancratistatin (PST), a natural compound from the *Hymenocallis littoralis* plant, specifically induces apoptosis in numerous cancer cell lines [9, 10]. Contrasting with many chemotherapeutic agents, PST is non-genotoxic and does not exhibit adverse effects in non-cancerous cells [9, 10]. Nonetheless, the availability of PST for preclinical and clinical work is limited as it is only present in its

natural source in very low quantities and there have been great difficulties associated with its chemical synthesis. We have synthesized and tested several synthetic derivatives of 7-deoxypancratistatin and have found a C-1 acetoxymethyl derivative, JC-TH-acetate-4 (JCTH-4), which has similar efficacy and selective apoptosis inducing activity to that of PST [11].

Tamoxifen (TAM) is commonly administered to estrogen receptor (ER) positive breast cancer patients [12]. In such cases, TAM binds the ER to induce apoptosis by preventing estrogen binding, which normally activates pro-survival signalling pathways. However, other studies have provided evidence of estrogen receptor independent mechanisms of apoptotic induction by TAM [13, 14]. More specifically, TAM has been shown to interact with the flavin mononucleotide site of Complex I of the mitochondrial respiratory chain (MRC) [14]. Interestingly, our past work also indicates PST to act on a mitochondrial target [10]. We have previously exploited this mitochondrial targeting and demonstrated a sensitization by TAM to PST-induced apoptosis in ER positive and negative breast cancer and melanoma cell lines [15, 16]. Another recent report has also shown enhancement of cytotoxic effects of nelfinavir, an HIV drug, by TAM in an ER independent manner in breast cancer cells [17]. As TAM has exhibited capabilities in enhancing the cytotoxicity of certain anti-cancer agents in cancer cells, we wanted to evaluate the combinatory effects of TAM and JCTH-4.

This study analyzes the combinatory treatment of TAM and JCTH-4 in both p53 positive HCT 116 and p53 negative HT-29 human CRC cell lines. Our results show JCTH-4 to be effective in inducing apoptosis and autophagy in both cell lines through mitochondrial targeting. Interestingly, when JCTH-4 was administered with TAM, there was an enhancement in the aforementioned effects in both cell lines. Minimal toxicity by JCTH-4 alone and with TAM was exhibited in a normal human fetal fibroblast (NFF) and a normal colon fibroblast (CCD-18Co) cell line. Thus, this study presents a therapeutic window in combination therapy using a novel synthetic analogue of PST with a clinically available drug to rival current cancer therapies.

Materials and methods

Cell culture

The human CRC cells lines HT-29 and HCT 116 (ATCC, Cat. No. CCL-218 & CCL-247, Manassas, VA, USA) were grown and cultured with McCoy's Medium 5a (Gibco BRL, VWR, Mississauga, ON, Canada) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 10 mg/ml gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada). The apparently normal human fetal fibroblast

(NFF) cell line (Coriell Institute for Medical Research, Cat. No. AG04431B, Camden, NJ, USA) was grown in Dulbecco's Modified Eagle's Medium, High Glucose (Thermo Scientific, Waltham, MA, USA) supplemented with 15% (v/v) FBS and 10 mg/mL gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada). The normal colon fibroblast (CCD-18Co) cell line (ATCC, Cat. No. CRL-1459, Manassas, VA, USA) was cultured with Eagle's Minimal Essential Medium supplemented with 10% (v/v) FBS and 10 mg/mL gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada). All cells were grown at 37°C and 5% CO₂.

Cell treatment

Cells were grown to 60%–70% confluence and treated with pancratistatin (PST), tamoxifen (TAM) citrate salt (Sigma-Aldrich, Cat. No. T9262, Mississauga, ON, Canada), JC-TH-acetate-4 (JCTH-4), JC-TH-acid-1 (JCTH-1), and other synthetic 7-deoxypancratistatin analogues at the indicated concentrations and time points. The C-1 analogues were produced by chemoenzymatic synthesis from bromobenzene as previously described [11].

Nuclear staining

Following treatment and incubation with the aforementioned drugs, nuclei were stained with Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA). Post drug treatment and incubation, cells were incubated with 10 µM Hoechst 33342 dye for 5 min. Images were captured at 400× magnification on a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany).

Annexin V binding assay

Subsequent to drug treatment and incubation, the Annexin V binding assay was performed to confirm apoptotic induction. Post drug treatment and incubation, cells were washed twice using phosphate buffered saline (PBS) and resuspended and incubated in Annexin V binding buffer (10 mM HEPES, 10 mM NaOH, 140 mM NaCl, 1 mM CaCl₂, pH 7.6) with Annexin V AlexaFluor-488 (1:50) (Sigma-Aldrich, Mississauga, ON, Canada) for 15 min. Images were captured at 400× magnification on a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany).

WST-1 assay for cell viability

The WST-1 based colorimetric assay for cell viability, an indicator of active cell metabolism, was conducted according to the manufacturer's protocol (Roche Applied Science,

Indianapolis, IN, USA). 96-well clear bottom tissue culture plates were seeded with approximately 2.0×10^3 HT-29 or HCT 116 cells/well, 5.0×10^3 NFF cells/well, or 4.0×10^3 CCD-18Co cells/well and treated with JCTH-4 and TAM at the indicated concentrations and durations. Subsequent to drug treatment and incubation, the WST-1 reagent, which is cleaved to formazan by cellular enzymes, was added to each well and incubated for 4 h at 37°C. The metabolic product formazan was quantified by taking absorbance readings at 450 nm on a Wallac Victor³™ 1420 Multilabel Counter (PerkinElmer, Woodbridge, ON, Canada). Absorbance readings were expressed as percentages of the untreated control groups.

Tetramethylrhodamine methyl ester (TMRM) staining

Tetramethylrhodamine methyl ester (TMRM) (Gibco BRL, VWR, Mississauga, ON, Canada) was used to detect MMP. Cells were grown on coverslips and treated with the indicated doses of drugs for the indicated durations. Following drug incubation, cells were incubated with 200 nM TMRM for 45 min at 37°C. Images were captured at 400× magnification on a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany).

Mitochondrial isolation

Mitochondria were isolated from untreated HT-29 and HCT 116 cells as per a previously published protocol [15]. In brief, cells were washed two times with cold PBS and resuspended in hypotonic buffer (1 mM EDTA, 5 mM Tris-HCl, 210 mM mannitol, 70 mM sucrose, 10 µM Leu-pep, 10 µM Pep-A, 100 µM PMSF). Cells were homogenized manually and subsequently centrifuged at 600×g for 5 min at 4°C. The resulting supernatant was centrifuged at 15,000×g for 15 min at 4°C. Subsequently, the cytosolic supernatant was discarded and the mitochondrial pellet was resuspended in cold reaction buffer (2.5 mM malate, 10 mM succinate, 10 µM Leu-pep, 10 µM Pep-A, 100 µM PMSF in PBS).

Amplex Red assay

The levels of ROS produced were quantified with Amplex Red (Molecular Probes, Eugene, OR, USA). Equal amounts of isolated mitochondria suspended in cold reaction buffer were loaded into an opaque 96-well plate (20 µg of protein/well) with the indicated concentrations of drugs. Paraquat (PQ) (Sigma-Aldrich, Mississauga, ON, Canada) was used as a positive control at 250 µM. In each well, Amplex Red reagent was added to a final concentration of 50 µM and horseradish peroxidase (HRP) (Sigma-Aldrich, Mississauga, ON, Canada) was added in the ratio of 6 U/200 µL. Plates

were read at Ex. 560 nm and Em. 590 nm on a spectrofluorometer (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, CA, USA) in 10 min intervals for 4 h. Fluorescence readings were expressed as relative fluorescence units (RFU).

Cellular lysate preparation

CRC cells were treated for 72 h with the indicated concentrations of JCTH-4 and TAM. Treated cells were mechanically homogenized in cold hypotonic buffer (10 mM Tris HCl at pH 7.2, 5 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1% Triton-X-100, 10 μM Leu-pep, 10 μM Pep-A, 100 μM PMSF). Cell lysates were stored at -20°C until use.

Western blot analyses

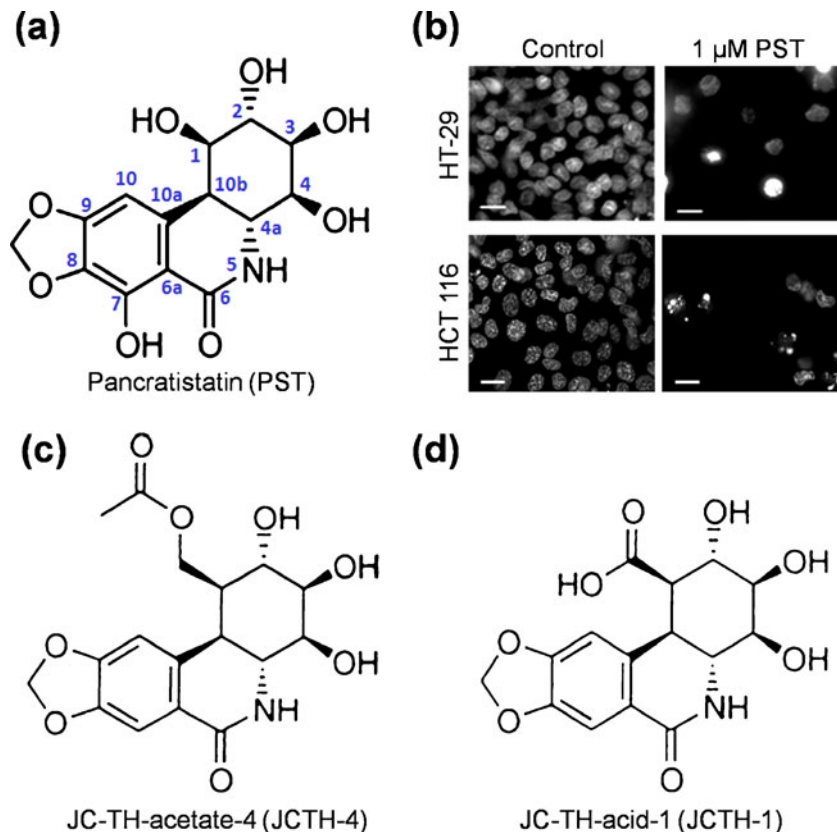
Protein samples (mitochondrial pellets, post mitochondrial supernatants, or cell lysates) were subjected to SDS-PAGE. Electrophoresed samples were transferred to a nitrocellulose membranes. Membranes were blocked with a 5% w/v milk TBST (Tris-Buffered Saline Tween-20) solution for 1 h. Membranes were probed with an anti-cytochrome c antibody (1:1000) (Abcam, Cat. No. ab13575, Cambridge, MA, USA), an anti-succinate dehydrogenase subunit A antibody (1:1000)

(Santa Cruz Biotechnology, Inc., Cat. No. sc-59687, Paso Robles, CA, USA), an anti-LC3 antibody (1:500) (Novus Biologicals, Cat. No. NB100-2220, Littleton, CO, USA), or an anti-β-Actin antibody (1:1000) (Santa Cruz Biotechnology, Inc., Cat. No. sc-81178, Paso Robles, CA, USA) overnight at 4°C. Subsequently, membranes were subjected to one 15 min and two 5 min washes in TBST and were incubated with an anti-mouse (1:2000) or an anti-rabbit (1:2000) horseradish peroxidase-conjugated secondary antibody (Abcam, Cat. No. ab6728 and ab6802, Cambridge, MA, USA) for 1 h at 25°C. Following three consecutive 5 min washes in TBST, bands were visualized with enhanced chemiluminescence reagent (Sigma-Aldrich, Cat. No. CPS160, Mississauga, ON, Canada). Densitometric analyses were performed using ImageJ software.

Z-VAD-FMK caspase inhibition

To determine the dependence of caspases in apoptosis induction, the broad spectrum caspase inhibitor Z-VAD-FMK was used. Approximately 2.0×10^3 HCT 116 cells were seeded in 96-well clear bottom tissue culture plates. After 24 h, cells were treated with 1 μM JCTH-4 alone and with 25 μM or 50 μM Z-VAD-FMK. Following 72 h, the WST-1 reagent was added to each well and incubated for 4 h at 37°C. Plates were read at 450 nm on a Wallac Victor³™ 1420

Fig. 1 PST induces apoptosis in CRC cells and structural comparison of PST to its synthetic 7-deoxy analogues. **a** Chemical structure of pancratistatin (PST). **b** Nuclear morphology of HCT 116 and HT-29 cells stained with Hoechst dye treated with 1 μM PST and the solvent control (Me₂SO) after 96 h. Scale bar=15 μm. **c** Chemical structure of JC-TH-acetate-4 (JCTH-4). **d** Chemical structure of JC-TH-acid-1 (JCTH-1)



Multilabel Counter (PerkinElmer, Woodbridge, ON, Canada). Absorbance readings were expressed as a percentage of untreated control groups.

Monodansylcadaverine (MDC) staining

Monodansylcadaverine (MDC) (Sigma-Aldrich, Mississauga, ON, Canada) staining was used to detect autophagic vacuoles. Cells were grown on coverslips and treated with the indicated doses of drugs for the indicated durations. Following drug incubation, cells were incubated with 0.1 mM MDC for 15 min. Images were captured at 400× magnification on a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany).

Long-term analysis on growth rate

To evaluate the long term effects of JCTH-4 and TAM on growth rate, cells were treated for 72 h at the indicated concentrations of drugs. Following drug incubation, cells were trypsinized and 2 plates per experimental group were seeded with approximately 5.0×10^5 live cells, quantified with Trypan Blue exclusion dye, in drug-free media. Subsequently, cells were trypsinized and the number of live cells was quantified using Trypan Blue exclusion dye after 48 and 96 h using 1 plate of cells per time point per experimental group.

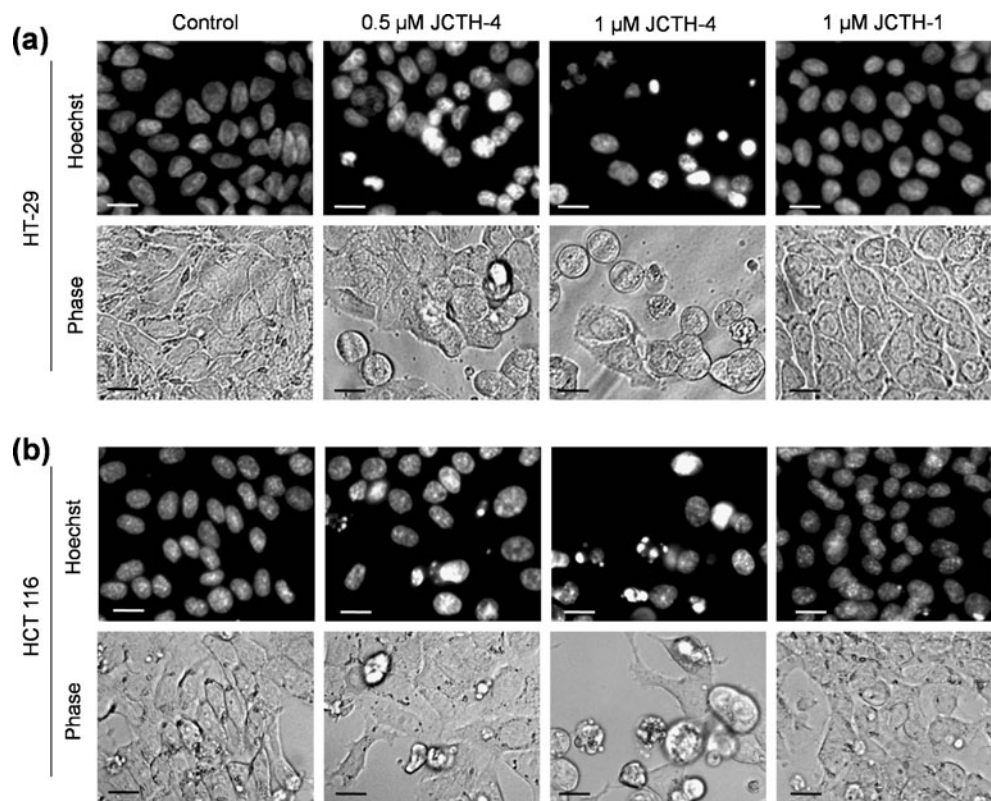
Results

JCTH-4 effectively induces apoptosis in human CRC cells

Natural PST (Fig. 1a) has been shown to induce apoptosis selectively in cancers cells [15, 16, 18]. As there are insufficient quantities of PST available for preclinical and clinical work, various synthetic analogues of PST were synthesized and screened for similar specific apoptosis inducing activity on both p53 positive HCT-116 and p53 negative HT-29 human CRC cell lines. As seen with Hoechst dye, 1 μ M natural PST after 96 h yielded condensed, brightly stained nuclei accompanied by apoptotic bodies, indicative of apoptosis in HT-29 and HCT 116 cells (Fig. 1b). Interestingly, one of the synthetic compounds, JCTH-4, was able to induce apoptosis with comparable efficacy to that of PST in both cell lines, while many other synthetic analogues (data not shown) of PST such as JCTH-1 did not (Fig. 2a,b).

The effect of JCTH-4 on both the HT-29 and HCT 116 cell lines was quantified using a WST-1 based colorimetric assay for cell viability, an indicator of active cell metabolism. JCTH-4 was able to reduce cell viability in both cell lines in a time and dose dependent manner and exhibited approximate half-maximal inhibitory concentration (IC_{50}) values of 1 μ M and 0.25 μ M in HT-29 and HCT 116 cells respectively after 96 h (Fig. 3a,b). Notably, JCTH-4 demonstrated selectivity

Fig. 2 JCTH-4 induces apoptosis in CRC cells. Nuclear morphology of **a** HT-29 and **b** HCT 116 cells stained with Hoechst dye treated with the indicated concentrations of JCTH-1 and JCTH-4 after 96 h. Control groups were treated with solvent (Me_2SO). Scale bar=15 μ m



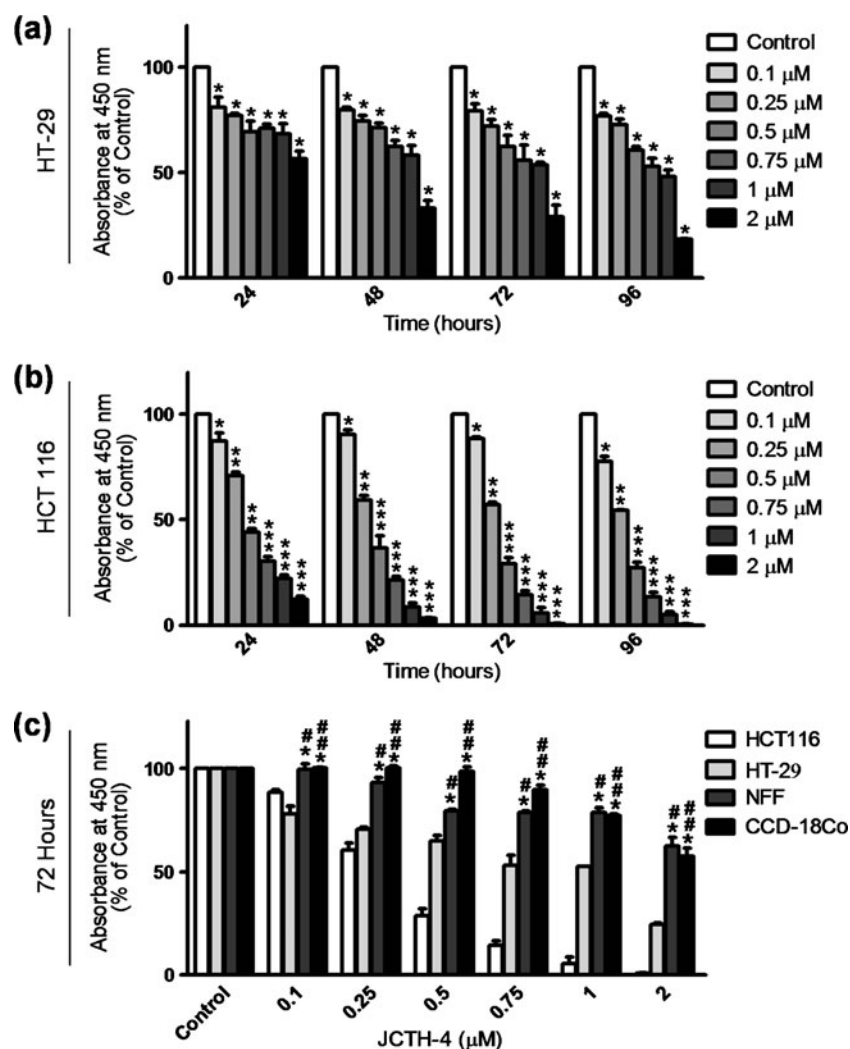


Fig. 3 JCTH-4 decreases viability of CRC cells in a time and dose-dependent manner. **a** 96-well plates were seeded with HT-29 cells and treated with JCTH-4 at concentrations ranging from 0.1 μM to 2 μM for 24, 48, 72, and 96 h. Subsequent to drug treatment and incubation, the WST-1 reagent was added to each well, the absorbance readings were taken at 450 nm, and expressed as a percentage of the control (Me_2SO). Statistics were performed using GraphPad Prism version 5.0. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. * p <0.005 versus control. **b** As performed with HT-29 cells, the WST-1 reagent was used to assess viability of

HCT 116 cells with the same concentrations of JCTH-4 and durations as previously described. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. * p <0.05, ** p <0.005, *** p <0.0005 versus control. **(c)** The WST-1 reagent was used to evaluate viability of NFF and CCD-18Co cells with the aforementioned concentrations of JCTH-4 after 72 h. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. Viability of HT-29 and HCT 116 cells presented correspond to 72 h of treatment with JCTH-4. * p <0.01 versus HT-29; # p <0.01, ### p <0.005 versus HCT 116

to CRC cell lines as non-cancerous normal human fetal fibroblasts (NFF) and normal colon fibroblasts (CCD-18Co) were significantly less sensitive compared to both CRC cell lines after 72 h (Fig. 3c).

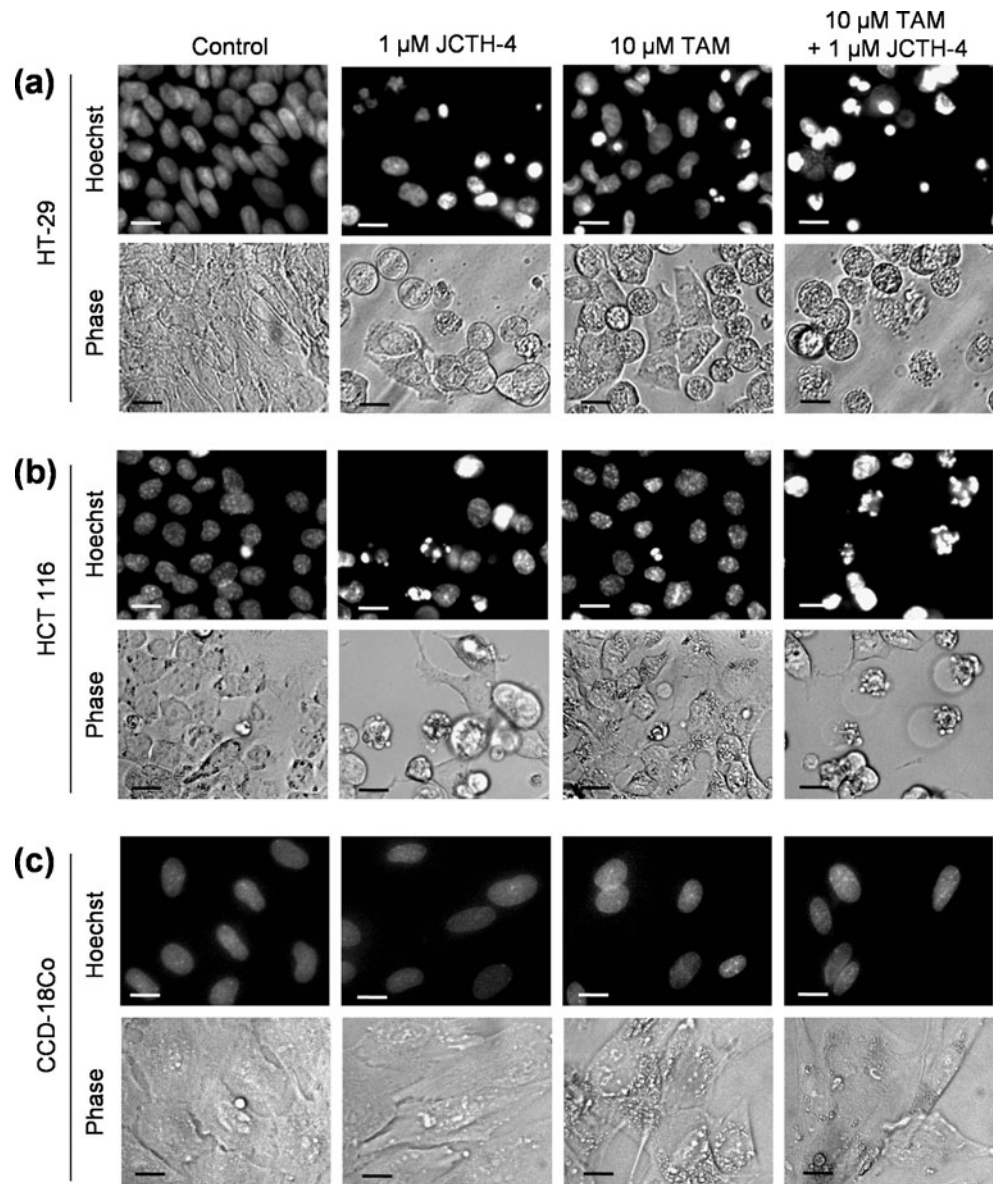
TAM enhances the apoptotic efficacy of JCTH-4 in CRC cells

We have previously shown PST and TAM to synergistically induce apoptosis in ER positive and negative breast cancer and melanoma cells [15, 16]. To investigate the potential of JCTH-4 to replicate the combinatorial effects of PST and

TAM, we treated HT-29 and HCT 116 cells with JCTH-4 and TAM at various doses. Enhanced induction of apoptosis with JCTH-4 was observed when administered with TAM as exhibited by Hoechst staining in both cell lines (Fig. 4a,b). No evident apoptosis induction was exhibited in CCD-18Co cells by JCTH-4 alone and in combination with TAM (Fig. 4c).

The WST-1 based colorimetric assay was used to quantify cell viability. This confirmed a greater decrease in viability of HCT 116 cells using 0.5 μM JCTH-4 with 5 μM and 10 μM TAM compared to 0.5 μM JCTH-4 alone, and a more prominent decrease in viability using 1 μM

Fig. 4 TAM enhances apoptosis-inducing activity of JCTH-4 selectively in CRC cells. Nuclear morphology of **a** HT-29, **b** HCT 116, and **c** CCD-18Co cells treated with the indicated concentrations of TAM and JCTH-4 for 96 h stained with Hoechst dye. Control groups were treated with solvent (Me_2SO). Scale bar=15 μm



JCTH-4 with 10 μM TAM compared to 1 μM JCTH-4 alone after 72 h (Fig. 5a). Similar results were observed with HT-29 cells (data not shown). After 72 h, the 1 μM JCTH-4 and 10 μM TAM combinational treatment was significantly less toxic to NFF (Fig. 5b) and CCD-18Co (Fig. 5c) cells.

Phosphatidylserine externalization, a biochemical marker of apoptosis, was detected by Annexin V binding to confirm apoptotic induction. JCTH-4 caused phosphatidylserine externalization indicated by the green fluorescence, confirming the induction of apoptosis in CRC cells (Fig. 6a, b). Such apoptotic induction was not exhibited in NFF cells (Fig. 6c). Thus, combinational treatment of JCTH-4 and TAM demonstrates cytotoxic selectivity towards CRC cell lines.

JCTH-4 and TAM target the mitochondria in HT-29 and HCT 116 cells

Although TAM is well characterized as an ER antagonist in the treatment of ER positive breast cancers, there have been studies which indicate that TAM can induce apoptosis independently of the estrogen receptor via mitochondrial targeting [14]. Moreover, we have previously provided evidence supporting the mitochondria as a target of PST and have shown TAM to strengthen the ability of PST to dissipate MMP and increase in ROS production [15, 16].

To assess the ability of JCTH-4 to dissipate MMP both alone and in combination with TAM, HT-29 and HCT 116 cells were treated for 96 h and stained with tetramethylrhodamine methyl ester (TMRM). Both 1 μM JCTH-4 and

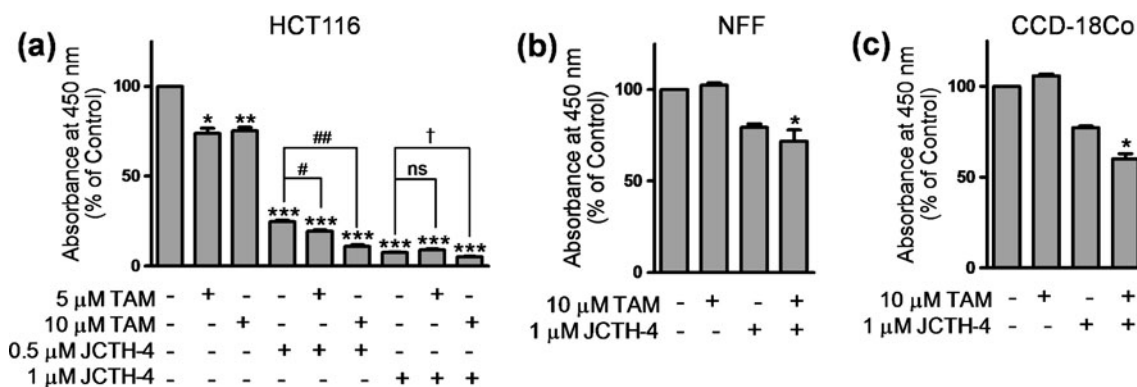


Fig. 5 TAM enhances viability decrease in CRC cells by JCTH-4. **a** As previously described, the WST-1 reagent was used to measure viability of HCT 116 cells treated with the indicated concentrations of TAM and JCTH-4 for 72 h. Absorbance readings were taken at 450 nm and expressed as a percentage of the control (Me_2SO). Statistics were performed using GraphPad Prism version 5.0. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. * p <0.05, ** p <0.005, *** p <0.0001 versus control; # p <0.05, ## p <0.005 versus 0.5 μ M JCTH-4; † p <0.05 versus 1 μ M JCTH-4. **b** The WST-1 reagent was used to measure viability of NFF

cells treated with the indicated concentrations of TAM and JCTH-4 for 72 h. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. * p <0.005 versus 10 μ M TAM+1 μ M JCTH-4 combinational treatment on HCT 116 cells (Fig. 5a). **c** The WST-1 reagent was used to measure viability of CCD-18Co cells treated with the indicated concentrations of TAM of JCTH-4 for 72 h. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. * p <0.005 versus 10 μ M TAM+1 μ M JCTH-4 combinational treatment on HCT 116 cells (Fig. 5a)

10 μ M TAM alone were able to dissipate MMP in both cell lines indicated by the decrease in positive TMRM staining; however, when used together, there was a greater dissipation in MMP (Fig. 7a,b). No evident dissipation in MMP was observed in CCD-18Co cells by JCTH-4 and TAM alone and in combination (Fig. 7c).

Increases in reactive oxygen species (ROS) production in mitochondria have been associated with both the cause and effect of mitochondrial membrane permeabilization, and the release of apoptogenic factors [19–21]. To assess the ability of JCTH-4 and TAM to increase generation of ROS in mitochondria directly, isolated mitochondria from HT-29 and HCT 116 cells were treated with 1 μ M JCTH-4 and 10 μ M TAM alone and in combination. ROS generation was monitored with Amplex Red dye over 4 h in 10 min intervals. Paraquat (PQ), a known inducer of mitochondrial ROS generation, was used as a positive control at 250 μ M [22]. In both cell lines, JCTH-4 and TAM alone caused a significant increase in ROS production compared to the control and with the combinational treatment the increase was significantly greater than both JCTH-4 or TAM alone in HT-29 and HCT 116 cells (Fig. 8a,b).

The release of apoptogenic factors such as Cyto c from the mitochondria following mitochondrial membrane permeabilization is important for apoptosis signalling and execution [23]. To assess the ability of both JCTH-4 and TAM to directly trigger release of Cyto c, isolated mitochondria from HT-29 cells were treated with the indicated concentrations of JCTH-4 and TAM for 2 h. Post treatment, the samples were centrifuged and western blot analyses were performed for Cyto c on the supernatants and succinate dehydrogenase

subunit A (SDHA) on the mitochondrial pellets. Both JCTH-4 and TAM alone were able to cause release of Cyto c but the effect was greatly enhanced with the combinatorial treatment, significantly releasing more Cyto c than both treatments alone (Fig. 9). Collectively these results are supportive of a mitochondrial target for both JCTH-4 and TAM.

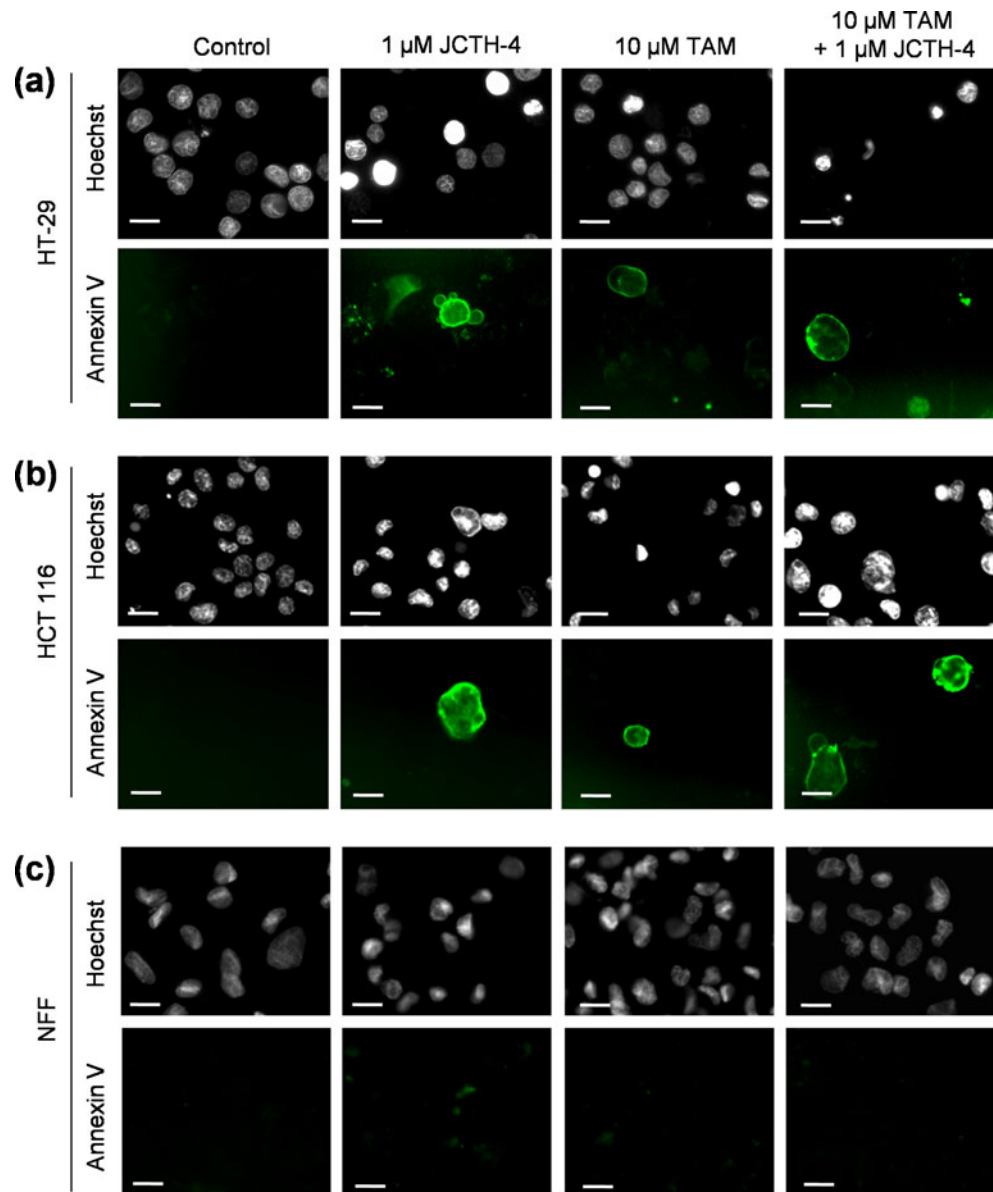
JCTH-4 induces apoptosis in a caspase-independent manner

Caspases are proteases well known for their participation in apoptosis signalling [24]. However, there are other pathways which are independent of these proteases. To determine the dependence on caspases in JCTH-4 induced apoptosis, HCT 116 cells were treated with JCTH-4 alone and in combination with the broad spectrum caspase inhibitor Z-VAD-FMK at 25 μ M and 50 μ M. Using the WST-1 based colorimetric assay to assess cell viability, there was no statistically significant difference observed in cell viability between the JCTH-4 treatment alone and the JCTH-4 treatment in combination with Z-VAD-FMK at both 25 μ M and 50 μ M, indicative of caspase independence in JCTH-4-induced apoptosis (Fig. 10).

JCTH-4 induces autophagy in HT-29 and HCT 116 cells

Autophagy, a process in which cells break down their own constituents, can be induced by various forms of cellular stress such as hypoxia, reactive oxygen species, protein aggregates, nutrient deprivation, growth factor deprivation, damaged organelles, and mutant proteins [25]. During this process, cytosolic material is taken up by autophagosomes, which are double-membraned vesicles that fuse to lyso-

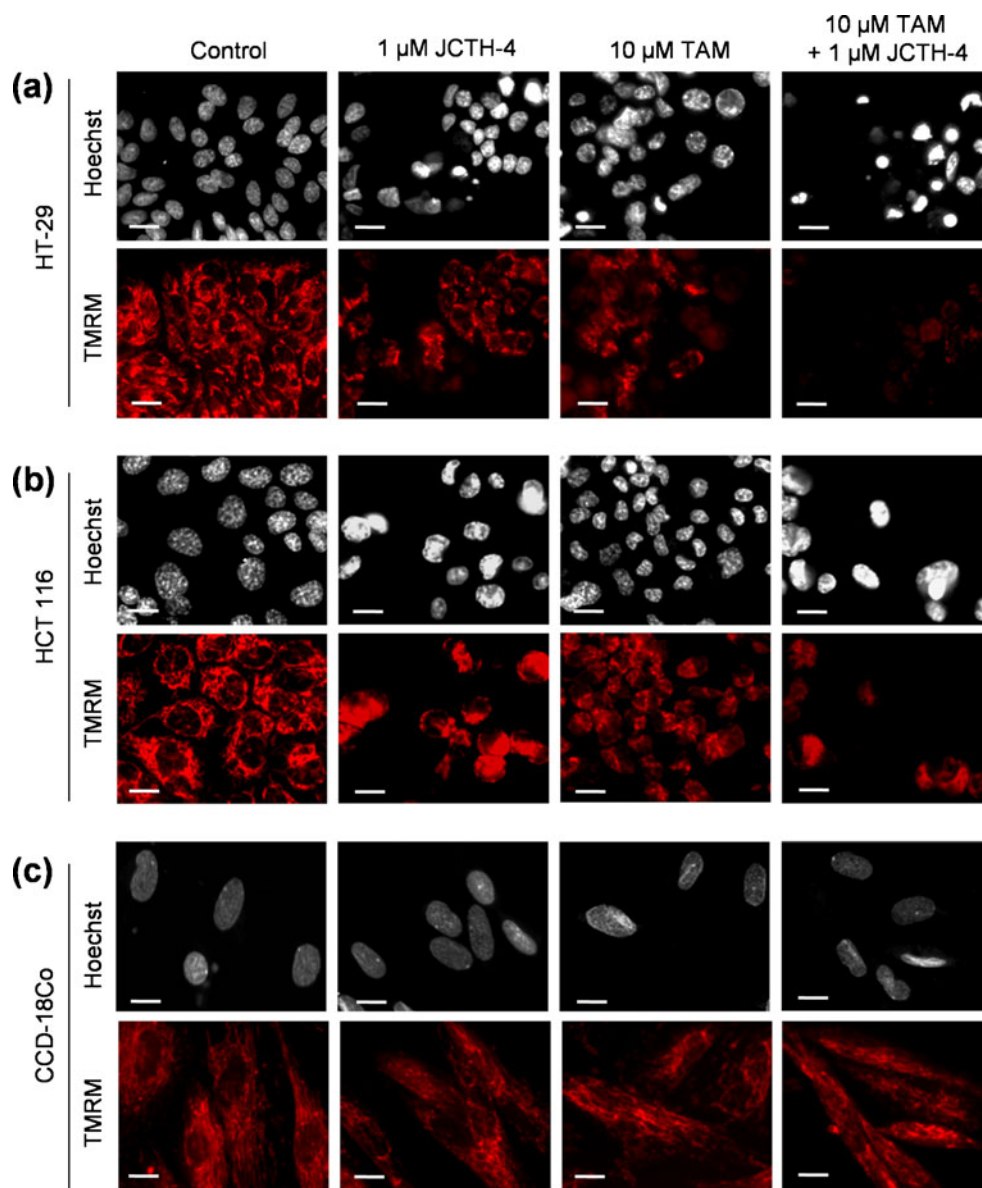
Fig. 6 JCTH-4 induces phosphatidylserine externalization alone and in combination with TAM in CRC cells. Annexin V binding to externalized phosphatidylserine was monitored to confirm apoptotic induction in **a** HT-29, **b** HCT 116, and **c** NFF cells treated with JCTH-4 and TAM for 72 h at the indicated concentrations. Control groups were treated with solvent (Me₂SO). Images were taken at 400× magnification on a fluorescent microscope. Scale bar=15 μm



somes to form autolysosomes. Subsequent to autolysosome formation, intra-autophagosomal contents are broken down by lysosomal enzymes [25]. To assess the induction of autophagy, HT-29 and HCT 116 cells were treated with 1 μM JCTH-4 and 10 μM TAM alone and in combination for 72 h and stained with monodansylcadaverine (MDC) (Fig. 11a,b). Bright punctate MDC staining was observed in JCTH-4 and in TAM treated HT-29 and HCT 116 cells, indicative of autophagosomes. Interestingly, the combinatorial treatment yielded the greatest induction of autophagosome formation, as indicated by the high intensity punctate MDC staining. However, after 72 h of treatment of NFF cells, minimal MDC staining was observed with JCTH-4, and bright punctate staining was observed with TAM (Fig. 11c). With the combinational treatment of JCTH-4 and TAM, NFF cells exhibited MDC staining intensity similar to that of TAM alone.

Upon autophagic induction, microtubule-associated protein 1 light chain 3 (LC3) localized in the cytosol, LC3-I, is conjugated to phosphatidylethanolamine, resulting in the lipidated protein LC3-II that is recruited to autophagosomal membranes [25]. Consequently, detection of LC3-II has been used as a marker of autophagic induction. To confirm the induction of autophagy, western blot analyses were performed using an anti-LC3 antibody on cell lysates from HT-29 and HCT 116 cells treated with the indicated concentrations of JCTH-4 and TAM for 72 h. Results confirmed that JCTH-4 induced autophagy in both CRC cell lines (Fig. 11d,e). Furthermore, the addition of TAM to JCTH-4 treatment enhanced the conversion of LC3-I to LC3-II. Therefore, these results indicate that JCTH-4 alone and in combination with TAM, triggers autophagic induction in CRC cell lines.

Fig. 7 JCTH-4 and TAM dissipate MMP in CRC cells. **a** HT-29, **b** HCT 116, and **c** CCD-18Co cells were grown on coverslips and treated with the indicated concentrations of drugs for 96 h and subsequently stained with TMRM to detect MMP. Control groups were treated with solvent (Me_2SO). Images were captured at $400\times$ magnification on a fluorescence microscope. Scale bar=15 μm



Long-term effect on human CRC cells post exposure to JCTH-4 and TAM

CRC is notorious for its aggressive nature, thus, the resilience of HT-29 and HCT 116 cells after exposure to 1 μM JCTH-4 and 10 μM TAM alone and in combination was examined. After the HT-29 and HCT 116 cells were initially exposed to the aforementioned treatments for 72 h, approximately 5.0×10^5 live cells were seeded in drug-free media and the number of live cells was monitored over an additional 96 h using Trypan Blue exclusion dye. As determined by quantifying the number of live cells, the cells initially treated with 1 μM JCTH-4 exhibited a significantly smaller growth rate in both the HT-29 and HCT 116 cells in drug-free media compared to the control group (Fig. 12a,b). The growth rate following drug removal after initial treatment was synergistically reduced in the

combination treatment group compared to both 1 μM JCTH-4 and 10 μM TAM alone in HT-29 and HCT 116 cells; however, the initial treatment of 10 μM TAM alone caused an increase in the growth rate in HCT 116 cells and had no significant effect on the growth rate in HT-29 cells after media replacement (Fig. 12a,b).

Discussion

There has been great difficulty in synthesizing PST (Fig. 1a). Many synthetic analogues of PST have been synthesized but demonstrated minimal anti-cancer activity [26, 27]. For the first time, we have demonstrated substantial cancer specific apoptosis inducing activity using a novel synthetic derivative of PST, JCTH-4, with comparable efficacy to that of PST, in

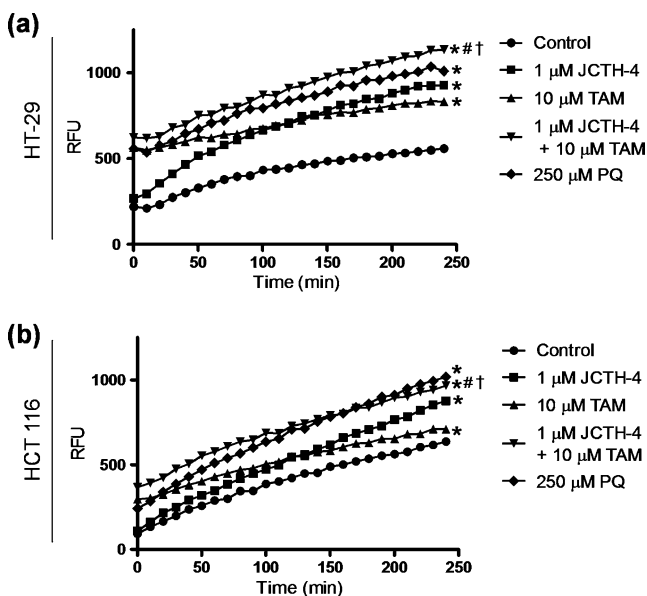


Fig. 8 JCTH-4 and TAM increase ROS generation in isolated mitochondria from CRC cells. Isolated mitochondria from **a** HT-29 and **b** HCT 116 cells were treated directly with JCTH-4 and TAM at the indicated concentrations and ROS was measured with Amplex Red substrate in presence of horseradish peroxidase (HRP). The control groups were treated with solvent (Me_2SO). Paraquat (PQ) was used at 250 μM as a positive control in both cell lines. Fluorescence readings were taken in 10 min intervals for 4 h at Ex. 560 nm and Em. 590 nm and expressed as relative fluorescence units (RFU). Statistics were performed using GraphPad Prism version 5.0. Images is representative of 3 independent experiments demonstrating similar trends. Values are expressed as mean \pm SD of quadruplicates of 1 independent experiment. * p <0.05 versus control; # p <0.05 versus 1 μM JCTH-4; † p <0.05 against 10 μM TAM

both p53 positive HCT 116 and p53 negative HT-29 human CRC cell lines. In both CRC cell lines, this C-1 acetoxymethyl derivative of 7-deoxypancratistatin was able to effectively induce apoptosis at 1 μM (Fig. 2a,b). NFF and CCD-18Co cells were significantly less sensitive to JCTH-4 than the CRC cells both alone (Fig. 3c) and in combination with TAM (Fig. 5a–c). Moreover, JCTH-4 alone and in combination with TAM did not yield any evident induction of apoptosis in NFF cells observed by Hoechst and Annexin V assays (Fig. 6c).

The various synthetic analogues of 7-deoxypancratistatin tested in this report are identical in structure with the exception of a varying functional group at C-1 (Fig. 1c,d). Specific anti-cancer activity was found only in JCTH-4 and not in the other synthetic analogues, such as JCTH-1. This suggests a critically specific binding of JCTH-4 to some particular protein domain or enzymatic pocket of a key cellular protein by the functional group at C-1. Furthermore, this study demonstrates an active compound in the PST series lacking the C-7 hydroxyl group, previously reported to mildly contribute to apoptotic activity, demonstrating the unimportance of this group to the structural

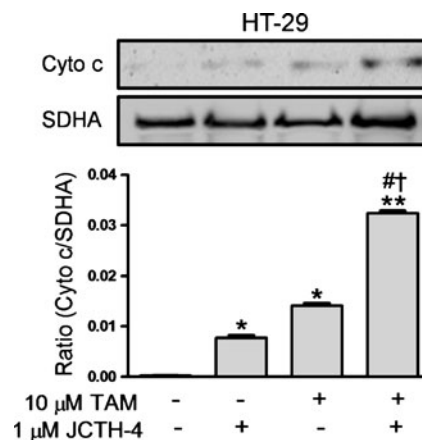


Fig. 9 JCTH-4 and TAM cause release of apoptogenic factor Cyto c from isolated mitochondria in CRC cells. Isolated mitochondria from HT-29 cells were directly treated with JCTH-4 and TAM at the indicated concentrations for 2 h. The control group was treated with solvent (Me_2SO). Following drug incubation, samples were resuspended and centrifuged at 15,000 \times g for 15 min at 4°C. Subsequently, western blot analyses were performed for Cyto c on the resultant supernatants and SDHA on the resultant mitochondrial pellets resuspended in reaction buffer. Densitometric analyses were performed using ImageJ software. Statistics were performed using GraphPad Prism version 5.0. Values are expressed as mean \pm SD. * p <0.005, ** p <0.0005 versus control; # p <0.005 versus 1 μM JCTH-4; † p <0.0005 versus 10 μM TAM

minimum pharmacophore for cancer specific cytotoxicity [26]. The minimum pharmacophore essential for specific anti-cancer activity suggests a specific target and mode of action of JCTH-4, as opposed to the non-specific binding to various cellular targets exhibited by many current chemotherapeutics. This report provides evidence of specific mitochondrial targeting.

The classification of mitocans collectively includes anti-cancer agents which directly or indirectly target mitochon-

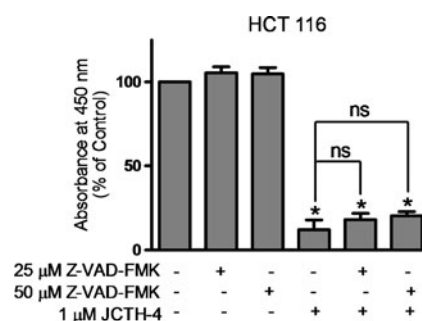


Fig. 10 JCTH-4 induces apoptosis in CRC cells independent of caspases. To determine the dependence of caspases in the induction of apoptosis by JCTH-4, HCT 116 cells were treated with JCTH-4 alone and in combination with the broad spectrum caspase inhibitor Z-VAD-FMK at the indicated concentrations. After 72 h, the WST-1 reagent was used to evaluate cell viability as previously described. Absorbance readings were taken at 450 nm and expressed as a percentage of the control (Me_2SO). Statistics were performed using GraphPad Prism version 5.0. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. * p <0.005 versus control

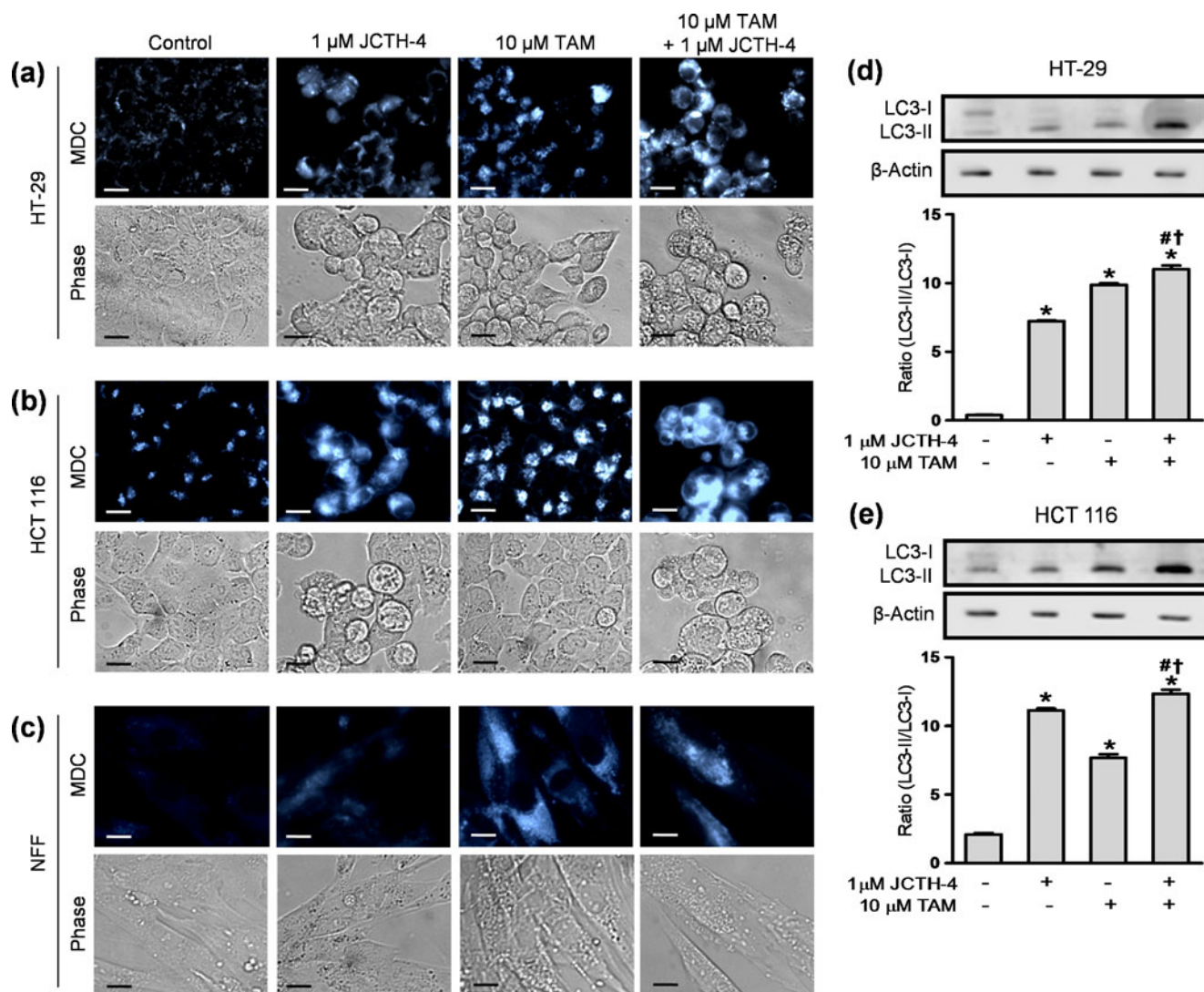


Fig. 11 JCTH-4 and TAM induce autophagy in CRC cells. Cells were grown on coverslips and treated with JCTH-4 and TAM at the indicated concentrations. The control group was treated with solvent (Me_2SO). After 72 h, **a** HT-29, **b** HCT 116, and **c** NFF cells were stained with MDC to detect autophagic vacuoles. Images were captured at 400 \times magnification on a fluorescence microscope. Scale bar=15 μm . Western blot analyses were performed for LC3 and β -Actin on cell lysates of CRC cells treated for 72 h with the indicated

concentrations of JCTH-4 and TAM. Densitometric analyses were performed using ImageJ software. Statistics were performed using GraphPad Prism version 5.0. Values are expressed as mean \pm SD. **d** HT-29: * p <0.0005 versus control; # p <0.005 versus 1 μM JCTH-4; † p <0.005 versus 10 μM TAM. **e** HCT 116: * p <0.0005 versus control; # p <0.05 versus 1 μM JCTH-4; † p <0.005 versus 10 μM TAM

drial functioning [28]. Both PST and JCTH-4 fall under this classification. Our previous reports provide evidence of mitochondrial targeting by PST [10, 15]. Similarly, this report presents data indicative of mitochondrial targeting by JCTH-4 in CRC cell lines. JCTH-4 was able to dissipate MMP (Fig. 7a,b), increase generation of reactive oxygen species in isolated mitochondria (Fig. 8a,b), and cause release the apoptogenic factor Cyto c from isolated mitochondria (Fig. 9) in both HCT 116 and HT-29 cells. Furthermore, the broad spectrum caspase inhibitor Z-VAD-FMK was unable to prevent JCTH-4-induced apoptosis (Fig. 10). This supports the action of JCTH-4 to be at the

level of mitochondrial destabilization, upstream of executioner caspase activation, to cause release of various apoptogenic factors involved in both caspase dependent and independent pathways of apoptotic induction.

As the Bcl-2 family of proteins serves crucial regulatory roles in apoptosis, there has been a growing interest in the development of small molecules capable of directly binding to and modifying the activity of Bcl-2 family proteins, such as the pro-apoptotic protein Bax [29]. However, we have evidence to rule out such direct binding to Bax specifically by PST and JCTH-4. We have previously stably transfected and expressed anti-Bax single-domain antibodies (sdAbs)

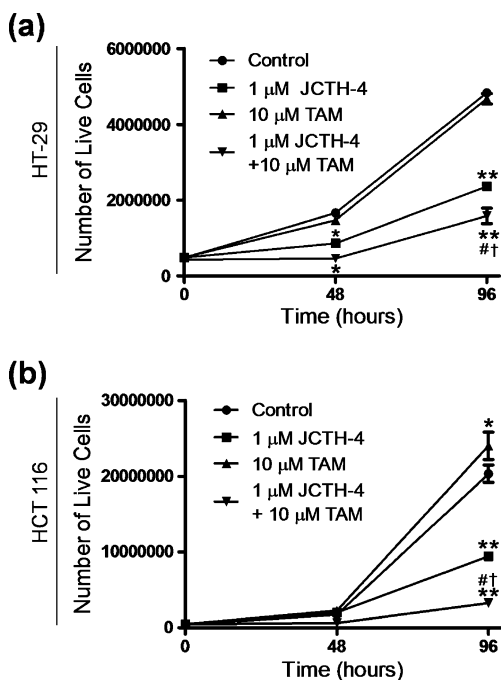


Fig. 12 Initial JCTH-4 treatment reduces resilience of CRC cells after drug removal. **a** Subsequent to 72 h of treatment with JCTH-4 and TAM at the indicated concentrations, approximately 5.0×10^5 live HT-29 cells were seeded in drug-free media and the number of live cells was counted after 48 and 96 h using Trypan Blue exclusion dye. Statistics were performed using GraphPad Prism version 5.0. Values are expressed as mean \pm SD ($n=3$). * $p < 0.005$, ** $p < 0.0005$ versus control (Me_2SO); # $p < 0.05$ versus 1 μM JCTH-4; † $p < 0.05$ versus 10 μM TAM. **b** The aforementioned procedure was performed with HCT 116 cells. Statistics were performed using GraphPad Prism version 5.0. Values are expressed as mean \pm SD ($n=3$). ** $p < 0.01$, *** $p < 0.005$ versus control (Me_2SO); # $p < 0.05$ versus 1 μM JCTH-4; † $p < 0.05$ versus 10 μM TAM

in human neuroblastoma cells (SH-SY5Y) capable of blocking Bax function [30]. These sdAbs however, were unable to prevent PST-induced apoptosis in the SH-SY5Y cells [18]. Moreover, the tumor suppressor p53 functions as a transcription factor for the pro-apoptotic proteins Bax, Noxa, and PUMA and also indirectly activates Bim, which all act to permeabilize the mitochondrial membrane to induce apoptosis [31–34]. In many aggressive cancers however, p53 is often mutated giving rise to resistance to many chemotherapeutics. Interestingly, both PST and JCTH-4 effectively induced apoptosis in both p53 positive and p53 negative human CRC cell lines. This suggests these compounds are capable of bypassing malfunctions in p53 and in a manner independent of p53 downstream proteins such as Bax.

In treating ER positive breast cancer, TAM is well known as an ER antagonist. In such circumstances, TAM interferes with estrogen binding to the ER which inhibits various signalling cascades resulting in apoptosis. The ER receptor was also found to be expressed in CRC cells. The CRC cell lines HT-29 and HCT 116 are both positive for

ER beta and in HT-29 cells in particular, previous work has proven TAM to be effective in inhibiting cell proliferation and inducing apoptosis [35, 36]. Independent of the ER, former studies have suggested TAM to act on Complex I of the MRC at the flavin mononucleotide site. Accordingly, we have demonstrated TAM to increase ROS generation (Fig. 8a,b) and cause release of Cyto c (Fig. 9) in isolated mitochondria from HT-29 and HCT 116 cells. As a proof-of-concept of mitochondrial targeting in both TAM and JCTH-4, combinatorial treatment with these compounds yielded greater increase in ROS production (Fig. 8a,b) and greater release of Cyto c (Fig. 9) in isolated mitochondria from HT-29 and HCT 116 cells than either of these compounds alone. Consequently, the enhanced mitochondrial targeting in these cell lines is most likely responsible for the greater apoptotic induction (Fig. 4a,b) and MMP dissipation (Fig. 7a,b) with the combinatorial treatment of TAM with JCTH-4. Furthermore, when these treatments were removed after 72 h of incubation, the HT-29 and HCT 116 cells initially treated with both 1 μM JCTH-4 and 10 μM TAM exhibited a synergistically reduced growth rate compared to both 1 μM JCTH-4 and 10 μM TAM alone in drug-free media (Fig. 12a,b). Notably, the CRC cells initially treated with 1 μM JCTH-4 alone grew much slower than the control group in drug-free media. However, 10 μM TAM alone had no significant effect on growth rate post-drug incubation in HT-29 cells and caused an increase in growth rate in HCT 116 cells after drug removal. Although previous work has shown TAM to exert moderate cytotoxicity against CRC cells at higher doses, TAM treatment alone at 10 μM appears to be ineffective and potentially detrimental in treating CRC unless utilized with JCTH-4 [35].

Consistent to the findings in this report, our previous work has shown TAM to sensitize ER positive and negative breast cancer and melanoma cell lines to PST-induced cell death via mitochondrial targeting independent of estrogen receptors [15, 16]. As we have shown specific mitochondrial targeting in cancer cells with PST and JCTH-4, there are numerous factors that may render cancer cells vulnerable to such targeting. Most cancer cells rely predominantly on glycolysis as the main source of energy production regardless of the abundance of oxygen present in the surrounding environment [37]. Furthermore, studies have proven cancer cells to exhibit higher levels of ROS which have been attributed to mitochondrial DNA (mtDNA) mutations in multiple types of cancers [38–41]. MRC complexes are encoded in mtDNA, and thus, mutations in these genes could give rise to errors in electron transport, and subsequent electron leakage, superoxide generation [42, 43]. Moreover, cancer cells possess elevated mitochondrial transmembrane potential, which can act to

supplement the generation of ROS by the MRC [28]. Increased levels of ROS have also been linked to the establishment aggressive and advanced malignancies [44, 45]. However, if the levels of ROS surpass the antioxidant capabilities of the cell, cytotoxicity and cell death may result [46]. Thus, it is possible for cancer cells to be more sensitive to compounds that abolish cellular antioxidant activity or promote ROS production.

The permeability transition pore (PTP) is a complex spanning the mitochondrial membranes and is proposed to be composed of multiple proteins including adenine nucleotide translocase (ANT), voltage-dependent anion channel (VDAC), and cyclophilin D (CypD). Under normal physiological conditions, small molecules are permitted in and out of the mitochondria via the PTP to maintain mitochondrial homeostasis. In the presence of apoptotic stimuli, the PTP can give rise to dissipation of MMP, ATP depletion, and expulsion of apoptogenic factors resulting in apoptosis [47]. Proteins associated with the PTP, which play roles in the regulation of its opening, are peripheral benzodiazepine receptor (PBR), creatine kinase (CK), and hexokinase (HK) [48]. In various types of cancer, PBR, HK, and CK have all been reported to be over-expressed [49–51]. Additionally, ANT, VDAC, and CypD are more abundant in malignant tissues and have been associated with inhibition of apoptosis [52–54]. Consequently, these mitochondrial abnormalities may serve as specific targets that can be exploited by PST and JCTH-4 to specifically induce apoptosis in cancer cells.

Autophagy exists as a stress response, allowing cells to survive under harsh environments [25]. However, extensive degradation of intracellular contents by this process can lead to autophagic cell death [55]. Recently, induction of autophagy has been implicated in both killing cancer cells, via autophagic cell death, as well as protecting cancer cells against chemotherapy [55]. Interestingly, TAM has been shown to induce autophagy in various cancer cell types [55]. We found strong autophagic induction in CRC (Fig. 11a,b,d,e) and NFF (Fig. 11c) cells treated with TAM but found minimal cell death (Fig. 11a–c). This may indicate that autophagic induction by TAM is a protective response in CRC and NFF cells. We also observed that JCTH-4 induced autophagy in CRC cells (Fig. 11a,b,d,e) with significant induction of apoptosis (Fig. 6a,b). Activation of autophagy in NFF cells however, appeared to be very minimal by JCTH-4 (Fig. 11c). As described previously, JCTH-4 causes mitochondrial dysfunction that leads to apoptosis; however, in response to mitochondrial dysfunction and oxidative stress, these CRC cells may trigger autophagy as a default mechanism. When JCTH-4 and TAM are used together, there was an enhanced induction of autophagy in CRC cells (Fig. 11a,b,d,e). More interestingly, the protective autophagic response induced by TAM

becomes lethal in the presence of JCTH-4, causing autophagic/apoptotic cell death (Figs. 4a,b, 5a, 6a,b, 11a,b,d,e). However, since mitochondria are permeabilized, the CRC cells ultimately undergo apoptosis. Such pathological autophagic processes were not observed in the NFF cell line as shown with MDC and bright field pictures (Fig. 11c). As TAM has been established to target complex I of the MRC, it can be hypothesized that such targeting can lead to the generation of oxidative stress which is insufficient to destabilize the mitochondria but sufficient to produce a protective autophagic response [14]. Nevertheless, such oxidative stress generated by TAM may act to sensitize cancer cell mitochondria to JCTH-4-induced dysfunction and permeabilization, ultimately yielding apoptosis. As both TAM and JCTH-4 are able to generate oxidative stress (Fig. 8a,b), the combinatorial production of such stress by both compounds may give rise to extensive activation of the autophagic pathway to ultimately yield a detrimental response.

In conclusion, we present a novel derivative of PST, JCTH-4, created by de novo synthesis, capable of specifically inducing apoptosis in human CRC cell lines via mitochondrial targeting. As a synthetic derivative of PST demonstrating similar efficacy and specificity towards cancer cells has been discovered, previous limitations on PST, including its low availability in its natural source and difficulties in its chemical synthesis, have been overcome. Moreover, TAM was able to enhance the efficacy of JCTH-4 and induce a pathological autophagic response in CRC cells in the presence of JCTH-4. Therefore, the novel synthetic compound JCTH-4 may serve as a safer and more effective alternative, both alone and in combination with TAM, to the chemotherapeutics currently available.

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References

1. Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. *CA Cancer J Clin* 55:74–108
2. Murphy JE, Ryan DP (2010) American Society of Clinical Oncology 2010 colorectal update. *Expert Rev Anticancer Ther* 10(9):1371–1373
3. de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, Cassidy J, Boni C, Cortes-Funes H, Cervantes A, Freyer G, Papamichael D, Le Bail N, Louvet C, Hendl D, de Braud F, Wilson C, Morvan F, Bonetti A (2000) Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol* 18:2938–2947

4. Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L, Rougier P (2000) Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 355:1041–1047
5. Maindrault-Goebel F, Louvet C, André T, Carola E, Lotz JP, Molitor JL, Garcia ML, Gilles-Amar V, Izrael V, Krulik M, de Gramont A (1999) Oxaliplatin added to the simplified bimonthly leucovorin and 5-fluorouracil regimen as second-line therapy for metastatic colorectal cancer (FOLFOX6): GERCOR. *Eur J Cancer* 35:1338–1342
6. André T, Louvet C, Maindrault-Goebel F, Couteau C, Mabro M, Lotz JP, Gilles-Amar V, Krulik M, Carola E, Izrael V, de Gramont A (1999) CPT-11 (irinotecan) addition to bimonthly, high-dose leucovorin and bolus and continuous-infusion 5-fluorouracil (FOLFIRI) for pretreated metastatic colorectal cancer: GERCOR. *Eur J Cancer* 35:1343–1347
7. Borst P, Rottenberg S (2004) Cancer cell death by programmed necrosis? *Drug Resist Updat* 7(6):321–324 [Epub 2005 Jan 11]
8. Earnshaw WC (1999) Apoptosis. A cellular poison cupboard. *Nature* 397(6718):387–389
9. Kekre N, Griffin C, McNulty J, Pandey S (2005) Pancreatistatin causes early activation of caspase-3 and the flipping of phosphatidyl serine followed by rapid apoptosis specifically in human lymphoma cells. *Cancer Chemother Pharmacol* 56(1):29–38
10. McLachlan A, Kekre N, McNulty J, Pandey S (2005) Pancreatistatin: a natural anti-cancer compound that targets mitochondria specifically in cancer cells to induce apoptosis. *Apoptosis* 10(3):619–630
11. Collins J, Rinner U, Moser M, Hudlicky T, Ghiviriga I, Romero AE, Kornienko A, Ma D, Griffin C, Pandey S (2010) Chemoenzymatic synthesis of Amariyllidaceae constituents and biological evaluation of their C-1 analogues. The next generation synthesis of 7-deoxypancratistatin and trans-dihydrolycoricidine. *J Org Chem* 75(9):3069–3084
12. Baum M (2005) Adjuvant endocrine therapy in postmenopausal women with early breast cancer: where are we now? *Eur J Cancer* 41:1667–1677
13. Mandlekar S, Kong ANT (2001) Mechanisms of Tamoxifen induced apoptosis. *Apoptosis* 6:469–477
14. Moreira P, Custodio J, Morena A, Oliveira C, Santos M (2006) Tamoxifen and estradiol interact with the flavin mononucleotide site of complex I leading to mitochondrial failure. *J Biol Chem* 281:10143–10152
15. Siedlakowski P, McLachlan-Burgess A, Griffin C, Tirumalai SS, McNulty J, Pandey S (2007) Synergy of pancreatistatin and tamoxifen on breast cancer cells in inducing apoptosis by targeting mitochondria. *Cancer Biol Ther* 7(3):376–384
16. Chatterjee SJ, McNulty J, Pandey S (2010) Sensitization of human melanoma cells by tamoxifen to apoptosis induction by pancreatistatin, a nongenotoxic natural compound. *Melanoma Res* 2010 Mar 17. [Epub ahead of print]
17. Bruning A, Friese K, Burges A, Mylonas I (2010) Tamoxifen enhances the cytotoxic effects of nelfinavir in breast cancer cells. *Breast Cancer Res* 12:R45
18. Griffin C, Karnik A, McNulty J, Pandey S (2011) Pancreatistatin selectively targets cancer cell mitochondria and reduces growth of human colon tumor xenografts. *Mol Cancer Ther* 10(1):57–68
19. Madesh M, Hajnóczky G (2001) VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. *J Cell Biol* 155(6):1003–1015 [Epub 2001 Dec 10]
20. Simon HU, Haj-Yehia A, Levi-Schaffer F (2000) Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 5(5):415–418
21. Batandier C, Leverve X, Fontaine E (2004) Opening of the mitochondrial permeability transition pore induces reactive oxygen species production at the level of the respiratory chain complex I. *J Biol Chem* 279(17):17197–17204 [Epub 2004 Feb 11]
22. Cochemé HM, Murphy MP (2008) Complex I is the major site of mitochondrial superoxide production by paraquat. *J Biol Chem* 283(4):1786–1798 [Epub 2007 Nov 26]
23. Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* 281:1309–1312
24. Stennicke HR, Salvesen GS (1999) Catalytic properties of the caspases. *Cell Death Differ* 6(11):1054–1059
25. Kroemer G, Mariño G, Levine B (2010) Autophagy and the integrated stress response. *Mol Cell* 40(2):280–293
26. McNulty J, Nair JJ, Griffin C, Pandey S (2008) Synthesis and biological evaluation of fully functionalized seco-pancratistatin analogues. *J Nat Prod* 71(3):357–363
27. McNulty J, Larichev L, Pandey S (2005) A synthesis of 3-deoxydihydrocoricidine: refinement of a structurally minimum Pancreatistatin pharmacophore. *Bioorg Med Chem Lett* 15:5315–5318
28. Chen G, Wang F, Trachootham D, Huang P (2010) Preferential killing of cancer cells with mitochondrial dysfunction by natural compounds. *Mitochondrion* 2010 Aug. 14 [Epub ahead of print]
29. Leber B, Geng F, Kale J, Andrews DW (2010) Drugs targeting Bcl-2 family members as an emerging strategy in cancer. *Expert Rev Mol Med* 12:e28
30. Gueorguieva D, Li S, Walsh N, Mukerji A, Tanha J, Pandey S (2006) Identification of singledomain, Bax-specific intrabodies that confer resistance to mammalian cells against oxidative-stress-induced apoptosis. *FASEB J* 20(14):2636–2638
31. Miyashita T, Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80(2):293–299
32. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 288:1053–1058
33. Nakano K, Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 7:683–694
34. Puthalakath H, Strasser A (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* 9:505–512
35. Fang YJ, Pan ZZ, Li LR, Lu ZH, Zhang LY, Wan DS (2009) MMP7 expression regulated by endocrine therapy in ERbeta-positive colon cancer cells. *J Exp Clin Cancer Res* 28:132
36. Janakiram NB, Steele VE, Rao CV (2009) Estrogen receptor-beta as a potential target for colon cancer prevention: chemoprevention of azoxymethane-induced colon carcinogenesis by raloxifene in F344 rats. *Cancer Prev Res Phila* 2(1):52–59
37. Warburg O (1956) On the origin of cancer cells. *Science* 123(3191):309–314
38. Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 51(3):794–798
39. Carew JS, Zhou Y, Albitar M, Carew JD, Keating MJ, Huang P (2003) Mitochondrial DNA mutations in primary leukemia cells after chemotherapy: clinical significance and therapeutic implications. *Leukemia* 17(8):1437–1447
40. Indo HP, Davidson M, Yen HC, Suenaga S, Tomita K, Nishii T, Higuchi M, Koga Y, Ozawa T, Majima HJ (2007) Evidence of ROS generation by mitochondria in cells with impaired electron transport chain and mitochondrial DNA damage. *Mitochondrion* 7(1–2):106–118
41. Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H, Nakada K, Honma Y, Hayashi J (2008) ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science* 320(5876):661–664

42. Adam-Vizi V, Chinopoulos C (2006) Bioenergetics and the formation of mitochondrial reactive oxygen species. *Trends Pharmacol Sci* 27(12):639–645
43. Brandon M, Baldi P, Wallace DC (2006) Mitochondrial mutations in cancer. *Oncogene* 25(34):4647–4662
44. Patel BP, Rawal UM, Dave TK, Rawal RM, Shukla SN, Shah PM, Patel PS (2007) Lipid peroxidation, total antioxidant status, and total thiol levels predict overall survival in patients with oral squamous cell carcinoma. *Integr Cancer Ther* 6(4):365–372
45. Kumar B, Koul S, Khandrika L, Meacham RB, Koul HK (2008) Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. *Cancer Res* 68(6):1777–1785
46. Fruehauf JP, Meyskens FL Jr (2007) Reactive oxygen species: a breath of life or death? *Clin Cancer Res* 13(3):789–794
47. Berridge MV, Herst PM, Lawen A (2009) Targeting mitochondrial permeability in cancer drug development. *Mol Nutr Food Res* 53(1):76–86
48. Halestrap AP (2009) What is the mitochondrial permeability transition pore? *J Mol Cell Cardiol* 46(6):821–831
49. Corsi L, Geminiani E, Baraldi M (2008) Peripheral benzodiazepine receptor (PBR) new insight in cell proliferation and cell differentiation review. *Curr Clin Pharmacol* 3(1):38–45
50. Meffert G, Gellerich FN, Margreiter R, Wyss M (2005) Elevated creatine kinase activity in primary hepatocellular carcinoma. *BMC Gastroenterol* 5:9
51. Palmieri D, Fitzgerald D, Shreeve SM, Hua E, Bronder JL, Weil RJ, Davis S, Stark AM, Merino MJ, Kurek R, Mehdorn HM, Davis G, Steinberg SM, Meltzer PS, Aldape K, Steeg PS (2009) Analyses of resected human brain metastases of breast cancer reveal the association between up-regulation of hexokinase 2 and poor prognosis. *Mol Cancer Res* 7(9):1438–1445
52. Kim GJ, Chandrasekaran K, Morgan WF (2006) Mitochondrial dysfunction, persistently elevated levels of reactive oxygen species and radiation-induced genomic instability: a review. *Mutagenesis* 21(6):361–367
53. Pedersen PL (2008) Voltage dependent anion channels (VDACs): a brief introduction with a focus on the outer mitochondrial compartment's roles together with hexokinase-2 in the “Warburg effect” in cancer. *J Bioenerg Biomembr* 40(3):123–126
54. Chen G, Izzo J, Demizu Y, Wang F, Guha S, Wu X, Hung MC, Ajani JA, Huang P (2009) Different redox states in malignant and nonmalignant esophageal epithelial cells and differential cytotoxic responses to bile acid and honokiol. *Antioxid Redox Signal* 11(5):1083–1095
55. Dalby KN, Tekedereli I, Lopez-Berestein G, Ozpolat B (2010) Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. *Autophagy* 6(3):322–329 [Epub 2010 Apr 26]