Coupling G2/M arrest to the Wnt/ β -catenin pathway restrains pancreatic adenocarcinoma

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Abstract

β -catenin plays a pivotal role in organogenesis and oncogenesis. Alterations in β -catenin expression are common in pancreatic cancer, which is an extremely aggressive malignancy with a notably poor prognosis. In this report, we analyzed the apoptotic activity of withanolide-D (witha-D), a steroidal lactone that was purified from an Indian medicinal plant, *Withania somnifera*, and its underlying mechanism of action. Witha-D induced apoptosis in pancreatic ductal adenocarcinoma cells by prompting cell-cycle arrest at the G2/M phase. This lactone abrogated β -catenin signaling in these cells regardless of disease grade, mutational status, and gemcitabine sensitivity. Witha-D also upregulated E-cadherin in most cells, thereby supporting the inversion of the epithelial–mesenchymal transition. Furthermore, the Akt/Gsk3 β kinase cascade was identified as a critical mediator of G2/M regulation and β -catenin signaling. Witha-D deactivated Akt, which failed to promote Gsk3 β deactivation phosphorylation. Consequently, activated Gsk3 β facilitated β -catenin destruction in pancreatic carcinoma cells. The knockdown of Chk1 and Chk2 further activated Akt and reversed the molecular signal. Taken together, the results of the current study represent the first evidence of β -catenin signal crosstalk during the G2/M phase by functionally inactivating Akt via witha-D treatment in pancreatic cancer cells. In conclusion, this finding suggests the potential identification of a new lead molecule in the treatment of pancreatic adenocarcinoma.

Key Words

- β -catenin
- Chk1/Chk2
- EMT
- G2/M
- pancreatic adenocarcinoma
- Wnt signaling

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Introduction

Aberrant Wnt/ β -catenin signaling is often observed in various cancers (Polakis 2000, Moon *et al.* 2002, Lustig & Behrens 2003). β -catenin is a ubiquitously expressed protein with multiple functions. Initially, this protein was known for its function in intercellular adhesion structures known as adherens junctions. Later, this protein was also found to act as a signaling molecule and cotranscription factor in the Wnt signaling pathway (Gumbiner 1996). However, the adhesion and signaling functions of this protein are often abrogated during

oncogenesis. Therefore, dysregulation of β -catenin may contribute to the development and progression of oncogenesis via distinct mechanisms. During cancer progression, the function of adherens junctions is generally compromised, thereby leading to the loss of cell–cell adhesion and increased cell proliferation (Zhurinsky *et al.* 2000, Livshits *et al.* 2012). In most of the solid cancer, the Wnt signaling pathway is constitutively active due to the mutations in canonical members of this pathway (Majid *et al.* 2012). Thus, this

pathway has become a hot target for cancer therapeutics. β -catenin also plays a major role as a coactivator of the transcription factors LEF and TCF. Stabilization of cytoplasmic β -catenin by activated Wnt signaling leads to its nuclear accumulation, binding with LEF/TCF transcription factors and transactivation of LEF/TCF target genes, eventually leading to cell proliferation (Eastman & Grosschedl 1999, Nusse 1999, Roose & Clevers 1999).

In the United States, pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer-related death. The incidence rate is equal to the mortality rate (Jemal et al. 2005). The poor diagnosis is due to the reduced response to chemotherapy and/or radiation therapy (Wang et al. 2011). PDA is considered to arise from highly proliferative premalignant ductal epithelium tissue through a series of genetic mutations (Hruban et al. 2000). Such genetic lesions include activating mutations primarily in the KRAS gene and the loss of the tumor suppressor genes CDKN2A, TP53, and SMAD4 (Bardeesy & DePinho 2002, Jaffee et al. 2002). Recent studies have shown that mutations in β -catenin/APC and aberrant canonical Wnt signaling pathway activity are found in acinar cell carcinoma (Abraham et al. 2002), PDA (Lowy et al. 2003, Al-Aynati et al. 2004, Zeng et al. 2006), pancreatoblastomas (Koesters & von Knebel Doeberitz 2003), and solid pseudopapillary neoplasm (Abraham et al. 2002, Cao et al. 2005, Min Kim et al. 2006, Nishimori et al. 2006). However, a direct fundamental association between Wnt pathway dysregulation and pancreatic tumor development has not been clearly confirmed to date.

Another equally important feature of all kinds of cancer is dysregulated cell division during eukaryotic cell cycle progression. The cells transit only if the regulatory signaling pathways with the successful completion of upstream cellular events before proceeding to the next phase. These regulatory signaling cascades are commonly known as cell-cycle checkpoints (Hartwell & Weinert 1989). Cyclin B1 which is frequently expressed during the G2 phase, translocates to the nucleus during early mitosis via a couple of intracellular events, and degrades at the last stage of mitosis (Takizawa & Morgan 2000). The intracellular pathways involved in G2/M arrest converge to inhibit the activation of Cdc2 and its complex formation with cyclin B1 (Hwang & Muschel 1998). Apoptosis, which is the outcome of a series of regulated cellular events that are often suppressed in tumors, can induce a variety of genes involved in cell-cycle inhibition by targeting the G2/M checkpoint (Jordan et al. 1998, Concin et al. 2003).

The efficacy of chemotherapy is frequently restricted due to the toxicity to other non-targeted tissues, as most cancer chemotherapeutics were developed only by

screening for cell growth inhibition without a clear understanding of the mode of action. Therefore, understanding the mechanism of action would be effective in designing chemotherapeutics with fewer side effects. Herbal anti-cancer agents are promising as they display reduced cytotoxicity (Newman et al. 2000, Mann 2002). While studying herbal compounds, we have demonstrated previously that mahanine, a purified carbazole alkaloid derived from the edible plant *Murraya koenigii*, was a potential activator of the cellular death cascade in a leukemic model (Bhattacharya et al. 2010). In another recent study, we have established that mahanine-induced reactive oxygen species-mediated functional inhibition of Hsp90 chaperone activity, resulting in Hsp90-Cdc37 disruption, leads to apoptosis in pancreatic adenocarcinoma (Sarkar et al. 2013).

Another ancient medicinal plant, *Withania somnifera*, has been found to be extensively used as an Indian traditional remedy and dietary supplement. It is also known for its efficacy in tumor cell growth inhibition and its anti-tumor effects on an *in vivo* mouse model (Diwanay et al. 2004). Withanolides are C₂₈-steroidal lactones-specific secondary metabolites of this plant. Withaferin A (WA) is one of the major and most predominant withanolides found in this plant. Our group has already demonstrated that WA induces apoptosis by activating the p38 MAPK signaling cascade in leukemic cells (Mandal et al. 2008). Withanolide-D (witha-D) is another pure herbal compound purified from this medicinal plant (Chaurasiya et al. 2008). We have previously shown that witha-D increases ceramide accumulation by activating neutral sphingomyelinase 2, altered JNK and p38 MAPK phosphorylation, while it also induced apoptosis in both myeloid and lymphoid cells (Mondal et al. 2010). In another study, we proposed that witha-D induced apoptosis in malignant cells via a Bax/Bak- and Bak-dependent pathway in p53-WT cells and p53-null cells respectively (Mondal et al. 2012). The main aim of this study was to assess the intracellular mechanism of witha-D-induced apoptosis in pancreatic adenocarcinoma cells. Therefore, we focused on the Wnt/ β -catenin signaling pathway and its association with the G2/M phase of the cell cycle.

In this study, we have successfully demonstrated that witha-D causes cell-cycle arrest in pancreatic adenocarcinoma cells at the G2/M checkpoint. Activation of Chk1/Chk2 and subsequent cell-cycle arrest at the G2/M checkpoint concurrently led to functional inhibition of Akt and successive activation of Gsk3 β . This further resulted in the inactivation of oncogenic β -catenin for proper arrangement with destruction complex. In conclusion, our observations demonstrate witha-D as a

novel promising herbal therapeutic to treat deadly pancreatic adenocarcinoma.

Materials and methods

Withanolide-D

Witha-D (Fig. 1A, molecular weight 470.6), a pure herbal C₂₈ steroidal lactone (C4b-hydroxyC5b,C6b-epoxy-1-oxo-,C20b,dihydroxy-20S,22R-witha-2,24-dienolide) was purified from the leaves of an extensively used Indian medicinal plant *W. somnifera* as described earlier (Chaurasiya et al. 2008). The purity of the compound was analyzed via IR, mass, ¹H-NMR, and ¹³C-NMR spectral analysis. The compound was dissolved in absolute ethanol and stored at -70 °C before use.

Cell lines and cell culture

Human PDA cell lines (purchased from ATCC, Manassas, VA, USA), such as MIA PaCa2, AsPC1, Panc10.05, BxPC3, were grown in RPMI-1640 medium; and Panc1 was cultured in IMDM medium. Vero (African green monkey kidney epithelial cells) and NIH3T3 (mouse embryonic

fibroblast cells) were cultured in IMDM medium. All cell cultures were supplemented with 10% heat inactivated FBS and 1% antibiotic-antimycotic and incubated in a humidified atmosphere at 37 °C with 5% CO₂.

Cell viability assay

The cells (1 × 10⁴) were exposed to witha-D (0–10 μM) for 48 h and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 100 μg/well) was then added to fresh cell-culture media for an additional 3 h. The formazane crystals that formed were dissolved in DMSO, and the optical density was measured using a plate ELISA reader (Thermo Scientific, USA) at 550 nm. Control cells were exposed to the highest concentration of the vehicle.

Flow cytometric detection of 7-aminoactinomycin D positive cells

The cells (1 × 10⁶) were exposed to witha-D (0–6 μM) for 24 h. Evaluation of apoptosis was carried out using the 7-aminoactinomycin D (7-AAD) fluorescent chemical compound according to the manufacturer's instructions. At least 10 000 cells were acquired, and the data

Figure 1

Witha-D induced apoptosis in pancreatic cancer cells. (A) Chemical structure of witha-D, a steroidal lactone. (B) Witha-D-induced anti-proliferative effects in five pancreatic adenocarcinoma cells as assessed via MTT assay after 48 h in a dose-dependent manner. Cell viability was represented as percentage viability. Each value is represented as the mean ± s.d. of at least three independent experiments ($P < 0.001$). * indicates a significant difference of $P < 0.001$. (C) Dose-dependent anti-proliferative activity in MIA PaCa2 cells (K-Ras^{mut}) and

BxPC3 cells (K-Ras^{wt}) after 24 h of witha-D treatment. Cell viability was represented as percentage viability. Each value is represented as the mean ± s.d. ($P < 0.001$) of at least three independent experiments. * indicates a significant difference of $P < 0.001$. (D) Witha-D treatment facilitated the positive regulation of apoptosis-related proteins in MIA PaCa2 and BxPC3 cells after 24 h incubation as assessed via western blotting. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-13-0315>.

were analyzed with CellQuest Pro software (BD FACSCalibur, NJ, USA).

Apoptosis assay

The cells (1×10^6) were treated with witha-D (0–6 μ M) for 24 h. Quantitative flow cytometric assessment of apoptotic cells was performed via TUNEL assay using a BD ApoDirect Kit (BD Biosciences, San Jose, CA, USA). At least 20 000 cells were acquired using a BD FACSCalibur and analyzed with CellQuest Pro software.

Flow cytometric assessment of Bcl-2 family proteins and caspase 9

The ratio of the mitochondrial proapoptotic protein, Bax, and antiapoptotic proteins, Bcl-xL and Bcl-2 were assessed via flow cytometry. The cells (1×10^6) were treated with witha-D (0–6 μ M) for 24 h, and after the indicated time point, the cells were washed and incubated with the proper antibodies. At least 10 000 cells were acquired and analyzed with CellQuest Pro software (BD FACSCalibur).

Caspase activity assay

The cells (1×10^6) were treated with witha-D (0–6 μ M) for 24 h, washed, and labeled with the FLICA assay solution (BioVision, Milpitas, CA, USA) by incubating for 2 h at 37 °C. The samples were analyzed spectrofluorimetrically at 400 nm (excitation)/505 nm (emission). The results are represented as the fold change in activity of caspase 3 and caspase 8.

Cell cycle analysis via flow cytometry

The cells (1×10^6 cells/well) were cultured in six-well plates and treated with witha-D (0–5 μ M) for 24 h. The cells were then harvested, washed in ice-cold PBS, and fixed with 70% ethanol/PBS at 4 °C. The fixed cells were incubated with 0.5 ml PBS containing 20 μ g/ml RNase A for 30 min at 37 °C, and stained with PI (50 μ g/ml) for 30 min in the dark at room temperature. Finally, the cells were analyzed using a FACSCalibur flow cytometer. A minimum of 1×10^4 cells per sample was evaluated, and the cell distribution percentage at each phase of the cell cycle was further calculated with CellQuestPro software.

Immunoblot and immunoprecipitation

The cells (1×10^6) were treated with or without witha-D (0–4 μ M) for 24 h, washed, and sonicated in chilled PBS.

Equal amounts (50 μ g) of protein were separated via SDS-PAGE (7.5–10%). Proteins were then electro-transferred into a nitrocellulose membrane, blocked with 2% TBS-BSA, and probed overnight with primary antibodies. Following an additional incubation with HRP-conjugated secondary antibodies, the membranes were washed and target proteins were identified using the West-pico ECL system.

For detection of the destruction complex, equal amounts of untreated and treated cell lysates (350 μ g) were incubated with anti- β -catenin antibody (1:100) overnight at 4 °C followed by an additional incubation with Protein A-Sepharose 4B. The immunocomplex was resolved via SDS-PAGE and subsequently identified using the specific antibodies.

Subcellular fractionation

The cells (1×10^6) were treated with witha-D (0–4 μ M) for 24 h and fractionated into cytosol and nuclear portions using a NE-PER Kit according to the manufacturer's procedure. Briefly, the cells were washed following witha-D treatment, incubated in cytosol extraction reagent, and then centrifuged. The supernatant served as the cytosolic fraction. The pellet was solubilized in nuclear extraction reagent and centrifuged, and the supernatant represented the nuclear fraction. Western blot analyses were performed with these subcellular fractions as described previously.

Confocal microscopy

BxPC3 cells (1×10^6) were seeded on cover slips 24 h before witha-D treatment (3 μ M, for the next 24 h). Untreated and treated cells were fixed in acetone-methanol, washed in 0.2 M PBS-glucose, blocked with 2% PBS-BSA, probed overnight with primary antibody, and washed with PBS-glucose. Immunocomplexes were identified with FITC-conjugated secondary antibody via a 2-h dark incubation period and mounted on DAPI-containing mounting medium (Molecular Probes, Invitrogen). The microscopic image was taken with 405 nm (DAPI) and 488 nm (FITC) using an Andor spinning disc confocal microscope (Andor Technology, Belfast, Northern Ireland, UK).

Semi-quantitative RT-PCR

Total RNA was extracted from witha-D-treated (0, 2, and 4 μ M) cells using an RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA with random primers using the

Im-Pro-II-Reverse transcription system (Promega) according to the manufacturer's protocol. The c-MYC and actin PCR assays were carried out with specific forward and reverse primers using a PTC-100 system (MJ Research, Waltham, MA, USA). The PCR products were electrophoresed on an agarose gel (1%), which was stained with ethidium bromide and visualized under u.v. light. The signal intensity of the respective DNA bands was measured with Quantity one version 4.1.1 software using a Bio Rad image analysis system.

esi-RNA-mediated silencing

MIAPaCa2 and BxPC3 cells (5×10^5) were preseeded for 18 h in a six-well plate, and control and esi-RNA (CHEK1, CHEK2) transfection reagents were subsequently prepared and added to the culture for 8 h according to the manufacturer's protocol. The cells were then supplemented with fresh complete RPMI-1640 medium for an additional 18 h in the presence and absence of witha-D. The cells were then processed for Western blot analysis and flow cytometric analysis.

Results

WA-related steroidal lactones derived from *W. somnifera* exhibited potent anti-proliferative activity in MIAPaCa2 cells

To determine the anti-proliferative effect of WA-related steroidal lactones, WA, withanolide A, and witha-D, the human pancreatic cancer cell line, MIAPaCa2, was treated for 48 h and assessed via MTT assay. The results demonstrated that witha-D and WA displayed similar levels of anti-proliferative activity.

Witha-D inhibited gemcitabine-resistant and gemcitabine-sensitive pancreatic cancer cell proliferation

All five pancreatic cancer cell lines were treated with witha-D (1–5 μ M) for 48 h. It displayed a cytotoxic effect against the gemcitabine-resistant MIAPaCa2, AsPC1, Panc10.05, Panc1 cells, and gemcitabine-sensitive BxPC3 (Arumugam *et al.* 2009) cells with an IC_{50} values of 1.6, 3.8, 2.8, 0.9, and 0.7 μ M respectively (Fig. 1B). Witha-D also inhibited, one of the cell lines from representative group, MIAPaCa2 and BxPC3 at 24 h and IC_{50} for this time point were 4.2 and 2.78 μ M respectively (Fig. 1C). No inhibition in cell proliferation was observed in the cell cultures treated with the vehicle control EtOH. No significant cell death was found in Vero cells or NIH3T3

cells. Morphological alteration was also shown in representative witha-D-treated MIAPaCa2 cells after 24 h in a dose-dependent manner with a gradual disintegration of cellular integrity, shape, and density.

Witha-D induced apoptosis in Kras^{mut} pancreatic cancer cells

As considerable cell death was observed in witha-D-treated MIAPaCa2 cells, we further quantitatively evaluated the cell death with 7-AAD staining. MIAPaCa2 cells were incubated with various concentrations of witha-D for 24 h. The proportion of 7-AAD-positive cells increased from 6.96% at 2 μ M to 44.46% at 6 μ M, thereby demonstrating that witha-D-decreased cell viability significantly.

TUNEL assay demonstrates witha-D-induced DNA fragmentation

To further validate the extent of apoptosis induced by witha-D (2–6 μ M) in MIAPaCa2 cells (24-h treatment), we performed a flow cytometric quantification of fragmented DNA by labeling with dUTP-FITC. Significant levels of TUNEL-positive cells were detected in a dose-dependent manner. The TUNEL-positive cell population increased from 14.94% (2 μ M) to 27.8% (6 μ M), thereby further confirming that witha-D induces cell death primarily via apoptosis.

The ratio of mitochondrial anti-and pro-apoptotic proteins altered by witha-D

After confirming that witha-D induces cell death primarily via apoptosis, we further assessed the major anti- and pro-apoptotic protein ratio alteration. A dose-dependent decrease in the Bcl-2:Bax ratio after 24 h of witha-D treatment in MIAPaCa2 cells was observed. In addition, the Bcl-xL:Bax ratio was also altered in dose-dependent manner.

Witha-D induced apoptosis through caspase-mediated pathway

As the cysteine–aspartic proteases or caspases are truly linked to the mitochondrial pro- and anti-apoptotic protein level changes and play a crucial role in apoptosis, we next evaluated the intensity of activation of these proteases. Fluorometric analysis revealed a dose-dependent increase in activated caspase 8 and caspase 3 at 3 and 4 μ M doses

respectively. Alternatively, flow cytometric analysis that showed pro-caspase 9 levels were considerably reduced after 24 h of witha-D treatment (4 μ M) in MIAPaCa2 cells. Western blot analysis also revealed that the key molecular mediators of apoptosis, such as Bcl-2, caspase 3, and PARP, positively regulated apoptosis in both MIAPaCa2 and BxPC3 cells (Fig. 1D).

Witha-D arrested MIAPaCa2 and BxPC3 cells at the G2/M phase

To determine whether growth inhibition induced by witha-D was associated with the arrest of cells in a particular phase, we analyzed the cell distribution at each phase of the cell cycle with increasing concentration of witha-D from 0 to 5 μ M using flow cytometry and representative data of doses 0, 2, and 4 μ M were given in Fig. 2A. In untreated MIAPaCa2 cells, 70.26% of cells were in the G1 phase, 12.2% cells were in the S phase, and 13.47% cells were in the G2/M phase. Witha-D treatment resulted in a significant accumulation of cells in the S and G2/M phase with a striking reduction of cells in the G1 phase. However, the peak of arrested cells in the G2/M phase was observed at the 2 μ M dose, whereas significant cell accumulation in the S phase following a 24-h treatment with witha-D (4 μ M). In parallel, in untreated BxPC3 cells, ~72.19% cells were in the G1 phase, 17.16% cells were in the S phase, and 9.83% cells were in the G2/M phase. Witha-D also resulted in a

considerable accumulation of cells in the G2/M phase (16.52% cells at 2 μ M and 30.73% cells at 4 μ M) with a remarkable reduction of cells in the G1 phase (70.21% cells at 2 μ M and 53.83% cells at 4 μ M).

Next, we assessed the regulators of the G2/M phase in MIAPaCa2 and BxPC3 cells to elucidate the molecular scenario (Fig. 2B). Western blot analysis demonstrated that witha-D activated ATM and ATR in a dose-dependent manner, which subsequently upregulated and phosphorylated Chk1 and Chk2. Activated Chk1 and Chk2 phosphorylated the cell-cycle regulatory phosphatases Cdc25A and Cdc25B and subsequently led to the degradation of these phosphatases. Cyclin B1 was also hyper-phosphorylated and probably destined for degradation. As a result, Cdc2 and cyclin B1 did not form complexes to transit the cells from the G2 to the M phase. The negative regulator of the G2/M cell-cycle checkpoint, Wee-1, was also upregulated in dose-dependent manner, which further promoted G2/M cell-cycle arrest.

In addition to the MIAPaCa2 and BxPC3 cells, two other cell lines were also assessed based on their CDKN2A and SMAD4 mutational status, AsPC1 cells (a highly aggressive metastatic pancreatic tumor cell line; KRAS, TP53, CDKN2A, and SMAD4 all are mutated) and Panc 10.05 cells (a noninvading primary tumor cell line; KRAS and TP53 are mutated where as CDKN2A and SMAD4 are WT). Both cell lines showed a similar G2/M

Figure 2

Witha-D treatment led to G2/M phase arrest in MIAPaCa2 and BxPC3 cells. (A) Flow cytometric analysis showed that witha-D arrested pancreatic adenocarcinoma cells irrespective of their KRAS mutational status in a dose- responsive manner at the G2/M phase. Representative data of three

individual sets of experiments. (B) The molecular signature of the G2/M-phase arrest after dose-dependent witha-D treatment in pancreatic adenocarcinoma cells as analyzed via Western blotting.

phase cell-cycle arrest profile via flow cytometry and intracellular molecular analysis.

Abrogation of the Wnt/ β -catenin pathway mediated by witha-D in MIAPaCa2 and BxPC3 cells

As the Wnt/ β -catenin signaling cascade is one of the most important developmental pathways in oncogenesis (Zhurinsky *et al.* 2000), we further examined this signaling pathway after witha-D treatment. This herbal compound perturbed various signaling elements of this pathway (Fig. 3A).

Witha-D treatment reduced Wnt3a expression in a dose-dependent manner in MIAPaCa2 and BxPC3 cells. In contrast, in BxPC3, Panc 10.05, and AsPc1 cells, witha-D treatment upregulated E-cadherin expression, an important molecular player in the epithelial to mesenchymal transition (EMT; Fig. 3A). However, in MIAPaCa2 cells, in which the E-cadherin gene is hypermethylated and protein production is subsequently turned off, witha-D failed to activate protein translation (Fig. 3A).

Similar to β -catenin, its cytoskeletal counter-part, α -catenin, also displayed a change in protein expression in both MIAPaCa2 and BxPC3 cells. However, another

protein of this family, γ -catenin, did not display a significant change in the expression following treatment.

Downregulation of the Dishevelled family protein, Dvl2, and upregulation of its inhibitor family protein Naked, Naked1, also demonstrated that the Wnt signaling cascade was disturbed by witha-D treatment (Fig. 3A). Other proteins of these two families (Dvl3 and Naked2) also showed similar alterations in expression. Casein kinase family proteins, CKII α/α' and CKI ϵ , were significantly upregulated in MIAPaCa2 cells within only 1.5 μ M dose treatment; although, this effect was not observed in BxPC3 cells.

The expression of the transcription factor and downstream target gene of the cotranscription factor β -catenin (TCF-1) was also decreased and hence reinforced this phenomenon (Fig. 3A). A known Wnt/ β -catenin pathway inhibitor, IWR1, also downregulated β -catenin and TCF-1 in all four pancreatic cancer cell lines (Fig. 3B). To further elucidate the reduction in transcriptional activity of β -catenin, the transcription levels of c-MYC, one of the transcriptional targets of β -catenin, were assessed. These results showed a significant downregulation in c-MYC transcripts in all the four cell lines (Fig. 3C). As anticipated, c-Myc protein was also downregulated in witha-D-treated MIAPaCa2 and BxPC3 cells (Fig. 3D). This further demonstrated that

Figure 3

Witha-D abrogated Wnt/ β -catenin signaling as reflected at the molecular level. (A) Most of the key regulatory proteins of the Wnt/ β -catenin signaling pathway were altered by dose-dependent witha-D treatment in both cell lines. (B) The lower panel shows IWR1, a known positive inhibitor of the Wnt/ β -catenin pathway, which downregulated β -catenin and TCF-1.

(C) Witha-D showed a dose-dependent decrease in c-MYC m-RNA levels (upper panel). Data are represented as fold change ($P < 0.001$) in bar diagram (lower panel). * indicates a significant difference of $P < 0.001$. (D) The decrease in c-Myc expression levels was confirmed via Western blotting in MIAPaCa2 and BxPC3 cells.

although β -catenin expression levels were increased in a dose-dependent manner in MIAPaCa2 cells, its transcriptional activity was inhibited by an unknown mechanism.

Witha-D mediated critical regulation of β -catenin turnover

β -catenin has been shown to play a crucial role in cell-cell adhesion by binding with E-cadherin. However, total β -catenin protein was upregulated exclusively in MIAPaCa2. This is not surprising as we observed transcriptional upregulation of β -catenin in witha-D-treated cells (data not shown). However, the mechanism is still unclear. We also found that witha-D treatment inhibited β -catenin migration from the cytosol to nucleus in both MIAPaCa2 and BxPC3 cells (Fig. 4A).

A decrease in total β -catenin in BxPC3 cells, as shown in Fig. 3A, was expectedly due to proteasomal degradation of β -catenin protein. Both MIAPaCa2 and BxPC3 cells were pretreated with MG132, a known proteasomal inhibitor, and were subsequently incubated with witha-D. Enhanced ubiquitinated β -catenin was observed in both cell lines (Fig. 4B), thereby suggesting that β -catenin was degraded after the treatment.

Moreover, β -catenin's association with the components of its destruction complex, viz. Axin1, and Gsk3 β was also assessed by immunoprecipitation analysis.

Enhanced association of Axin1 with β -catenin was observed in a dose-dependent manner; however, no significant change was visibly seen in the association of β -catenin and Gsk3 β (Fig. 4C). These observations illustrate the outline of the β -catenin inactivation in pancreatic cancer cells.

The confocal microscopic images also revealed that the intracellular pool of β -catenin dramatically decreased after witha-D treatment (3 μ M) after 24 h in BxPC3 cells (Fig. 4D).

Witha-D downregulated functionally active β -catenin

Since its discovery, β -catenin has been observed to execute a crucial signaling role as a component of the Wnt pathway (Gumbiner 1996). Akt, which is the key regulator for the inhibitory phosphorylation (S^9) of Gsk3 β , was drastically downregulated both in its total and active phosphorylated (S^{473}) states (Fig. 5A). This phenomenon resulted in the downregulation of Gsk3 β in its functional inhibitory (S^9) phosphorylation. Simultaneously, upregulation of its activation phosphorylation (Y^{216}) activated this kinase. These chronological phenomena potentiated the sequential phosphorylation of β -catenin at the $S^{33/37}T^{41}$ sites by active Gsk3 β . This phosphorylation triggered the enhanced degradation of this protein.

Figure 4

Critical regulation of β -catenin turnover after witha-D treatment. (A) The transmigration of β -catenin from the cytosol to the nucleus was also inhibited by witha-D treatment in MIAPaCa2 and BxPC3 cells as demonstrated by a subcellular fractionation study. (B) Ubiquitinated β -catenin in both cell lines was identified by a 1-h preincubation with MG132 in a witha-D dose-responsive manner. (C) Witha-D-mediated

destruction complex formation on β -catenin was also identified via immunoprecipitation assay in BxPC3 cells after 24 h. (D) Confocal microscopic images show that witha-D treatment led to a decrease in intracellular β -catenin levels after 24 h. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-13-0315>.

Inhibition of Chk1/Chk2 reiterated the activation of Akt resulting in cell survival

To further understand the regulation of the β -catenin pathway, we examined the role of Chk1/Chk2. Accordingly, we knocked down endogenous Chk1 via esi-RNA (Fig. 5B). The results showed that this silencing led to the activation of Akt by increasing the phosphorylation at S⁴⁷³. Subsequently, Gsk3 β was hyper-phosphorylated at position S⁹ and therefore Gsk3 β was functionally inhibited. Inactive Gsk3 β failed to phosphorylate β -catenin at S^{33/37}T⁴¹ as observed in both cell lines. Similar observations were demonstrated by knocking down endogenous Chk2 by esi-RNA (Fig. 5C).

As Akt activation and β -catenin accumulation are often associated with cell survival, we further confirmed the effect of Chk1 and Chk2 silencing in cell survival.

Knockdown of Chk1 and Chk2 also resulted in the inhibition of cell death. The 7-AAD-positive cells decreased ~ 8 and $\sim 5\%$ after silencing Chk1 and Chk2, respectively, in MIA PaCa2 cells, whereas only a $\sim 4\%$ decrease was observed in BxPC3 cells (Fig. 5D). The results confirmed that the inhibition of Chk1 and Chk2 led to a decrease in cell mortality.

Discussion

Dysregulation of Wnt/ β -catenin signaling is frequently reported in pancreatic adenocarcinoma (Karayiannakis et al. 2001, Lowy et al. 2003), although most pancreatic cancers do not display any mutations in the β -catenin gene (Gerdes et al. 1999). However, loss of cadherins and catenins or their critical regulations have implications in

Figure 5

Witha-D downregulated functionally active β -catenin: the inverse relationship of Chk1/Chk2 and Akt facilitated the abrogation of the Wnt/ β -catenin pathway. (A) Inhibition of Akt exerted GSK 3 β -mediated β -catenin degradation after a 24-h witha-D treatment. Total β -catenin protein was upregulated in MIA PaCa2 cells, whereas total β -catenin protein was downregulated in BxPC3 cells. However, the increased phosphorylation at the S^{33/37}T⁴¹ sites tagged this oncogenic protein for

further degradation in both cell lines. (B) Knockdown of Chk1 activated Akt and functionally inhibited GSK3 β , thereby resulting in the inhibition of β -catenin destruction. (C) Knockdown of Chk2 also inhibited β -catenin destruction. (D) Knockdown of Chk1 and Chk2 led to a decrease in cell mortality as demonstrated by the reduction in 7-AAD-positive cells. Each value is represented as the mean $\pm$ s.d. of at least three independent experiments ($P < 0.0001$). * indicates a significant difference of $P < 0.0001$.

pancreatic tumor progression and metastasis (Toyoda *et al.* 2005, Winter *et al.* 2008). The critical association of cell-cycle regulation and β -catenin signaling remains unclear. Some studies have shown that β -catenin plays a positive role in the G1/S transition (Orford *et al.* 1999) or is directly involved in apoptosis (Kim *et al.* 2000). However, the function of β -catenin in relation to the G2/M phase is not well established. A study has shown a steady increase in soluble β -catenin levels in cells with a maximum accumulation at the G2/M phase, although the exact mechanism was not identified (Olmeda *et al.* 2003). In this report, we assessed a plausible mechanism of G2/M cell-cycle arrest and abrogation of the Wnt/ β -catenin pathway using witha-D, a herbal steroidal lactone derived from an Indian medicinal plant, *W. somnifera*, in pancreatic adenocarcinoma cells.

This study is the first of its kind to provide evidence of crosstalk of β -catenin signaling in the G2/M phase via functionally inactive Akt via witha-D treatment in pancreatic cancer cells. The primary achievement of this investigation was the demonstration that witha-D treatment led to G2/M cell-cycle arrest by positive regulation of several molecular players. In parallel, this treatment dysregulated the Wnt/ β -catenin pathway in such a manner that eventually led to apoptosis. Akt was shown to be the crucial negotiator of G2/M regulation and β -catenin signaling (Fig. 6).

Loss of E-cadherin is often associated with noncohesive and highly metastatic pancreatic cancer as shown with the EMT (Winter *et al.* 2008). In this study, among the five pancreatic cancer cell lines, only MIAPaCa2 was E-cadherin^{null} and was thus characterized as highly metastatic. β -catenin, one of the partners of E-cadherin in adhesion, was also greatly downregulated in this cell line. Strikingly, witha-D treatment increased β -catenin levels in a dose-responsive manner in MIAPaCa2 cells. However, it is not clear whether the actual intention of this augmentation was for the reversal of the EMT (although witha-D incubation failed to activate E-cadherin in MIAPaCa2 cells) or to arrest cells in the G2/M phase (Olmeda *et al.* 2003). However, upregulated β -catenin was not transcriptionally active due to the critical regulation of the Akt-Gsk3 β kinase cascade in MIAPaCa2 cells. In contrast, witha-D treatment downregulated β -catenin in the rest of the three pancreatic cancer cell lines, in which its levels were already elevated. Thus, the strategic modulation of β -catenin via witha-D treatment ultimately facilitated cell death in an array of pancreatic cancer cells. Reversal of the EMT with witha-D incubation was confirmed by the elevation of E-cadherin levels in these cells. In this

context, cadherins have been recently found to control cell proliferation by altering β -catenin signaling independent of the cell adhesion manner (Gottardi *et al.* 2001, Lowy *et al.* 2002).

All cells were arrested in the G2/M phase irrespective of their mutational status. Though TP53 is mutated in both MIAPaCa2 and BxPC3, still the activation phosphorylation of TP53 occurred after witha-D treatment. However, what is the exact function of these phosphorylated p53^{mu} in the cell apoptosis is yet to be proved.

A recent study has shown that overexpression of N-cadherin downregulated β -catenin, which was found to inhibit the transactivation of p21 expression (Kamei *et al.* 2003). We also observed a similar phenomenon. Functionally inactive β -catenin could not inhibit the augmentation of p21 in both MIAPaCa2 and BxPC3 cells. Another study indicated that the phosphorylation of Chk1 by ATR is antagonized by protein phosphatase 2 α (PP2 α) in unperturbed cell-cycle division (Leung-Pineda *et al.* 2006). In this study, we have also observed that the activation of Chk1 occurred earlier than the activation of the PP2 α catalytic subunit in a dose-dependent manner. However, activated Chk1 most likely induced cells to accumulate in the G2/M phase before activation of PP2 α , which may have downregulated Chk1 phosphorylation and acted by other unidentified means. Recently, it was shown that Akt can phosphorylate Chk1 at S²⁸⁰ and subsequently inactivate the protein (King *et al.* 2004). Strikingly, witha-D treatment functionally inactivated Akt (the catalytic subunit (p110 α) of PI3K also reduced by witha-D incubation, suggesting a direct relationship between blockage of PI3K and subsequent blocking of Akt kinase activity) and subsequently activated Chk1 by enhancing phosphorylation at S³⁴⁵ and S²⁹⁶. Knocking down Chk1 or Chk2 separately via esi-RNA activated Akt as demonstrated by an increase in S⁴⁷³ phosphorylation levels. This observation correlates with a recent study, in which the inhibition of the PI3K/Akt pathway is associated with an increase in Chk1 kinase activity (Shtivelman *et al.* 2002). Downregulation of Chk1 and Chk2 also led to functional inactivation of Gsk3 β via the upregulation of S⁹ phosphorylation and ultimately the accumulation of β -catenin.

We have recently shown that witha-D elicited Bax/Bak-mediated apoptosis in malignant cells (Mondal *et al.* 2012). In this context, a report has shown that β -catenin promotes cell survival by inhibiting Bax in a PI3K/Akt-dependent manner in renal epithelial cells (Wang *et al.* 2009). Therefore, the eventual inactivation of β -catenin by witha-D could also elicit Bax in pancreatic

Figure 6

Proposed pathways of witha-D mediated apoptosis. Witha-D treatment induces ATM/ATR and subsequent Chk1/2 activation and finally leads to G2/M arrest both in MIAPaCa2 and BxPC3 cells. It also inactivates Akt, and successive activation of Gsk3 β ultimately downregulates functionally active

β -catenin. However, the overexpression of β -catenin, particularly in MIAPaCa2 cells, occurs via an unknown mechanism. Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-13-0315>.

cancer. Witha-D also activates the JNK/p38 MAPK pathway (Mondal *et al.* 2010). Furthermore, JNK was shown to be associated with the phosphorylation and degradation of β -catenin (Lee *et al.* 2009). Whether the JNK/p38 MAPK pathway also modulates β -catenin signaling remains to be explored.

Conclusions

Our study successfully demonstrated that the herbal steroidal lactone, witha-D, perturbed Wnt/ β -catenin signaling, one of the major oncogenesis pathways, by arresting pancreatic adenocarcinoma cells at the G2/M phase of the cell-cycle checkpoint. To the best of our knowledge, this study presents the first description of the intracellular mechanism by which witha-D exerts its apoptotic effects in pancreatic adenocarcinoma, and these findings may support the development of a new drug with greater efficacy in the management of this deadly disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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