In vitro and *in vivo* evaluation of paclitaxel-induced release of apoptotic biomarker ccCK18 to guide treatment optimization in ovarian cancer

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Abstract

Background: Cytokeratins hold potential as biomarkers due to their epithelial specificity, abundance and cleavage by caspases during apoptosis. We evaluated paclitaxel-induced circulating caspase-cleaved (cc) cytokeratin 18 (CK18) as potential apoptotic cell-death marker to guide treatment optimization in ovarian cancer.

Methods: Six ovarian cancer cell lines (SK-OV-3, SK-OV-3lucIP1, Caov-3, NIH:OVCAR-3, PA-1 and PM-LGSOC-01) were exposed in vitro to paclitaxel (PTX, 0 to 1000 nM) for 24h. Extracellular levels of ccCK18 were measured until 5 days after drug exposure. Cell count and ccCK18 release data were analyzed using a phase-nonspecific pharmacodynamic model implemented in NONMEM®. PA-1 and SK-OV-3lucIP1 xenografted female SCID/Beige mice received a placebo or single dose of 50 mg/kg PTX intraperitoneally. Response to PTX was evaluated in vivo using tumor volume and released ccCK18 levels.

Results: In vitro, the correlation between cell count and released ccCK18 levels was present in all cell lines (Spearman's rank correlation coefficient > 0.64). Tumor volume and ccCK18 longitudinal dynamics were markedly different for controls and PTX-treated PA-1 xenografts with changes in ccCK18 release preceding changes in tumor volume. For SK-OV-3lucIP1 xenografts, no differences were found between controls and PTX-treated mice.

Conclusions: An association between PTX-induced ccCK18 release and cell count was demonstrated in vitro. The in vivo study supported the presence of an early-apoptotic peak in ccCK18 levels compared to a later observed effect on tumor volume in PTX-sensitive xenografts. Given the heterogeneous character of ovarian cancer, application and implementation of ccCK18 in a clinical setting to optimize or personalize cancer treatment needs further refinement.

Background

As a member of the intermediate filament protein family, cytokeratin 18 (CK18) belongs to the type 1 acidic keratins and is expressed in a variety of single-layered and simple epithelia (1). Besides its role in tumor cell behavior and numerous cellular processes such as apoptosis and cell cycle progression, CK18 is also involved in providing the intracellular scaffolding that structures the cytoplasm, supports normal mitochondrial structures and resists stress applied from the outside of the cell (2, 3). Furthermore, this keratin is also used as an epithelial marker in tumor pathology (4).

In contrast to its filament partner cytokeratin 8 (CK8), CK18 is a substrate for caspase cleavage during epithelial cell apoptosis (5). This characteristic makes ccCK18 interesting as a tumor marker that provides information on the extent of ongoing apoptotic tumor cell death. Caspases, a family of aspartate-specific cysteine proteases, contribute frequently to the process of apoptosis and are the key effectors responsible for the characteristic morphological aspects of apoptosis, i.e. rounding up of the cell, reduction of cellular and nuclear volume, nuclear fragmentation, plasma membrane swelling etc. (6).

Caulin et al. observed that CK18 is cleaved by caspase-6, -3 and -7, resulting in the appearance of a specific neo-epitope, which can be measured using an enzyme-linked immunosorbent assay (ELISA) (5).

A number of studies already investigated the value of total and caspase-cleaved CK18 as tumor marker in gastro-intestinal(7), breast(8) and testicular(9) cancer and showed that the measurement of ccCK18, as a marker of apoptosis, and uncleaved CK18, released during both apoptosis and necrosis, allows to distinguish between different cell death modes (10).

In this work, we studied the correlation between ccCK18 levels and PTX treatment efficacy in vitro and in vivo. Pharmacodynamic modelling was used to characterize the longitudinal changes in cell count and released ccCK18 levels from the in vitro experiments.

Methods

1. IN VITRO STUDY

a. Cell lines and culture conditions

The human ovarian cancer cell lines PA-1, Caov-3 and SK-OV-3 were obtained from ATCC (LGC Standards S.a.r.l., France). SK-OV-3LucIP1 cells were selected in vivo (11) and together with the NIH:OVCAR-3 cell line provided by the LECR research group (Ghent University, Belgium). This cell panel was extended with the patient-derived xenograft-derived low-grade serous ovarian cancer cell line PM-LGSOC-01(12). SK-OV-3 cells were maintained in ATCC-formulated McCoy's 5A medium, SK-OV-3LucIP1 and Caov-3 cells in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) and PA-1 together with PM-LGSOC-01 cells in ATCC-formulated Eagle's Minimum Essential medium (EMEM). NIH:OVCAR-3 cells were maintained in RPMI-1640 Medium (Life Technologies, Belgium). All growth media were completed by adding 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin), and incubated at 37 °C with 5% CO₂ in air.

SK-OV-3 represents the parental cell line of SK-OV-3lucIP1, the SK-OV-3 cell line was established based on ascitic fluid from a 64-year-old woman. SK-OV-3 cells were derived from an adenocarcinoma and carries mutations in TP53 and PIK3CA. The SK-OV-3lucIP1 cells were established after one intraperitoneal passage of SK-OV-3luc cells in mice, as described by De Vlieghere et al. (11). The PA-1 cell line presents with an NRAS mutation and is derived from a 12-year-old girl diagnosed with a teratocarcinoma or ovarian mixed-germ cell tumor. Both Caov-3 and NIH:OVCAR-3 were derived from an ovarian adenocarcinoma. Caov-3 cells were established from a 54-year-old woman and carry mutations in TP53 and PIK3CA whereas NIH:OVCAR-3 cells were derived from a 60-year-old woman and present with a TP53 mutation. The PM-LGSOC-01 cells were derived from a peritoneal metastasis-patient derived xenograft model established based on fresh tumor tissue from a 60-year-old woman suffering from low grade serous ovarian cancer, as described by De Thaye et al. (12). These cells do present a KRAS mutation.

b. Drug treatments

Cells were suspended in culture medium and added to a 96-well plate in a volume of 200 μ L with a concentration of 6 000 cells. Cells were allowed to attach for 72 hours before treating them with paclitaxel (PTX, Enzo Life Sciences BVBA, Belgium). A 1% EtOH-solution in complete growth medium was used as control solution (0 nM) and vehicle for PTX-containing solutions. PTX concentrations in the clinically relevant concentration range of 1 to 1 000 nM(13) were studied. After incubating the cells with 0, 1, 10, 100 or 1000 nM PTX for 24 hours, wells were washed with phosphate buffered saline and complete growth medium before adding 200 μ L of fresh complete growth medium. Every 24h, up to 120h after PTX treatment, medium was collected, levels of released ccCK18 biomarker were measured and an MTS assay was performed. For every concentration and time point, the experiment was performed in fourfold.

c. MTS assay

The MTS assay was performed according to the manufacturer's instructions, using the CellTiter 96® AQueous One Solution cell proliferation assay (Promega, USA). Absorbance was measured at 490 nm using the Infinite® M200 PRO NanoQuant plate reader (TECAN, Switzerland). Cell counts were assessed by performing MTS assays in the presence of standard curves using increasing cell amounts.

d. Assessment of ccCK18 by ELISA

The cell culture medium aspirated from the wells was first centrifuged for 5 min at 1000 rpm and then aliquoted and stored at -80°C until analysis. In order to be certain that only cell-free supernatant was collected, an examination under the AE2000 binocular microscope (Motic Instruments Inc., Canada) was performed. Levels of ccCK18 were quantitatively determined, according to the manufacturer's instructions, using the M30 Apoptosense® ELISA kit (PEVIVA, Sweden).

e. Cell lysates and Western blot analysis

Proteins were extracted from untreated cancer cells using the Laemmli lysis buffer (0.125 M Tris-HCl, 10% glycerol, 2.3% sodium dodecyl sulfate (SDS), pH 6.8). Cell lysates were suspended in reducing sample buffer (1 M Tris-HCl, 30% glycerol, 6% SDS, 3% β -mercaptoethanol, 0.005% bromophenol blue, pH 6.8) and boiled for 5 minutes at 95°C . 25 μ g proteins of each cell line were exposed to a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After blocking the membranes using 5% non-fat milk in phosphate-buffered saline (PBS) with 0.5% Tween® 20 (Sigma-Aldrich, Belgium), the blot was incubated overnight at 4°C with mouse monoclonal anti-human cytokeratin 18 (R&D Systems, United Kingdom). After washing the membrane, incubation with HRP-conjugated anti-mouse IgG secondary antibody was performed at room temperature for 1 hour. The WesternBright Quantum HRP substrate (Advansta, Menlo Park, CA, USA) was added to the membranes to capture the luminescent signal using the Proxima 2850 Imager (IsoGen Life Sciences, De Meern, The Netherlands). Equal loading of samples was verified by primary antibodies mouse monoclonal anti-GAPDH (clone GAPDH-71.1, Sigma-Aldrich, Belgium).

f. Phase-nonspecific pharmacodynamic model for cell count

Cell count data were analyzed using a phase-nonspecific pharmacodynamic model with NONMEM® (version 7.3.0, ICON, Hanover, MD, USA) based on the work by Mouton et al. (1997)(14), Nielsen et al. (2007)(15) and Rao et al. (2016) (16).

See formula 1 in the supplementary files.

where N refers to the cell number, t is time, k_{ng} is the net growth rate constant and E_{max} refers to the maximum PTX-induced treatment effect. $Conc$ refers to the concentration of PTX to which cells were exposed and $C50$ denotes the concentration at which half of the maximum effect is reached.

There was also a term *base_count* included in this model to take into account the estimated cell numbers present before treatment with PTX. Population parameters and error variance were estimated using the first-order (FO) estimation routine.

g. Phase-nonspecific pharmacodynamic model for released ccCK18 levels

The ccCK18 release data were also analyzed using a phase-nonspecific pharmacodynamic model with NONMEM® (version 7.3.0, ICON, Hanover, MD, USA). The term k_{prod} was used here as the net production rate constant of extracellular ccCK18. Parameters such as the maximum amount of released marker (A_{max}) and the Hill coefficient γ , to describe the steepness of the relationship between PTX concentration and response, were added. A refers here to the extracellular amount of ccCK18.

See formula 2 in the supplementary files.

Also here the *base_amount* term was included in the model to estimate the initial amount of released ccCK18 before exposure to PTX. Population parameters and error variance were estimated using the first-order (FO) method.

2. IN VIVO STUDY

a. PA-1 and SK-OV-3LucIP1 xenograft models

The human ovarian cancer cell lines PA-1 and SK-OV-3LucIP1 cells were used to xenograft SCID/Beige mice (mice were commercially obtained from Envigo, The Netherlands). SK-OV-3LucIP1 cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) and PA-1 cells in ATCC-formulated Eagle's Minimum Essential medium (EMEM). All growth media were completed by adding 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). Cells were incubated at 37 °C with 5% CO₂ in air. 7 to 9-week-old female SCID/Beige mice were unilaterally subperitoneally injected with 1×10^6 cancer cells (1:1 serum free medium:Matrigel (Corning, The Netherlands). In total, 20 PA-1-xenografted mice and 30 SK-OV-3LucIP1-xenografted mice were studied. The control groups were based on 10 PA-1- and 15 SK-OV-3LucIP1-xenografted mice, respectively. The maximum caging density was 6

from the same experimental group. All mice were maintained on a regular diurnal lighting cycle (12:12 light:dark) with ad libitum access to food and water. Mice were monitored once every day.

b. Single dose study

Mice were randomly assigned to the placebo or treatment group after subperitoneal tumor cell injection. PTX (Abraxane®, Celgene, US) was intraperitoneally (ip, 50 mg/kg) administered to the treatment group 2.5 weeks after tumor cell injection whereas the control group received an intraperitoneal injection of 0.9% NaCl (placebo) in an equal volume at the same time. Whole blood was collected via cardiac puncture in K3EDTA-treated Sarstedt (B.V.B.A. Berchem, Belgium) Microvette® 200 tubes, prior to obtain plasma. Samples were collected up to 2 weeks after PTX administration to measure ccCK18 levels. Blood collection via cardiac puncture was considered a terminal procedure before mice were euthanized using an isoflurane overdose prior to cervical dislocation. Every individual mouse was considered an experimental unit in this study. The animals were excluded from the study if no tumor was present 2.5 weeks after tumor cell injection, which was not the case here.

c. Measurement of tumor volume

Tumor volume in xenografted mice was assessed using ultrasound imaging. Transparent ultrasound transmission Polaris II gel (Ondes & Rayons Medical, France) was applied to bare skin and a MicroScan™ MS550D (22–55 MHz, VisualSonics Inc., Canada) transducer with the Vevo® 2100 imaging system (VisualSonics Inc., Canada) was used to analyze the tumor volume in Vevo LAB 1.7.1 (VisualSonics Inc., Canada). Tumor volumes (in mm³) were measured before receiving the single dose of PTX and before the cardiac puncture at the time of sacrifice.

d. Tissue processing and immunohistochemistry

Tumor tissues collected from mice were fixed overnight in neutral buffered 10% formalin solution (Sigma-Aldrich, Belgium) and processed in the tissue core facility at Ghent University Hospital.

e. Assessment of caspase-cleaved CK18 by ELISA

Collected plasma samples were aliquoted and stored at –80 °C until analysis. Levels of ccCK18 were quantitatively determined, according to the manufacturer's instructions, using the M30 Apoptosense® ELISA kit (PEVIVA, Sweden).

Results

1. IN VITRO STUDY

a. Effect of paclitaxel on ovarian cancer cell panel

Compared to the control solution, PTX concentrations of 1 to 1 000 nM were found to affect cell counts over time for all tested cell lines. Table 1 shows the estimated parameters, their relative standard errors (RSE) and 95% confidence intervals (CIs) for the phase-nonspecific models on cell count.

All timepoints, up to 120h post treatment, were taken into account in this model for all cell lines, except for SK-OV-3. As for the SK-OV-3 profiles over time only a clear difference between PTX concentrations was noticed starting 96h post treatment, only the last two timepoints were included in the model.

From Table 1 we see that all parameters are estimated with good precision except for C50 in SK-OV-3 and SK-OV-3lucIP1 cells as the large confidence intervals (CIs) are indicative for a high level of uncertainty. The estimated C50s for SK-OV-3lucIP1 and SK-OV-3 were 85.2 nM and 6 080 nM, respectively, suggesting that SK-OV-3lucIP1 is less sensitive compared to the other studied cell lines but with an estimated C50 within the tested concentration range whereas SK-OV-3 is rather insensitive to PTX with an estimated C50 outside the tested concentration range. To avoid difficulties with the estimation fitted, a net growth rate parameter was included for each cell line instead of separate rate constants for natural cell growth and cell death. The estimated net growth rate constant was lowest in SK-OV-3 (0.0036 h^{-1}) and highest in SK-OV-3lucIP1 (0.0164 h^{-1}). For SK-OV-3, we were not able to separately estimate E_{max} and C50 due to the limitations of the data, hence E_{max} was fixed to the mean E_{max} of all five other cell lines. The estimated E_{max} parameter was lowest in PM-LGSOC-01 (0.0151 h^{-1}) compared to similar estimates of about 0.025 h^{-1} for the other cell types. We also found that the estimated base_count parameter, representing the amount of cells that were present prior to exposure to PTX, was different across cell lines. Based on these estimates, cell division and growth is considered being slower in SK-OV-3lucIP1 in contrast to the other cell lines with PM-LGSOC-01 having the highest amount of cells at the start of the therapy.

b. Western blotting

Figure 1 illustrates the differences in CK18 expression at PTX-free cell level. These results show that expression of CK18 is lower in SK-OV-3lucIP1 compared to the other cell lines.

c. In vitro release of ccCK18

The estimated values for the fitted phase-nonspecific pharmacodynamic model on released ccCK18 levels are presented in Table 2, accompanied by the percent relative standard error (RSE) and 95% confidence intervals (CIs) for all parameters. This table illustrates a slower release rate for ccCK18 (K_{prod}) in PM-LGSOC-01 (0.0155 h^{-1}) and PA-1 (0.0177 h^{-1}) cells compared to the other cell lines. The estimated E_{max} , the effect of PTX on release of ccCK18 was highest in PM-LGSOC-01 (0.0428 h^{-1}) followed by PA-1 (0.02 h^{-1}), SK-OV-3 (0.0128 h^{-1}), NIH:OVCA-3 (0.0087 h^{-1}), Caov-3 (0.008 h^{-1}) and SK-OV-3lucIP1 (0.0076 h^{-1}). An almost 11-fold difference in C50 value was observed between NIH:OVCA-3 (8.8 nM) and Caov-3 (0.796 nM) cells. A Hill coefficient of 8.02 was estimated for PM-LGSOC-01 compared to 2.21 for SK-OV-3, indicating a steeper drug concentration-effect relationship in PM-LGSOC-01 compared to SK-OV-3. Based on the base_amount and A_{max} parameters, SK-OV-3lucIP1 and SK-OV-3 had the lowest initial extracellular

ccCK18 levels and also the lowest levels of maximum releasable amounts of ccCK18. For SK-OV-3lucIP1, the lower estimated $A_{\max}$ value of 907 was also in line with the western blot result as expression of CK18 was demonstrated to be lower compared to the other cell types.

d. Relationship between cell count data and released ccCK18 levels

Figure 2 illustrates the relationship between cell count and released ccCK18 levels. Here, the model predictions for both cell count and released levels of ccCK18 are plotted over time. The calculated Spearman's rank correlation coefficient was highest for SK-OV-3lucIP1 (0.89, 95% CI: 0.75-0.95), followed by PA-1 (0.87, 95% CI: 0.71-0.94), NIH:OVCAR-3 (0.76, 95% CI: 0.51-0.89), PM-LGSOC-01 (0.73, 95% CI: 0.45-0.87), SK-OV-3 (0.71) and Caov-3 (0.64, 95% CI: 0.32-0.83). Changes in cell count are associated with proportionally larger increases in ccCK18 for PM-LGSOC-01 and SK-OV-3. This is seen from Figure 2 where most data points lie above the identity line. In contrast, reductions in cell count, at higher PTX levels, are accompanied with less than proportional increases in ccCK18 for NIH:OVCAR-3. As observed from Figure 2, at later timepoints, ccCK18 levels start decreasing or reach a plateau.

2. IN VIVO STUDY

a. Relationship between tumor volume and released ccCK18 levels

Due to the observed differences in sensitivity to PTX and ccCK18 release in vitro, xenografts based on PA-1 and SK-OV-3lucIP1 cells were selected to be studied in vivo. Figure 3 illustrates the in vivo observed relationship between tumor volume and released ccCK18 levels. Regarding tumor volume, it is interesting to see that it takes about 1 week before a drop in tumor volume was observed in the PA-1 xenografts whereas no clear drop in tumor volume was observed for the SK-OV-3lucIP1 xenografts. When looking at the released ccCK18 levels, SK-OV-3lucIP1 tumors present with more similar released ccCK18 levels over time between control and treatment group whereas the PTX-treated PA-1 tumors clearly show an initial peak of ccCK18 after PTX treatment before normalizing after about 1 week.

b. Immunohistochemistry

From both the control and 50 mg/kg intraperitoneally treated PA-1 and SK-OV-3lucIP1 groups, tissue samples were stained for H&E, proliferation marker Ki67 and CK8-18 marker. The latter was only studied in the control groups. In addition, it is important to note that both cancer cell lines do represent a very different type of ovarian cancer (ovarian serous cystadenocarcinoma SK-OV-3lucIP1 versus ovarian mixed germ cell tumor PA-1). In Figure 4, expression of Ki67 confirmed the ongoing tumor cell proliferation in both cell line-derived xenografts post therapy. Expression of CK8-18 in the control group illustrated the basis for later potential caspase cleavage caused by PTX.

Discussion

As many chemotherapeutic agents cause apoptosis, the potential of ccCK18 as a quantitative pharmacodynamic biomarker and hence, a helpful decision tool in treatment optimization was evaluated here. In this study, next to observed differences in sensitivity to PTX, the correlation between cell count and apoptotic cell death marker ccCK18 was shown in ovarian cancer cell lines SK-OV-3lucIP1, PA-1, NIH:OVCAR-3, PM-LGSOC-01, SK-OV-3 and Caov-3. Based on the in vitro results, SK-OV-3lucIP1 and PA-1 xenografts were studied in vivo representing, respectively, a model less sensitive and sensitive to PTX. In vivo, a correlation between released ccCK18 levels and tumor volume was observed in PA-1 xenografts whereas no clear effect of PTX was found on tumor volume or ccCK18 levels over time in the SK-OV-3lucIP1 xenografted mice.

The C50 values observed, based on the in vitro model, are hard to compare with previously published work due to differences in experimental conditions (such as drug concentration and exposure time). Compared to our C50 of about 6 000 nM for the SK-OV-3 cells, Au et al. (17) found a C50 equal to 5 nM for SK-OV-3 exposing the cells 24h to PTX and measuring the drug effect after 96h. Possible reasons for the variability in observed C50 value for SK-OV-3 in both studies could be related to cell cycle, confluency and/or passage effects (18). It is also important to highlight that in this experiment C50 was estimated considering a 1 week period whereas commonly only one single timepoint is used. In addition, the difference in C50 values reported between cell types can also be explained by heterogeneous responses to anticancer treatments (19).

Brandt and colleagues (2010)(7) concluded that circulating levels of ccCK18 might be a useful biomarker to monitor treatment response in patients with gastrointestinal cancers. A correlation between intact and caspase-cleaved forms of CK18 levels and clinical response to therapy in breast cancer was observed by Olofsson *et al.* (2007)(8). In patients with disseminated testicular germ cell cancer, de Haas *et al.* (2008) observed changes in total and caspase-cleaved CK18 during chemotherapy(9). As a result, measuring soluble keratin protein fragments in the clinic is believed to be of great value for the early detection of tumor progression, metastasis formation and for a quick evaluation of the therapeutic response in epithelial malignancies (20). Outside the field of oncology, serum CK18 levels have also been studied as cell death markers in (alcoholic) hepatitis(21, 22) and in patients with cirrhosis(23) or drug-induced liver injury(24) .

In our in vitro study, at later time points, deviating biomarker release patterns were observed which can be explained by the loss of M30 reactivity as reported in apoptotic cells(25) or by the different ongoing processes during cell death(26). As PTX induces cell death processes, Lieu at al. (27) described the apoptogenic mechanisms after PTX treatment to be mitotic arrest and microtubule damage, the first one induced at PTX concentrations below 200 nM and the last one at higher PTX concentrations. As a result, high concentrations of PTX are able to induce apoptosis independently of mitotic arrest at any phase of the cell cycle.

Next to differences in CK18 expression at the cell level(28), also variation in population doubling time between these different cell lines may explain our observed differences in ccCK18 release post therapy.

As a result, a higher number of cell divisions will be accompanied by a longer duration of drug present to a larger number of cells. Next to an extended drug effect, the previously mentioned phenomenon might also cause an increase in the specific cell fraction subjected to PTX toxicity (17). In addition, the cell fraction passing through the mitotic phase will also play a crucial role with regard to the release of ccCK18, since PTX-induced cell cycle arrest and apoptosis are mainly occurring in the mitotic phase (29). We did not discriminate between the G1, S, G2 and M phase in this study in accordance with the clinical setting in which equally no cellular analysis of the complete tumor is possible before treating the patient.

Based on our *in vivo* observations, it can be concluded that the release of ccCK18 into the blood circulation happens early, before the effect of PTX on tumor volume was observed in PA-1 xenografts. This is also in agreement with the early-stage apoptosis reflected by the M30 ELISA as M30 does not measure late apoptosis stages including generation of secondary CK18 caspase cleavage products(30). Induction of apoptosis by chemotherapeutic agents is typically characterized by slow kinetics over 24 hours. Regarding paclitaxel, as this compound is retained in tumors for over 5 days (31), CK18 is typically cleaved after more than 12 hours of drug exposure (32). Levels of ccCK18 are likely a better marker to inform on PTX-induced tumor cell death for the PA-1 group compared to the SK-OV-3lucIP1 group. Nevertheless, the small number of animals used did not allow to draw firm conclusions on the potential of ccCK18 to provide significant information regarding chemotherapeutic responses.

Given the complexity of a disease like ovarian cancer and the variety of cell death processes, we believe another potential explanation for our *in vivo* findings, i.e. to consider ccCK18 a better marker for effects of PTX on PA-1 cells compared to SK-OV-3lucIP1 cells, might be related to the involvement of Akt(33). The serine-threonine kinase Akt is known to exert anti-apoptotic effects through several downstream targets. It is also known that Akt is cleaved during mitochondrial-mediated apoptosis in a caspase-dependent manner. The observation that cleavage of Akt occurs during apoptosis suggests that either a level of baseline Akt signaling is vital for cell survival or that Akt activation occurs during apoptosis and acts as a 'brake' on this process (34). Since it is known that Akt inactivation sensitizes human ovarian cancer cells to paclitaxel, Kim and colleagues (2007)(33) explored the difference in Akt phosphorylation levels between PTX-sensitive PA-1 cells and PTX-resistant SK-OV-3 cells. In their study, a higher level of phosphorylated Akt in SK-OV-3 cells compared to PA-1 cells was shown. As a result, the link between Akt activity, PTX resistance and paclitaxel-induced apoptosis was hypothesized(35). Our *in vivo* findings are also in line with this as we concluded that SK-OV-3lucIP1 is associated with less ccCK18 release as a result of less paclitaxel-induced caspase cleavage of CK18 compared to PA-1. Taking into account the higher Akt activity in SK-OV-3 cells observed by Kim *et al.* (2007)(33), mediation of survival signals by Akt might preserve SK-OV-3lucIP1 cells from apoptotic pathways.

In order to further substantiate these findings, future studies focusing on different chemotherapeutic agents, both single drugs and combination therapies, will be of great interest. In addition, also the focus on other types of cancer, besides ovarian cancer, is needed to study the broader applicability of the apoptotic biomarker ccCK18 in its role to optimize individual chemotherapeutic strategies.

Conclusion

In conclusion, our in vitro study showed evidence of an association between PTX-induced cell toxicity and released ccCK18 levels. Based on our in vivo study with PTX-sensitive xenografts, we found that response to PTX was immediately shown as a peak in released ccCK18 whereas an observed decrease in tumor volume was rather delayed. Overall, these experiments indicate the potential of ccCK18 to inform on drug-induced cell death which can be informative to optimize cancer treatment.

Declarations

Ethics approval and consent to participate

Animal experiments were conducted in accordance with the local ethics committee (ECD 16/11, Ghent University Hospital). The reported study does not involve human participants, human data or human tissue.

Consent for publication

Not applicable as this manuscript does not contain data from any individual person.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

AV is an employee of Johnson & Johnson and holds stock/stock options in the company. All other authors declare no conflict of interest.

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Authors' contributions

EDT acquired and analyzed in vitro and in vivo data, wrote the manuscript. KVdV and JVD performed pathological studies. AV and PC participated in data analysis. JVB participated in coordination of the project. Critical revision of the manuscript and approval of the final version: all authors.

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Abbreviations

CK18 : cytokeratin 18

ccCK18: caspase-cleaved cytokeratin 18

PTX: paclitaxel

CK8: cytokeratin 8

ELISA: enzyme-linked immunosorbent assay

ATCC: American Type Culture Collection

DMEM: Dulbecco's Modified Eagle's Medium

EMEM: Eagle's Minimum Essential medium

FBS: fetal bovine serum

CI: confidence interval

RSE: relative standard error

K_{ng}: net growth rate constant

E_{max}: maximum effect

C₅₀: concentration at 50% effect

K_{prod}: net production rate constant

A_{max}: maximum amount of released marker

References

1. M. B. Omary, N. O. Ku, J. Liao, Price D. Keratin modifications and solubility properties in epithelial cells and in vitro. Sub-cellular biochemistry. 1998;31:105-40.
2. E. Fuchs, Cleveland DW. A structural scaffolding of intermediate filaments in health and disease. Science. 1998;279(5350):514-9.

3. H. Kumemura, M. Harada, C. Yanagimoto, H. Koga, T. Tawaguchi, S. Hanada, et al. Mutation in keratin 18 induces mitochondrial fragmentation in liver-derived epithelial cells. *Biochemical and Biophysical Research Communications*. 2008;367(1):33-40.
4. R. Moll. Cytokeratins as markers of differentiation in the diagnosis of epithelial tumors. *Sub-cellular biochemistry*. 1998;31:205-62.
5. C. Caulin, G.S. Salvesen, Oshima RG. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *Journal of Cell Biology*. 1997;138(6):1379-94.
6. Nancy A. Thornberry, Lazebnik Y. Caspases: Enemies Within. *Science*. 1998;281(5381):1312-6.
7. Brandt D, Volkmann X, Anstätt M, Länger F, Manns MP, Schulze-Osthoff K, et al. Serum biomarkers of cell death for monitoring therapy response of gastrointestinal carcinomas. *European Journal of Cancer*. 2010;46(8):1464-73.
8. Olofsson MH, Ueno T, Pan Y, Xu R, Cai F, van der Kuip H, et al. Cytokeratin-18 Is a Useful Serum Biomarker for Early Determination of Response of Breast Carcinomas to Chemotherapy. *Clinical Cancer Research*. 2007;13(11):3198.
9. de Haas EC, di Pietro A, Simpson KL, Meijer C, Suurmeijer AJH, Lancashire LJ, et al. Clinical Evaluation of M30 and M65 ELISA Cell Death Assays as Circulating Biomarkers in a Drug-Sensitive Tumor, Testicular Cancer. *Neoplasia*. 2008;10(10):1041-8.
10. S. Linder, A. M. Havelka, T. Ueno, Shoshan MC. Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. *Cancer Letters*. 2004;214(1):1-9.
11. Elly De Vlieghere, Charlotte Carlier, Wim Ceelen, Marc Bracke, Wever OD. Data on in vivo selection of SK-OV-3 Luc ovarian cancer cells and intraperitoneal tumor formation with low inoculation numbers. *Data in Brief*. 2016;6:542-9.
12. De Thaye E, Van de Vijver K, Van der Meulen J, Taminiau J, Wagemans G, Denys H, et al. Establishment and characterization of a cell line and patient-derived xenograft (PDX) from peritoneal metastasis of low-grade serous ovarian carcinoma. *Scientific Reports*. 2020;10(1):6688.
13. Jang SH, Wientjes MG, Au JLS. Determinants of Paclitaxel Uptake, Accumulation and Retention in Solid Tumors. *Investigational New Drugs*. 2001;19(2):113-23.
14. Mouton JW, Vinks AA, Punt NC. Pharmacokinetic-pharmacodynamic modeling of activity of ceftazidime during continuous and intermittent infusion. *Antimicrob Agents Chemother*. 1997;41(4):733-8.
15. Nielsen EI, Viberg A, Löwdin E, Cars O, Karlsson MO, Sandström M. Semimechanistic pharmacokinetic/pharmacodynamic model for assessment of activity of antibacterial agents from time-kill curve experiments. *Antimicrob Agents Chemother*. 2007;51(1):128-36.
16. Rao GG, Ly NS, Tsuji BT, Bulitta JB, Forrest A. Translational Modeling of Antibacterial Agents. In: Mager DE, Kimko HHC, editors. *Systems Pharmacology and Pharmacodynamics*. Cham: Springer International Publishing; 2016. p. 371-402.
17. J. L. S. Au, D. Li, Y.B. Gan, X. Gao, A.L. Johnson, J. Johnston, et al. Pharmacodynamics of immediate and delayed effects of paclitaxel: Role of slow apoptosis and intracellular drug retention. *Cancer*

Research. 1998;58(10):2141-8.

18. Wang Jin, Catherine J. Penington, Scott W. McCue, Simpson MJ. A computational modelling framework to quantify the effects of passaging cell lines. *PLOS ONE*. 2017;12(7):e0181941.
19. Benjamin Haibe-Kains, Nehme El-Hachem, Nicolai Juul Birkbak, Andrew C. Jin, Andrew H. Beck, Hugo J. W. L. Aerts, et al. Inconsistency in large pharmacogenomic studies. *Nature*. 2013;504(7480):389-93.
20. V. Barak, H. Goike, K. W. Panaretakis, Einarsson R. Clinical utility of cytokeratins as tumor markers. *Clinical Biochemistry*. 2004;37(7):529-40.
21. Parfieniuk-Kowerda A, Lapinski TW, Rogalska-Plonska M, Swiderska M, Panasiuk A, Jaroszewicz J, et al. Serum cytochrome c and m30-neoepitope of cytokeratin-18 in chronic hepatitis C. *Liver Int*. 2014;34(4):544-50.
22. Bissonnette J, Altamirano J, Devue C, Roux O, Payance A, Lebrech D, et al. A Prospective Study of the Utility of Plasma Biomarkers to Diagnose Alcoholic Hepatitis. *Hepatology*. 2017;66(2):555-63.
23. Macdonald S, Andreola F, Bachtiger P, Amoros A, Pavesi M, Mookerjee R, et al. Cell death markers in patients with cirrhosis and acute decompensation. *Hepatology*. 2018;67(3):989-1002.
24. Church RJ, Kullak-Ublick GA, Aubrecht J, Bonkovsky HL, Chalasani N, Fontana RJ, et al. Candidate biomarkers for the diagnosis and prognosis of drug-induced liver injury: An international collaborative effort. *Hepatology*. 2019;69(2):760-73.
25. Tao GZ, Li DH, Zhou Q, Toivola DM, Strnad P, Sandesara N, et al. Monitoring of epithelial cell caspase activation via detection of durable keratin fragment formation. *J Pathol*. 2008;215(2):164-74.
26. Yeung TK, Germond C, Chen X, Wang Z. The Mode of Action of Taxol: Apoptosis at Low Concentration and Necrosis at High Concentration. *Biochemical and Biophysical Research Communications*. 1999;263(2):398-404.
27. C. H. Lieu, Y. N. Chang, Lai YK. Dual cytotoxic mechanisms of submicromolar taxol on human leukemia HL-60 cells. *Biochemical Pharmacology*. 1997;53(11):1587-96.
28. Stimpfl M, Schmid BC, Schiebel I, Tong D, Leodolter S, Obermair A, et al. Expression of mucins and cytokeratins in ovarian cancer cell lines. *Cancer letters*. 1999;145(1-2):133-41.
29. M.A. Jordan, R. J. Toso, D. Thrower, Wilson L. Mechanism of mitotic block and inhibition of cell-proliferation by taxol at low concentrations. *Proceedings of the National Academy of Sciences of the United States of America*. 1993;90(20):9552-6.
30. Ku N-O, Strnad P, Bantel H, Omary MB. Keratins: Biomarkers and modulators of apoptotic and necrotic cell death in the liver. *Hepatology*. 2016;64(3):966-76.
31. Mori T, Kinoshita Y, Watanabe A, Yamaguchi T, Hosokawa K, Honjo H. Retention of paclitaxel in cancer cells for 1 week in vivo and in vitro. *Cancer Chemother Pharmacol*. 2006;58(5):665-72.
32. Gerhard H. Cytokeratin 18 (CK18) and Caspase-Cleaved CK18 (ccCK18) as Response Markers in Anticancer Therapy, Cytokeratins - Tools in Oncology IntechOpen. 2012.

33. Kim S-H, Juhnn Y-S, Song Y-S. Akt Involvement in Paclitaxel Chemoresistance of Human Ovarian Cancer Cells. *Annals of the New York Academy of Sciences*. 2007;1095(1):82-9.
34. Tang D, Okada H, Ruland J, Liu LQ, Stambolic V, Mak TW, et al. Akt is activated in response to an apoptotic signal. *Journal of Biological Chemistry*. 2001;276(32):30461-6.
35. Page C, Lin H, Jin Y, Castle V, Nunez G, Huang M, et al. Overexpression of Akt/AKT can modulate chemotherapy-induced apoptosis. *Anticancer research*. 2000;20:407-16.

Tables

Table 1. Phase-nonspecific model parameters on cell count

	PM-LGSOC-01	Caov-3	NIH:OVCAR-3	PA-1	SK-OV-3lucIP1	SK-OV-3
Kn _g (h ⁻¹)	0.0077	0.0038	0.0089	0.0055	0.0164	0.0036
(RSE%)	(8%)	(20%)	(7%)	(10%)	(7%)	(27%)
(95% CI)	(0.007-0.009)	(0.002-0.005)	(0.008-0.01)	(0.004-0.007)	(0.014-0.019)	(0.002-0.005)
E _{max} (h ⁻¹)	0.0151	0.0236	0.0299	0.0288	0.0253	0.0245 FIX
(RSE%)	(4%)	(6%)	(3%)	(3%)	(14%)	
(95% CI)	(0.014-0.016)	(0.021-0.026)	(0.028-0.031)	(0.027-0.031)	(0.018-0.032)	
C50 (nM)	4.02	4.85	7.24	22.3	85.2	6 080
(RSE%)	(14%)	(27%)	(15%)	(10%)	(56%)	(55%)
(95% CI)	(2.905-5.135)	(2.263-7.437)	(5.143-9.337)	(17.733-26.867)	(-7.9-178.3)	(-505.6-12665.6)
Base_count	89 322	10 615	15 522	36 316	3 905	44 356
(RSE%)	(0%)	(1%)	(0%)	(0%)	(1%)	(1%)
(95% CI)	(80 018-99 708)	(9 653-11 673)	(14 705-16 384)	(33 423-39 459)	(3 492-4 368)	(37 161-52 945)

Table 2. Phase-nonspecific model parameters on released ccCK18

	PM-LGSOC-01	Caov-3	NIH:OVCAR-3	PA-1	SK-OV-3lucIP1	SK-OV-3
k_{prod} (h ⁻¹)	0.0155	0.0267	0.0333	0.0177	0.0228	0.0237
(RSE%)	(5%)	(3%)	(3%)	(4%)	(4%)	(8%)
(95% CI)	(0.014-0.017)	(0.025-0.028)	(0.031-0.035)	(0.016-0.019)	(0.021-0.025)	(0.02-0.027)
k_{max} (h ⁻¹)	0.0428	0.008	0.0087	0.02	0.0076	0.0128
(RSE%)	(3%)	(9%)	(9%)	(3%)	(5%)	(9%)
(95% CI)	(0.041-0.045)	(0.007-0.009)	(0.007-0.01)	(0.019-0.021)	(0.007-0.008)	(0.011-0.015)
IC_{50} (nM)	8.13	0.796	8.8	1.49	7.37	4.53
(RSE%)	(2%)	(3%)	(6%)	(3%)	(5%)	(28%)
(95% CI)	(7.752-8.508)	(0.756-0.836)	(7.724-9.876)	(1.407-1.573)	(6.659-8.081)	(2.06-7)
kill factor γ	8.02	5.46	6.71	5.31	6.58	2.21
(RSE%)	(8%)	(16%)	(24%)	(5%)	(3%)	(30%)
(95% CI)	(6.83-9.21)	(3.751-7.169)	(3.515-9.905)	(4.747-5.873)	(6.249-6.911)	(0.928-3.492)
use_amount (pM)	137	203	102	150	20.5	22.2
(RSE%)	(2%)	(1%)	(1%)	(1%)	(2%)	(4%)
(95% CI)	(119-159)	(185-222)	(91.4-113)	(136-165)	(17.8-23.5)	(17.5-28.2)
k_{max} (pM)	2 345	3 395	2 644	4 024	907	838
(RSE%)	(1%)	(1%)	(1%)	(1%)	(2%)	(3%)
(95% CI)	(2 156-2 551)	(2 097- 3 722)	(2 390-2 925)	(3 419-4 737)	(733-1 122)	(590-1 190)

Figures

Figure 1

Western blot of CK18 expression. Western blot to evaluate expression of CK18 at cell level. GAPDH was used as loading control. The original blot is presented in Supplementary Figure S1.

Figure 2

In vitro relationship between cell count and released ccCK18 levels. Relationship between cell count and released ccCK18 levels. These graphs are based on the predicted data from the different phase-nonspecific pharmacodynamic models. On the x-axis cell count is represented as the following ratio: prediction for control (0 nM) over prediction for a specific PTX level, M30 on the y-axis represents ccCK18 using the ratio calculated from prediction for a specific PTX level over prediction for control (0 nM). The grey line in all graphs illustrates the line of identity. From left to right, the symbols are directly related to

the specified time points studied (0, 24, 48, 72, 96, 120 and 144h). This figure was made using GraphPad Prism 8 (GraphPad Software, Inc., USA).

Figure 3

In vivo relationship between tumor volume and released ccCK18 levels. Relationship between tumor volume and released ccCK18 levels. The left panels represent data from PA-1 xenografts whereas the right panels illustrate the SK-OV-3lucIP1 xenografts. These graphs are based on measured tumor volume (mm³, upper panel) and measured ccCK18 levels (pM, lower panel) using the M30 Apoptosense® Elisa (PEVIVA, Sweden). Grey triangles and lines represent the PTX-treated group of cell line-derived xenografts whereas the black triangles and lines are illustrating the non-PTX-treated control groups.

Figure 4

Immunostaining of tumor tissue. Tumor tissue samples stained for H&E, proliferation marker Ki67 and CK8-18 marker (only control group) at day 5 of the experiment. Scale bars represent 1mm, except for the PTX-treated SK-OV-3lucIP1 group where the scale bar represents 200 μ m.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [figureS1.png](#)
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