research article

Phytotherapeutics oridonin and ponicidin show additive effects combined with irradiation in pancreatic cancer *in vitro*

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Background. Chemoradiation of locally advanced non-metastatic pancreatic cancer can lead to secondary operability by tumor mass reduction. Here, we analyzed radiomodulating effects of oridonin and ponicidin in pancreatic cancer *in vitro*. Both agents are ent-kaurane diterpenoids, extracted from *Isodon rubescens*, a plant that is well known in Traditional Chinese Medicine. Cytotoxic effects have recently been shown in different tumor entities for both agents.

Materials and methods. Pancreatic cancer cell lines AsPC-1, BxPC-3, Panc-1 and MIA PaCa-2 were pretreated with oridonin or ponicidin and irradiated with 2 Gy to 6 Gy. Long-term survival was determined by clonogenic assay. Cell cycle effects and intensity of γH2AX as indicator for DNA double-strand breaks were investigated by flow cytometry. Western blotting was used to study the DNA double-strand break repair proteins Ku70, Ku80 and XRCC4.

Results. Oridonin and ponicidin lead to a dose-dependent reduction of clonogenic survival and an increase in γH2AX. Combined with irradiation we observed additive effects and a prolonged G2/M-arrest. No relevant changes in the levels of the DNA double-strand break repair proteins were detected.

Conclusions. Pretreatment with oridonin or ponicidin followed by irradiation lead to an additional reduction in survival of pancreatic cancer cells *in vitro*, presumably explained by an induced prolonged G2/M-arrest. Both agents seem to induce DNA double-strand breaks but do not interact with the non-homologous end joining (NHEJ) pathway.

Key words: oridonin; ponicidin; irradiation; pancreatic cancer; γH2AX; radiosensitivity

Introduction

Pancreatic cancer is the fourth leading cause of cancer related deaths in the United States.[1] Surgical resection is still the most important treatment modality and prognostic factor, but in the late stage of the disease, only 10–20% of all primary tumours are still operable.[2] Beside primary metastasized and thus palliative stage IV disease 30-40% of all cases are locally advanced but non-metastasized tumors that could benefit from neoadjuvant treatment.[3] We recently reported that approximately one third of these locally advanced tumors become resectable by a neoadjuvant combined chemoradiation and thus regain a curative therapy option.[4] To further increase the efficacy of radiotherapy in pancreatic cancer, new chemotherapeutics, that can sensitize pancreatic cancer tissue to radiation, are needed.

The diterpenoids oridonin and ponicidin are both extracts from Isodon rubescens, a plant used in Traditional Chinese Medicine that is known for its anti-inflammatory and antitumor properties in the treatment of esophageal and cardiac cancer.[5] Recently, antineoplastic properties of oridonin and ponicidin have been published.[6-8] In pancreatic cancer oridonin induces apoptosis and leads to an arrest in the G2/M-phase of the cell cycle.[9-12] Further, oridonin inhibits nuclear factor-kB.[13] Ponicidin is also known to induce apoptosis[14-17] but to our knowledge, there is no data for ponicidin in pancreatic cancer.

Potential radiosensitization could be observed in chinese-hamster-V79 cells by oridonin.[18] Apart from this study we found no publication concerning oridonin or ponicidin induced radiomodulation.

As natural occurring products for both substances no serious side effects in humans are known which qualifies them as potential new chemotherapeutic drugs. Data of an *in vivo* trial for oridonin used in acute leukemia seems to confirm this hypothesis.[19] Here, we analyzed radiomodulating effects of oridonin and ponicidin in pancreatic cancer cells *in vitro* by clonogenic assay, cell cycle analysis, γH2AX expression and DNA repair proteins expression analysis.

Material and methods

Cell culture and irradiation

The pancreatic cancer cell lines AsPC-1, BxPC-3 and MIA PaCa-2 were cultured in RPMI-1640 medium, the cell line Panc-1 in Dulbecco's modified Eagle's Medium (DMEM). Media included 10% FBS and 1% Penicillin/Streptomycin. The cells were cultured at 37°C in 5% CO_2. AsPC-1 and Panc-1 cell lines were purchased from CLS cell line service GmbH, Germany and BxPC-3 as well as MIA PaCa-2 cells from LGC Standards GmbH (ATCC), Germany. Oridonin was obtained from Sigma-Aldrich Corporation, USA and ponicidin from Shanghai Zhanshu Chemical Technology Co., Ltd., China. Both reagents were dissolved in DMSO and stored at -80°C.

Cells were X-irradiated (X-RAD 320, Precision X-Ray, Inc., USA) operated at 320 kV and 12.5 mA. The average dose rate was 1.1 Gy/min. The used filter consisted of 1.5 mm Al, 0.25 mm Cu and 0.75 mm Sn. The tissue culture flasks, in which the cells were cultured, were placed on a PMMA-plate of 1 cm height.

Clonogenic assay

To assess long-term cell survival, a defined number of cells were seeded in 25 cm^2 tissue culture flasks and incubated for 24 hours. Cell counts were adjusted for the different treatment schemes. Cell lines AsPC-1, BxPC-3 and Panc-1 were treated with oridonin, ponicidin or DMSO (control) for 24 hours as indicated and irradiated afterwards once with 2 Gy or 6 Gy. The dose of each substance and the intensity of irradiation were established through pre-experiments to achieve a survival rate of 20-70% after single treatment alone. MIA PaCa-2 cells were pretreated with lower doses of oridonin and ponicidin due to higher sensitivity to the reagents. 8-9 days (time differs among cell lines) after the treatment cells were fixed in 70% ethanol and stained with 0.2% methylenblue. Colonies, defined as containing at least 50 cells, were counted manually under a light microscope and the plating efficiency, defined as the quotient of counted colonies to plated cells, was determined. The surviving fraction was defined as the ratio of each experiment's plating efficiency to its control. Cells were plated in triplets to compensate for pipetting errors and at least three independent experiments were performed.

Determination of radiosensitivity

To analyze combined treatments, surviving fractions were normalized to the values of corresponding single reagent treatment using a ratio of the combined treatment's plating efficiency to the single reagent treatment one's. Dose-response curves were created using the linear-quadratic model.[20]

Combination effects were analyzed defining additivity according to Steel and Peckham.[21] For this purpose a dose-response curve was calculated that represents the course of the measured control curve at a pre-effect similar to the single reagent treatment's effect („theoretical control-curve"). Additive effects were defined as area between the measured control curve and the theoretical one. Supraadditive effects were defined as beneath the theoretical control curve and radioprotective effects as above the measured control curve. Independent toxicity was defined as identical curve to the measured control curve (for further information see Suppl. Figure 1).

Cell cycle analysis

Since impact on cell cycle distribution was regressing 24 hours after pretreatment a shorter exposi-
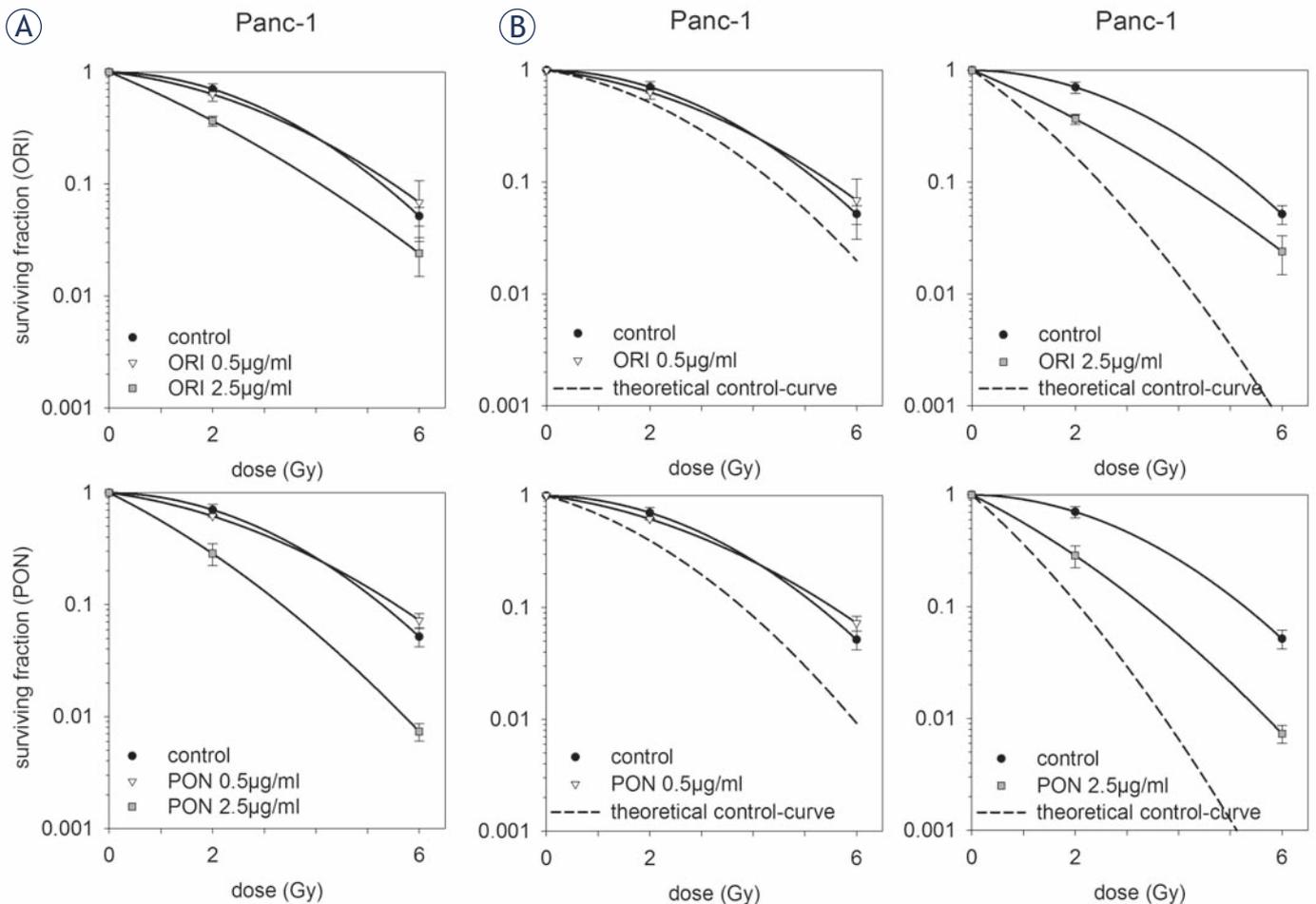
FIGURE 1. Clonogenic survival of Panc-1 cells treated with either irradiation and/or oridonin (ORI) and ponicidin (PON) as indicated. **(A)** survival curves and **(B)** radiosensitivity to different dosages with measured and theoretical control curve, according to Steel and Peckham for Panc-1 cells. Results are presented as mean values +/- standard deviation. At least three independent experiments were performed.

tion time to oridonin and ponicidin compared to the other experiments was chosen. Simultaneously the dosage was augmented to compensate for shortage in exposition time. Cell lines Panc-1 and MIA PaCa-2 were pretreated for two hours with oridonin, ponicidin or DMSO (control) and irradiated as indicated. To induce a less lethal damage than one would expect of 6 Gy, an irradiation dose of 4 Gy was chosen. Cells were then fixed in ice-cold ethanol and RNA-strands were cleaved by RNase A (AppliChem GmbH, Germany) at the following time points: beginning of the treatment, before irradiation, 12 and 24 hours after irradiation. Then cells were stained with propidium iodide (Sigma-Aldrich Corporation, USA) and the amount of propidium iodide per cell was quantified in a flow cytometer (FACScan, Becton Dickinson and Company, USA). At least 10^4 ungated cells were analyzed by BD CellQuest Pro 4.0.2 (Becton

Dickinson and Company, USA) and the different phases of the cell cycle were determined using the software ModFit LT 3.0 (Verity Software House, Inc., USA). At least two independent experiments were performed.

γH2AX-quantification

Since clonogenic assays revealed different sensitivity between AsPC-1, BxPC-3 and Panc-1 cells on the one hand and MIA PaCa-2 cells on the other hand further experiments were done with the cell lines Panc-1 and MIA PaCa-2. To quantify the induction of DNA double-strand breaks Panc-1 and MIA PaCa-2 cells were pretreated with oridonin, ponicidin or DMSO (control) for 24 hours and were irradiated once as indicated. At different time points after irradiation MIA PaCa-2 and Panc-1 cells were fixed using 1% PFA and ice-cold ethanol. Cells

were then stained with a γH2AX-antibody (art. no. 16-202A, Merck KGaA, Darmstadt, Germany) and pan-nuclear intensity was measured using a flow cytometer. Results were normalized by creating a ratio of the measured intensity to the intensity of the control probes. At least three independent experiments were performed.

Western Blot

Cell lines Panc-1 and MIA PaCa-2 were treated with oridonin, ponicidin or DMSO (control) for 24 hours. Then lysates of the cells were gained using lysis buffer (10 mM HEPES pH 7.4, 5 mM CHAPS, 5 mM DTT) and inhibitors (PMSF, Pepstatin, Leupeptin, Aprotinin). The protein concentration was determined in a Bradford protein assay and 20 μg to 30 μg Protein were used for Western Blots. A SDS-polyacrylamide-gel-electrophoresis was run using 12% polyacrylamide-gels with a voltage of 160 V and amperage of 250 mA. A wet transfer was performed (80 V, 60 mA) to transfer the proteins on a polyvinylidenfluoride membrane. The membrane was blocked using Roti-Block (Carl Roth. GmbH & Co. KG, Germany). b-Actin (art. no. 4967), GAPDH (art. no. 2118), Ku70 (art. no. 4588) and Ku80 (art. no 2753) primary antibodies were purchased from Cell signaling Technology Inc., USA. XRCC4 (art. no. sc-271087) and secondary antibodies (art. no. sc-2380, sc-2379) were obtained from Santa Cruz Biotechnology, Inc., USA. Dilution was done according to manufacturer's instructions. X-Ray films were scanned and the density of the protein bands were determined with the software Image J 1.47V (National Institute of Health, USA). Density was normalized by creating a ratio of protein band to house-keeping protein's one to avoid minimal differences in the amount of protein load. Then another ratio of the treatment's value to its control was calculated and allowed to quantify densities and treatment differences. At least two independent experiments were performed.

Statistical analysis

The results of each experiment were generated as described above. Statistical analysis of the results was done with an unpaired two-tailed t-test using Microsoft® Excel® 2008 for Mac 12.3.6 (Microsoft Corporation, USA). The dose control curves and the figures were calculated and generated with SigmaPlot 12.0 (Systat Software Inc., USA). Results are presented as mean values +/- standard deviation. P < 0.05 was considered as a significant differ-ence between the compared results. *P < 0.05, **P < 0.005, ***P < 0.001.

Results

Oridonin and ponicidin reduce clonogenic survival and show additive effects when combined with irradiation

To investigate long-term survival cells were treated with oridonin or ponicidin in a low dosage of 0.5 μg/ml and high dosage of 2.5 μg/ml (Figure 1A). Since MIA PaCa-2 cells exposed a higher sensitivity to both oridonin and ponicidin, we used 0.1 μg/ml and 0.5 μg/ml as the low and the high dosage in these cells, respectively. MIA PaCa-2 cells showed a significant reduction in clonogenic survival to a fraction of 51% when treated with the lower dosage of oridonin and thus were much more sensitive than the other cell lines, with a mean surviving fraction of 88% in AsPC-1, BxPC-3 and Panc-1 cells (Suppl. Figure 2). Interestingly, ponicidin was more effective than oridonin: 2.5 μg/ml of ponicidin lead to a mean surviving fraction of 12%. The same amount of oridonin showed an almost three-fold higher surviving fraction of 32% in AsPC-1-, BxPC-3- and Panc-1 cells (Suppl. Figure 3).

A further dose-dependent reduction in survival could be observed by combination with irradiation. The mean surviving fraction of 88% after single treatment with low dosage of oridonin could be further attenuated by additional irradiation with 2 Gy or 6 Gy to mean 63% and 13%, respectively.

Using these results we investigated the radiosensitivity according to the model of Steel and Peckham as described in the methods section.[21] The higher dose of oridonin and ponicidin lead to additive effects in almost all investigated cell lines when combined with 2 Gy or 6 Gy irradiation, whereas the lower dose just showed independent toxicity (Figure 1B and Suppl. Figure 4). Radioprotectivity as well as supraadditivity could not be observed.

G2/M-arrest after combined treatment of radiation and oridonin or ponicidin

To determine changes in cell cycle, the four pancreatic cancer cell lines were treated with oridonin or ponicidin alone as well as in combination with irradiation. Cell cycle phases were analyzed 12 and 24 hours after irradiation. Pretreatment with oridonin (Figure 2A) or ponicidin (Figure 2B) induced a clear G2/M-arrest of the cell lines AsPC-1 and Panc-1 after 12 hours with up to 36% (Figure 2 and Suppl.

Figure 5). The G2/M- fraction of combined treatments was not as high as the one observed after irradiation alone but was augmented in time leading to the fact that 24 hours after irradiation there still was a clear G2/M-arrest in the cell lines AsPC-1, BcPC-3 and Panc-1 when treated with the combined therapy. MIA PaCa-2 cells showed a clear G2/M-arrest 24 hours after combined treatment with ponicidin but not with oridonin (Suppl. Figure 5). We did not observe a sub-G1 fraction as surrogate of apoptosis.

Oridonin and poncidin induce an increased pan-nuclear γH2AX-intensity

The potential influence of oridonin or ponicidin on the DNA double-strand break repair was analyzed by measuring the pan-nuclear γH2AX-intensity of pretreated cells via flow cytometry. Both oridonin (Figure 3A) and poncidin (Figure 3B) lead to an approximately 2.5-fold increase of the γH2AX-intensity shortly after treatment, independent of the particular dose level (7.5 μg/mL $vs.$ 15 μg/mL). There was an increase of γH2AX after irradiation alone but highest γH2AX-intensities could be observed in combined treatments. The Intensity of γH2AX already declined 10 hours posttreatment. We could not observe a difference in the relative reduction of the γH2AX-intensity between irradiation alone and combined treatments.

Oridonin and ponicidin do not influence the expression of DNA repair proteins related to the non-homologous end joining (NHEJ) pathway

To determine the influence of oridoinin or ponicidin on the repair of DNA double-strand breaks, Panc-1- and MIA PaCa-2-cells were treated with either agent and three main proteins of the NHEJ pathway, namely XRCC4, Ku70 and Ku80 were detected by Western Blots. Pretreatment with oridonin or ponicidin lead to a slight reduction of XRCC4-expression but there was no considerable change in Ku70 and Ku80 protein expression in MIA PaCa-2 cells. Panc-1 cells showed no stringent effect in XRCC4 protein expression but a dose-dependent increase of Ku70 and Ku80 after pretreatment with oridonin or ponicidin (Figure 4).

Discussion

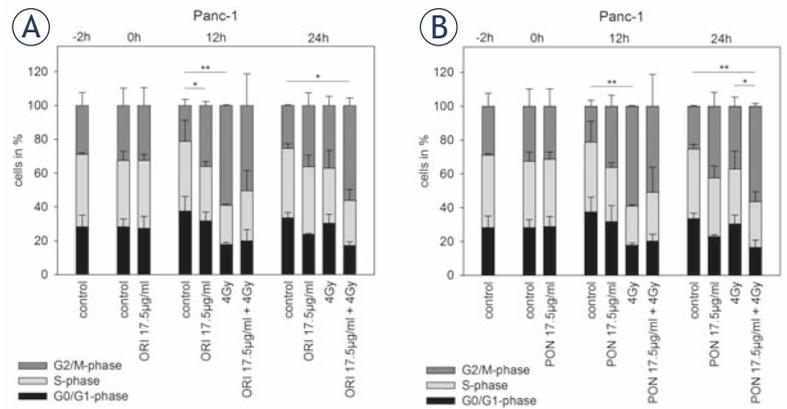The aim of this study was to investigate the two phytotherapeutics oridonin and ponicidin for their

FIGURE 2. Cell cycle assay of Panc-1 cells treated with irradiation and/or **(A)** oridonin (ORI) and **(B)** ponicidin (PON) as indicated. Results are presented as mean values +/- standard deviation. *P<0.05, **P<0.005, asterisk referring to the amount of cells in G2/M-phase. At least two independent experiments were performed.
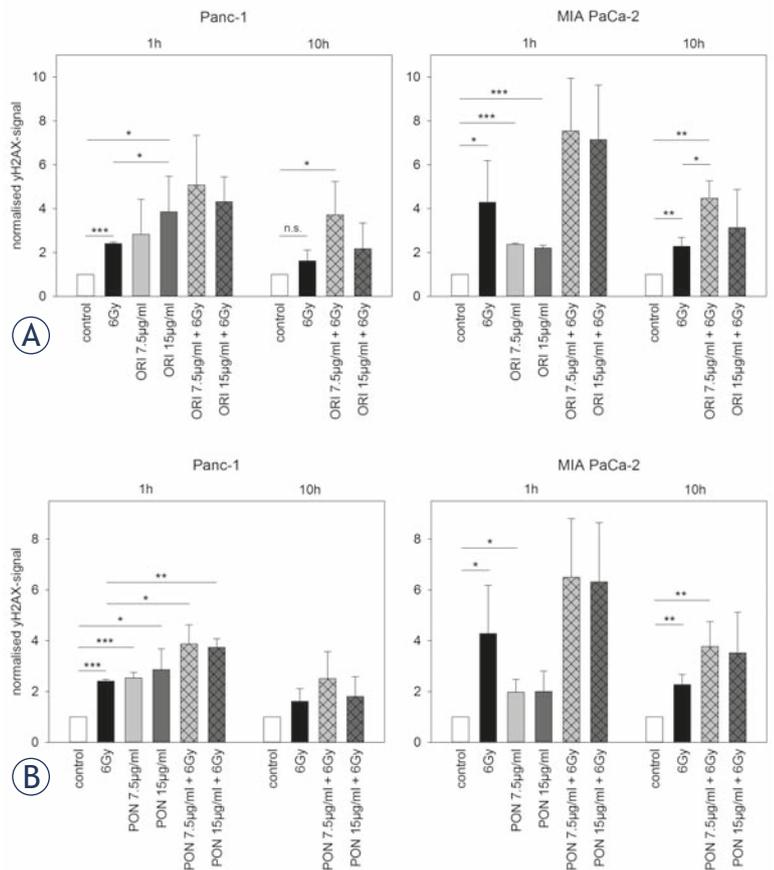
FIGURE 3. γH2AX-intensity of Panc-1 and MIA PaCa-2 cells treated with irradiation and/or **(A)** oridonin (ORI) and **(B)** ponicidin (PON) as indicated. Results are presented as mean values +/- standard deviation. *P<0.05, **P<0.005, ***P<0.001, n.s.=not significant. At least three independent experiments were performed.
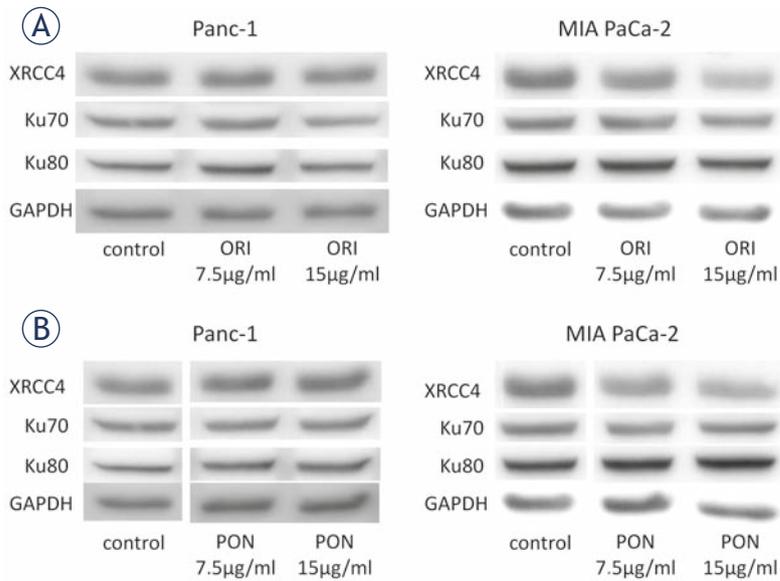
FIGURE 4. Western Blots of Panc-1 and MIA PaCa-2 cells treated with either **(A)** oridonin (ORI) and **(B)** ponicidin (PON) as indicated. One representative Western Blot is shown. At least two independent experiments were performed. NHEJ = non-homologous end-joining.

capability to enhance effects of irradiation in pancreatic cancer cells. Compared to other tumor entities pancreatic cancer cells are more resistant to chemo- and radiotherapy and thus radiosensitizing agents are highly clinically relevant.

To investigate treatment efficacy long-term survival was determined by clonogenic assays. Changes in cell cycle distribution and intensity of γH2AX as indicator for DNA double-strand breaks were investigated by flow cytometry, since effects of radiotherapy base predominantly on induction of DNA double-strand breaks.[22] Western blotting was used to study key proteins of the NHEJ pathway of the DNA double-strand break repair. To our knowledge we are the first who tested ponicidin in combination with irradiation whereas potential radiosensitzing effects of oridonin could already be observed in chinese-hamster-V79 cells.[18]

After treatment with either oridonin or ponicidin we observed a dose-dependent reduction of clonogenic survival in all tested pancreatic cancer cell lines AsPC-1, BxPC-3, MIA PaCa-2 and Panc-1 after treatment with either oridonin or ponicidin. Compared to oridonin, equal doses of ponicidin were more effective in reducing clonogenic survival. Similar results were observed by Hsieh et al. in clonogenic survival assays of the breast cancer cell line MCF-7, who also tested both agents in proliferation assays with comparable results of oridonin and ponicidin.[23] Based on these data we infer that

ponicidin might be a more potent cytostatic agent than oridonin.

After combined treatment with irradiation and different concentrations of oridonin or ponicidin we observed an increased reduction of clonogenic survival. The analysis of potential interactions revealed a trend towards additive effects for the higher concentration of oridonin as well as ponicidin. Supraadditive effects that provide evidence for actual radiosensitization could not be observed.

Cell cycle assays of the present study showed that both oridonin and ponicidin lead to an accumulation of cells in the G2/M-phase of the cell cycle. The combination of irradiation and oridonin or ponicidin leads to an even prolonged G2/M-arrest. Cells that are stuck in this phase usually present DNA-lesions, such as DNA double-strand breaks or blocked DNA replication forks, which have to be repaired before passing the G2/M-checkpoint.[24] An oridonin-induced G2/M-arrest was previously reported by Qi et al. for the pancreatic cancer cell line Panc-1 along with a suppression of cell cycle regulating cyclin B1 and cdc2.[10] In our study, we focused on DNA double-strand breaks as underlying mechanism especially after combination with irradiation. Therefore we did not investigate any changes in proteins of the cell cycle regulation but further experiments should pinpoint the precise mode G2/M-phase arrest induced by oridonin and ponicidin. Since induction of apoptosis was reported for both oridonin and ponicidin[9-12,14-17], we expected a sub-G1 peak in cell cycle analysis but could not observe any peak in the sub-G1 noise. Interestingly, the cell cycle assays of Qi et al. for PANC-1 cells treated with oridonin also did not show any sub-G1 peak.[10]

The analyzed pan-nuclear γH2AX-quantification revealed a two-fold higher γH2AX-intensity after treatment with oridonin or ponicidin. This increase in γH2AX level was independent of the particular dose level of oridonin and ponicidin. Combined with irradiation both oridonin and ponicidn lead to an even higher amount of γH2AX. Pan-nuclear determination of γH2AX is just an indirect indicator of DNA double-strand break induction, but it is comparable to the quantification of γH2AX-foci[25] which is known to be highly suspect for an induction of DNA double-strand breaks.[26] Accordingly, oridonin and ponicidin as well as irradiation seem to induce DNA double-strand breaks. Beside possible effects on cell cycle phase proteins as suggested by Qi et al.[10] the induction of DNA double-strand breaks are a good explanation for the observed G2/M-arrest. An already described augmentation

of γH2AX in oridonin-treated BxPC-3 cells and an increased γH2AX-concentration in breast cancer cells exposed to irradiation are in line with our present data.[9,27]

By comparison of γH2AX-intensities at different time points the level of γH2AX of the combined treatment schemes decreased quite similar to that of the irradiation treatment alone. We therefore have to conclude that oridonin and ponicidin do not significantly lower the DNA double-strand break repair capacity. To verify this, proteins of the DNA double-strand break repair were detected by Western Blot. Since pancreatic cancer cells are known to show only weak activity of the homologous recombination pathway[28], we concentrated on the key proteins of the NHEJ. There were no relevant changes in the amount of Ku70, Ku80 or XRCC4 in oridonin- or ponicidin-treated cells supporting the hypothesis that oridonin and ponicidin do not reduce the DNA double-strand break repair mechanisms. Further studies should investigate if oridonin or ponicidin interfere with proteins of the homologous recombination pathway of DNA double-strand break repair, such as RAD51 or MRE11/RAD50/NBS1 complex.

Recently, Wang *et al.* could show synergistic effects of an oridonin derivative and paclitaxel through increased reactive oxygen species (ROS) levels and subsequent apoptosis induction.[29] Cao *et al.* observed a synergistic effect of cetuximab and oridonin through increased ROS levels and epidermal growth factor receptor (EGFR) inhibition.[30] Activation of ROS is known to be one of the main effects of irradiation in cancer treatment. These findings strengthen the idea of combined treatment schemes with oridonin and irradiation.

In the next step, both substances should be tested in combination with the recent standard chemotherapies such as gemcitabine or FOLFIRINOX. Finally, *in vivo* data are needed to reveal if efficacy of irradiation in pancreatic cancer patients can be improved by oridonin and ponicidin.

Conclusions

The current data demonstrate new aspects of the two interesting phytotherapeutics oridonin and poncidin. *In vitro* both substances are highly effective against pancreatic cancer cells especially when combined with irradiation. Therefore both oridonin and ponicidin are promising agents in pancreatic cancer therapy. Our data warrant further *in vivo* investigations especially in combination

with irradiation or standard chemotherapy such as FOLFIRINOX or gemcitabine and nab-paclitaxel.

Acknowledgements

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