The inhibition of growth and metastasis of human pancreatic ductal adenocarcinoma by MART-10

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Abstract

Previous studies demonstrated that MART-10, an analog to the active form of vitamin D3, has anti-cancer properties in anaplastic thyroid cancer (ATC). It can inhibit ATC's metastasis by altering cadherin protein expression and preventing the EMT process. This study aims to investigate the effect of MART-10 in a different system, pancreatic ductal adenocarcinoma (PDAC), in both in vitro and in vivo conditions. Methods: The study will use two known PDAC cell lines. The cells will be treated with increasing MART-10 for various durations. In vitro metastasis will be measured by trans-well migration and Matrigel invasion assay, in vitro proliferation will be measured by CCK-8 assay, and the interaction between MART-10 and PDAC will be investigated with Western blot. Mice will be injected with tumor cells and treated with increasing MART-10; in vivo tumor growth and metastasis will be recorded weekly. The positive control for the experiments is ADH-1 (Exherin), and the negative control is PBS in DMSO. Possible results: There are three main possible results: (1) MART-10 inhibits the growth and metastasis of PDAC cells; (2) MART-10 acts as a stimulant for PDAC growth and metastasis; (3) MART-10 has no significant effect on the growth and metastasis of PDAC. Conclusion: The result of the study will provide important insight into the preclinical effectiveness of MART-10 in PDAC; it also sets the basis for future clinical studies of the drug. Future studies should investigate the mechanism underlying MART-10's effectiveness in PDAC or search for drug combinations with MART-10 that synergize the disease.

Keywords: pancreatic ductal adenocarcinoma, MART-10, cadherin, orthotopic xenograft

1. Introduction

As one of the most aggressive solid exocrine malignancies and the fourth leading cause of cancer-related death [1], pancreatic ductal adenocarcinoma (PDAC) induces a significant and rapidly escalating incidence rate around the world. In the last decades, considerable improvements have been achieved in the screening and therapy of various types of solid cancers, drastically increasing patients' survival and recovery rate. However, despite the advancements in research on pancreatic cancer, the mortality rate for patients with PDAC has not experienced significant improvement in the last few decades, with a one-year overall survival rate of around 24%, and a five-year survival rate of less than 9% [2].

This detrimental outcome of PDAC is associated with delayed diagnosis, which is often a consequence caused by a lack of visible and distinctive symptoms and reliable biomarkers for prognosis. Furthermore, PDAC is associated with a high potency of metastasis to adjacent organs such as the liver and gallbladder, thus escalating the difficulty of prognosis and treatment [3]. Therefore, by inhibiting its metastatic potential, a more suitable therapy for PDAC may be developed, potentially benefiting the clinical prognosis and treatment of PDAC patients.

Epithelial-to-mesenchymal transition (EMT) is a process that provides cancer cells with a metastatic phenotype and thus plays a vital role in the progression and chemoresistance of cancers [4]. For cancer cells to metastasize, they must lose their cell-cell adhesiveness and detach from the original tumor. One factor contributing to such a transition is the change in the expression of cadherins, a family of membrane glycoproteins that mediate calcium-dependent cell-cell adhesion [5,6]. E-cadherins are down-regulated during cancer EMT, usually expressed by epithelial cells. In contrast, N-cadherins, represented by mesenchymal cells, is upregulated, thus resulting in a loss of epithelial phenotypes and a *de novo* acquisition of mesenchymal phenotypes, rendering the cancer cells motile [7].

As a type of cancer with high metastatic potency, it is curious to investigate if inhibiting the EMT process or altering the cadherin composition of PDAC cells would inhibit PDAC growth and metastasis. Previous research has shown that a synthetic peptide ADH-1, which serves as an N-cadherin antagonist in PDAC cells, significantly reduced the progression and metastasis of PDAC [8]. The active form of vitamin D_3, calcitriol, is also shown to be effective against various types of metastatic cancers [9-11]. However, the clinical application of calcitriol to treat

cancer is still limited because the effective concentration of calcitriol to carry out its anticancer function exceeds the physiologically sustainable concentration of calcitriol, thus inducing severe side effects such as hypercalcemia [12,13].

More recently, a synthetic analog for calcitriol, MART-10 (19-nor-2α-(3-hydroxypropyl)-1α,25$(OH)_2D_3$), was discovered to be less hypercalcemia-inducing. At the same time, MART-10 can alter the expression of cadherins on cancer cells through transcriptional and translational controls, thus inhibiting cancer cell growth [14]. An experimental study by Chiang's group investigated the efficacy of MART-10 in anaplastic thyroid cancer (ATC) [15]. With treatment of MART-10 at different concentrations, both the migration and invasion capacity of ATC cells was shown to be decreased to different extents. Furthermore, treated ATC cells had upregulated E-cadherin count and downregulated N-cadherin count, and reduced F actin formation. Chiang's results suggest that MART-10 can inhibit the metastatic potential of ATC via the attenuation of the EMT process in cancer cells, therefore supporting the anti-cancer function of MART-10 *in vitro*. Additionally, MART-10 is shown to be effective at inhibiting various types of cancer growth and metastasis both *in vitro* and *in vivo* by several other studies [16-18].

However, despite being a type of cancer with a high likelihood of metastasis and a high mortality rate, the investigation of MART-10's efficacy in PDAC remains unclear. Therefore, this study aims to elucidate the effect of MART-10 treatment in PDAC both *in vitro* and *in vivo*.

Hypothesis: Due to the similarity between PDAC and ATC, such that both types of cancers pathogenically display high potency of metastasis and elevated N-cadherin count, it is predicted that MART-10 would be effective in reducing or inhibiting metastasis of PDAC cells, just as has been seen for ATC. Further, it is also expected that the expression of E-cadherin will be increased while the expression of N-cadherin will be decreased in PDAC cells under treatment. Finally, the viability and growth of PDAC cells will be reduced both *in vitro* and *in vivo*.

2. Materials

2.1 *Reagents*

19-nor-2α-(3-hydroxypropyl)-1α,25$(OH)_2D_3$ (MART-10) will be synthesized and obtained via Julia olefination as described [19], ADH-1 (Exherin) will be purchased from companies.

2.2 *Cell lines*

Two human PDAC cell lines (BxPC-3 and PANC-1) will be used to compare MART-10's efficacy. BxPC-3 cells will be grown in RPMI 1640 medium, and PANC-1 cells will be produced in the DMEM medium. Both cultures will be supplemented with 2mM glutamine, 10% fetal bovine serum (FBS), 1% penicillin, and streptomycin and will be incubated at 37°C with 5% CO_2 and 95% humidity; the culture medium will be changed three times per week. All required cell lines, medium, and supplemental chemicals will be purchased from companies.

2.3 *Laboratory animals*

40 male BALB/c Nude mice, age 4 weeks old with an average weight of 15 - 20g, will be obtained; mice will be fed with a routine rodent diet and water ad libitum. The mice will be housed in a sterile environment and allowed to acclimatize for ten days before any experiments [20]. All animal experiments and care will be by the guidelines of institutional authorities.

3. Methods

3.1 *In vitro migration and invasion assays*

Trans-well migration and Matrigel invasion assays will be performed using trans-well plates (24-well plates, 8μm-pore-size polycarbonate membrane); the membrane for trans-well migration assays will be left uncoated. In contrast, for Matrigel invasion assays, the membrane will be coated with a layer of Matrigel extracellular matrix proteins. BxPC-3 and PANC-1 cells will be pre-treated with MART-10 (10^{-8}, 10^{-7}, 10^{-6} M), ADH-1 (0.2mg/ml), or phosphate-buffered saline (PBS) in dimethyl sulfoxide (DMSO) for two days. RPMI 1640 medium (or DMEM medium, corresponding to the cell culture) with 20% FBS will be added to the lower chamber of the plates as a chemoattractant, and 4.0×10^4 pre-treated cells suspended in the serum-free medium will be seeded to the upper section of each well. Dishes will be incubated at 37°C, 5% CO_2, and 95% humidity for 24 hours in migration assay and 48 hours in invasion assay. After incubation, cells remaining in the upper chamber will be removed by sterile swap, and cells in the lower section will be fixed and stained with 0.1% crystal violet dye for 30min. Three independent visual fields from each membrane will be examined via microscopic observations, and the number of stained cells will be counted [21, 22]. Both trans-well migration and Matrigel invasion assays will be performed in triplicate.

3.2 *Western blot*

After two days of pre-treatment with MART-10, ADH-1, or PBS, cells will be collected, rinsed with PBS once, and lysed with RIPA lysis buffer. Cell lysates will then be centrifuged at 12000rpm for 10min at 4°C. The

concentration of the protein content in the resulting supernatant will be quantified using BCA Protein Assay Kit to ensure equal loading of samples. Proteins will then be electrophoresed through sodium dodecyl sulfate/polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After incubation with 4% non-fat milk in Tris-buffered saline and Tween-20 for 1 hour at room temperature to block non-specific binding, the membrane will be rinsed and incubated with rabbit monoclonal primary antibodies against E-cadherin, N-cadherin, and tubulin overnight at 4°C with agitation. The membrane will then be washed with TBST 3 times for 5min each and incubated with HRP-conjugated anti-rabbit secondary antibody for 1 hour at room temperature. Bands will be visualized through the ECL system after 3 washes with TBST for 5min each [15, 23]. The expression of targeted proteins relative to tubulin (as the loading control) will be calculated.

3.3 *CCK-8 cell proliferation assay*

BxPC-3 and PANC-1 cell viability and proliferation under MART-10's effect will be assessed with CCK-8 assay. After two days of pre-treatment, cells will be played at a 96-well plate with a density of 2.0×10^3 cells/well. After culturing for 24, 48, 72, and 96 hours, a complete medium containing 10% CCK-8 reagents will be added to respective wells at the indicated time points. After 1 hour of incubation in the dark at 37°C, the optical density of the corresponding wells will be measured at 450nm absorbance using a microplate reader [24]. The experiment will be performed in triplicate.

3.4 *Animal studies*

The mice will be randomly assigned into two groups of 20; each group will receive an orthotopic injection of a different tumor cell line. BxPC-3 and PANC-1 cells will be trypsinized and suspended in Matrigel. After midline laparotomy of the mice, 25µl of Matrigel suspension containing 1.0×10^5 tumor cells will be injected into the tail of the pancreas [25]. After acclimatization, mice will be randomly assigned into four groups of 5: two treatment groups (0.15µg/kg and 0.3µg/kg of MART-10), one positive control (ADH-1), and one negative control (PBS in DMSO). Treatments will be given via intraperitoneal (IP) injection starting 3 days after tumor cell inoculation and will be repeated twice a week for 5 weeks. All mice will be inspected daily for complications. Tumor size and growth will be monitored and recorded non-invasively by ultrasound imaging using Vevo 2100 Imaging Station every week [20, 26, 27]. 5 weeks after tumor cell injection, all mice will be euthanized [24].

3.5 *Flow cytometry*

Cell density will be measured before the corresponding experiment to ensure an accurate number of cells used in each of the above experiments using flow cytometry. Tumor cells will be trypsinized and then washed and suspended in PBS. The cell suspension will then be incubated with APC anti-mouse CD274 monoclonal antibody for 30min at 4°C. Flow cytometry analyses will then be performed on BD FACSCalibur [28].

3.6 *Statistical analysis*

The statistical significance of all numerical data acquired from migration/invasion assays, western blot, CCK-8 assay, and animal studies will be analyzed using the student's T-test, with the level of significance set to $p < 0.05$.

4. Possible Results

4.1 *Description of each combination*

Combination of possible results 1 (CR1): There are fewer cells in the lower chamber after the invasion and migration assay for the MART-10 treated groups than for the negative control group. The optical density measured for MART-10 treated groups in the CCK-8 assay is lower than that for the negative control group. On the western blot, the band of E-cadherin is thicker, while the crew of N-cadherin is lighter for the MART-10 treated groups than for the negative control group. The growth rate of the orthotopic tumor in MART-10 treated mice is lower than in negative control mice.

Combination of possible results 2 (CR2): There are fewer cells in the lower chamber after the invasion and migration assay for the MART-10 treated groups than for the negative control group. The optical density measured for MART-10 treated groups in the CCK-8 assay is also lower than that for the negative control group. On the western blot, the band of E-cadherin is thicker, while the crew of N-cadherin is lighter for the MART-10 treated groups than for the negative control group. The growth rate of the orthotopic tumor in MART-10 treated mice is higher than in negative control mice, or there is no significant difference between the two groups.

Combination of possible results 3 (CR3): There are fewer cells in the lower chamber after the invasion and migration assay for the MART-10 treated groups than for the negative control group. The optical density measured for MART-10 treated groups in the CCK-8 assay is lower than that for the negative control group. On the western blot, the band of E-cadherin is lighter. At the same time, the crew of N-cadherin is thicker for the MART-10 treated groups than for the negative control group, or there is no

significant difference between the two groups. The growth rate of the orthotopic tumor in MART-10 treated mice is lower than in negative control mice.

Combination of possible results 4 (CR4): There are fewer cells in the lower chamber after the invasion and migration assay for the MART-10 treated groups than for the negative control group. The optical density measured for MART-10 treated groups in the CCK-8 assay is significantly higher than that for the negative control group, or there is no significant difference between the two groups. On the western blot, the band of E-cadherin is thicker, while the crew of N-cadherin is lighter for the MART-10 treated groups than for the negative control group. The growth rate of the orthotopic tumor in MART-10 treated mice is lower than in negative control mice.

Combination of possible results 5 (CR5): The number of cells in the lower chamber after the invasion and migration assay is significantly higher for the MART-10 groups than for negative control, or there is no significant difference between the two groups. The optical density measured for MART-10 treated groups in the CCK-8 assay is lower than that for the negative control group. On the western blot, the band of E-cadherin is thicker, while the crew of N-cadherin is lighter for the MART-10 treated groups than for the negative control group. The growth rate of the orthotopic tumor in MART-10 treated mice is lower than in negative control mice.

Combination of possible results 6 (CR6): There are fewer cells in the lower chamber after the invasion and migration assay for the MART-10 treated groups than for the negative control group. The optical density measured for MART-10 treated groups in the CCK-8 assay is lower than that for the negative control group. On the western blot, the band of E-cadherin is lighter. At the same time, the crew of N-cadherin is thicker for the MART-10 treated groups than for the negative control group, or there is no significant difference between the two groups. The growth rate of the orthotopic tumor in MART-10 treated mice is higher than in negative control mice, or there is no significant difference between the two groups.

Combination of possible results 7 (CR7): There are fewer cells in the lower chamber after the invasion and migration assay for the MART-10 treated groups than for the negative control group. The optical density measured for MART-10 treated groups in the CCK-8 assay is significantly higher than that for the negative control group, or there is no significant difference between the two groups. On the western blot, the band of E-cadherin is thicker, while the band of N-cadherin is lighter for the MART-10 treated groups than for the negative control group. The growth rate of the orthotopic tumor in MART-10 treated mice is higher than in negative control mice, or there is no significant difference between the two groups.

Combination of possible results 8 (CR8): There are fewer cells in the lower chamber after the invasion and migration assay for the MART-10 treated groups than for the negative control group. The optical density measured for MART-10 treated groups in the CCK-8 assay is significantly higher than that for the negative control group, or there is no significant difference between the two groups. On the western blot, the band of E-cadherin is lighter. At the same time, the crew of N-cadherin is thicker for the MART-10 treated groups than for the negative control group, or there is no significant difference between the two groups. The growth rate of the orthotopic tumor in MART-10 treated mice is lower than in negative control mice.

Combination of possible results 9 (CR9): The number of cells in the lower chamber after the invasion and migration assay is significantly higher for the MART-10 groups than for negative control, or there is no significant difference between the two groups. The optical density measured for MART-10 treated groups in the CCK-8 assay is lower than that for the negative control group. On the western blot, the band of E-cadherin is lighter. In comparison, the crew of N-cadherin is thicker for the MART-10 treated groups than for the negative control group, or there is no significant difference between the two groups. The growth rate of the orthotopic tumor in MART-10 treated mice is lower than in negative control mice.

Combination of possible results 10 (CR10): The number of cells in the lower chamber after the invasion and migration assay is significantly higher for the MART-10 groups than for negative control, or there is no significant difference between the two groups. The optical density measured for MART-10 treated groups in the CCK-8 assay is significantly higher than that for the negative control group, or there is no significant difference between the two groups. On the western blot, the band of E-cadherin is thicker, while the band of N-cadherin is lighter for the MART-10 treated groups than for the negative control group. The growth rate of the orthotopic tumor in MART-10 treated mice is lower than in negative control mice.

Combination of possible results 11 (CR11): The number of cells in the lower chamber after the invasion and migration assay is significantly higher for the MART-10 groups than for negative control, or there is no significant difference between the two groups. The optical density measured for MART-10 treated groups in the CCK-8 assay is lower than that for the negative control group. On the western blot, the band of E-cadherin is thicker, while the crew of N-cadherin is lighter for the MART-10 treated groups than for the negative control group. The

growth rate of the orthotopic tumor in MART-10 treated mice is higher than in negative control mice, or there is no significant difference between the two groups.

Combination of possible results 12 (CR12): There are fewer cells in the lower chamber after the invasion and migration assay for the MART-10 treated groups than for the negative control group. The optical density measured for MART-10 treated groups in the CCK-8 assay is significantly higher than that for the negative control group, or there is no significant difference between the two groups. On the western blot, the band of E-cadherin is lighter. In comparison, the crew of N-cadherin is thicker for the MART-10 treated groups than for the negative control group, or there is no significant difference between the two groups. The growth rate of the orthotopic tumor in MART-10 treated mice is higher than in negative control mice, or there is no significant difference between the two groups.

Combination of possible results 13 (CR13): The number of cells in the lower chamber after the invasion and migration assay is significantly higher for the MART-10 groups than for negative control, or there is no significant difference between the two groups. The optical density measured for MART-10 treated groups in the CCK-8 assay is lower than that for the negative control group. On the western blot, the band of E-cadherin is lighter. In comparison, the crew of N-cadherin is thicker for the MART-10 treated groups than for the negative control group, or there is no significant difference between the two groups. The growth rate of the orthotopic tumor in MART-10 treated mice is higher than in negative control mice, or there is no significant difference between the two groups.

Combination of possible results 14 (CR14): The number of cells in the lower chamber after the invasion and migration assay is significantly higher for the MART-10 groups than for negative control, or there is no significant difference between the two groups. The optical density measured for MART-10 treated groups in the CCK-8 assay is significantly higher than that for the negative control group, or there is no significant difference between the two groups. On the western blot, the band of E-cadherin is thicker, while the band of N-cadherin is lighter for the MART-10 treated groups than for the negative control group. The growth rate of the orthotopic tumor in MART-10 treated mice is higher than in negative control mice, or there is no significant difference between the two groups.

Combination of possible results 15 (CR15): The number of cells in the lower chamber after the invasion and migration assay is significantly higher for the MART-10 groups than for negative control, or there is no significant difference between the two groups. The optical density measured for MART-10 treated groups in the CCK-8 assay is significantly higher than that for the negative control group, or there is no significant difference between the two groups. On the western blot, the band of E-cadherin is lighter. At the same time, the crew of N-cadherin is thicker for the MART-10 treated groups than for the negative control group, or there is no significant difference between the two groups. The growth rate of the orthotopic tumor in MART-10 treated mice is lower than in negative control mice.

Combination of possible results 16 (CR16): The number of cells in the lower chamber after the invasion and migration assay is significantly higher for the MART-10 groups than for negative control, or there is no significant difference between the two groups. The optical density measured for MART-10 treated groups in the CCK-8 assay is significantly higher than that for the negative control group, or there is no significant difference between the two groups. On the western blot, the band of E-cadherin is lighter. At the same time, the crew of N-cadherin is thicker for the MART-10 treated groups than for the negative control group, or there is no significant difference between the two groups. The growth rate of the orthotopic tumor in MART-10 treated mice is higher than in negative control mice, or there is no significant difference between the two groups (Table 1).

Table 1: Table of a combination of possible results

Variety of possible outcomes (CR)	Decrease in vitro metastasis?	Decrease in vitro growth?	Increase the E to N cadherin ratio.	Decrease in vivo growth?	Does it support the hypothesis?
CR1	+	+	+	+	**Yes**
CR2	+	+	+	-	**Partially**
CR3	+	+	-	+	**Partially**
CR4	+	-	+	+	**Partially**
CR5	-	+	+	+	**Partially**
CR6	+	+	-	-	**Partially**

Variety of possible outcomes (CR)	Decrease in vitro metastasis?	Decrease in vitro growth?	Increase the E to N cadherin ratio.	Decrease in vivo growth?	Does it support the hypothesis?
CR7	+	-	+	-	**Partially**
CR8	+	-	-	+	**Partially**
CR9	-	+	-	+	**Partially**
CR10	-	-	+	+	**Partially**
CR11	-	+	+	-	**Partially**
CR12	+	-	-	-	**Partially**
CR13	-	+	-	-	**Partially**
CR14	-	-	+	-	**Partially**
CR15	-	-	-	+	**Partially**
CR16	-	-	-	-	**No**

Note. "+" represents the result of the experiment conducted supports the hypothesis, "-" represents the result contradicts the hypothesis

5. Discussion

5.1 *Discussion about differential effects in two cell lines*

The two cell lines that were originally chosen (BxPC-3 and PANC-1) have different responses when treated with MART-10 in any of the experiments conducted. This may be due to the different mechanistic nature of causing PDAC in the two tumor cell lines, or perhaps the two cell lines innately have different chemoresistance against drugs like MART-10. Any following experiments demonstrating such differential results would only partially support the hypothesis, and the experiment should be repeated with other PDAC cell lines to confirm MART-10's efficacy.

5.2 *Discussion about combinations of possible results*

Previous studies frequently demonstrated that MART-10 exhibits anti-cancer properties in various metastatic cancers [15-18]. However, little is known about the effect of MART-10 in pancreatic cancer systems. Therefore, to test the therapeutic preclinical impact of MART-10 in PDAC systems, this study applies MART-10 treatment to two well-studied PDAC cell lines to establish an *in vitro* model and use orthotopically transplanted mice to establish an *in vivo* PDAC model under MART-10 treatment.

A combination of Possible results (CR) 1 is consistent with the findings of previous studies investigating the *in vitro* effect of MART-10 in ATC [15]. There are fewer cells present after the invasion and migration assay for the MART-10 treated groups than for the negative control groups, indicating that the metastatic potential of PDAC cells is reduced *in vitro* by MART-10. Similarly, a lower optical density as observed through the CCK-8 assay for the MART-10 groups than for the negative control groups indicate fewer MART-10-treated cells survived after the assay, therefore suggesting MART-10 also inhibits PADC growth *in vitro*. Through western blot, an increase in the E- to N- cadherin ratio in the MART-10 treated groups suggest MART-10 upregulated the expression of E-cadherin and downregulated the expression of N-cadherin and as a result, potentially participates in the attenuation of EMT process in PDAC. Growth of PDAC is also reduced *in vivo* as shown by the animal experiments, the size of the orthotopic tumor would be growing at a lower rate for the MART-10 groups than for the negative control groups. Taken together, the results from CR1 would fully support the hypothesis and support an anti-metastasis and anti-proliferative role of MART-10 in PDAC.

In CR2, MART-10 has a complete *in vitro* effect; it can inhibit both growth and metastasis of PDAC cells *in vitro* and increase the E- to N- cadherin ratio, as demonstrated by the western blot. The *in vivo* effect of MART-10, however, is not observed. This could be explained if MART-10 indeed does not have any *in vivo* effect for PDAC, or this could be due to the dosage given to mice being too low for MART-10 to carry out its anti-cancer function. The animal experiment should be repeated with mice given higher dosages of MART-10. Meanwhile, raising MART-10 concentration would also increase the possibility of overdose toxicity; the mice treated with higher MART-10 dosages should thus be monitored extra carefully for any signs of side effects, such as hypercalcemia. Similar to CR2, in CR7 MART-10 does

not inhibit the growth of PDAC either *in vitro* or *in vivo*, this could be explained as MART-10 having no anti-proliferative effect against PDAC, it only serves to inhibit the metastasis of PDAC cells.

CR3 demonstrates that MART-10 is capable of inhibiting both the growth and metastasis of PDAC cells *in vitro*, as well as inhibiting the growth of PDAC tumors *in vivo*, however, the expected increase in E- to N- cadherin ratio is not observed in this combination. In principle, performing a western blot against E- and N- cadherins in this study aims to investigate whether the treatment of MART-10 can induce a reversal of the cadherin switch in PDAC cells, as seen in previous studies on ATC cells [15]. The results from this western blot could also serve as an indirect measure of the attenuation in the EMT process. In this case, although the results from the western blot contradict the hypothesis, the rest of the results in this combination still supports the hypothesis. Thus, the EMT process in PDAC is likely attenuated but through a different mechanism than expected.

On the contrary, while the result of the western blot in CR6, CR9, and CR12 contradicts the hypothesis, there are other parts of the developments in those combinations that also reject the idea; for instance, in CR6, MART-10 does not inhibit PDAC tumor growth *in vivo*, in CR9, MART-10 does not inhibit PDC cell metastasis *in vitro*, and in CR12, MART-10 does not inhibit PDAC cell growth *in vitro*. Those combinations not only do not support that MART-10 is not causing a cadherin switch, but also do not support an attenuation in the EMT process. As a result, those combinations can only partially support the hypothesis.

In CR4, it is curious to observe that when MART-10 does not inhibit PDAC growth *in vitro*, it does inhibit PDAC tumor growth *in vivo*. This pattern is also observed in various other combinations of results, such as CR8, CR10, and CR15. Such observations where an effect is not seen *in vitro* but seen *in vivo* are not expected as it opposes the common knowledge, and therefore contradict the hypothesis. One explanation for such results would be that there are errors in performing the animal experiment, one probability is that the number of tumor cells injected into each mouse is not equalized, leading to different tumor sizes in the first place. In this case, the experiment should be repeated with the same setup and extra caution.

In CR5, MART-10 does not inhibit metastasis of PDAC cells *in vitro*, while it is still capable of inhibiting the growth of PDAC cells both *in vitro* and *in vivo*. Similarly, CR5, CR11, and CR14 also show no *in vitro* anti-metastatic effect. However, in this case, it is not able to conclude that MART-10 has no anti-metastatic effect for PDAC in general because whether MART-10 can still inhibit PDAC tumor metastasis *in vivo* is not being tested in this study. As a result, CR4, CR5, CR11, and CR14 can only partially support the hypothesis as their data on the anti-metastatic ability of MART-10 on PDAC is inconclusive.

CR16 is the most minor expected outcome in all the possible combinations, it demonstrates that the results of every experiment conducted contradict the hypothesis. It would suggest that although the anti-cancer effect of MART-10 is supported by various evidence in other systems, MART-10 does not have any effect in treating PDAC.

6. Conclusion

In general, this study explores the effect of MART-10 in two different PDAC cell lines and xenografted mice. The results of this study will indicate whether or not the treatment with MART-10 is capable of inhibiting the proliferation and metastasis of pancreatic cancer *in vitro* and *in vivo*, and whether or not a change in the expression of cadherin membrane proteins mediates this inhibition. Such a favorable therapeutic effect of MART-10 would thus prepare the basis for its transition to clinical trials. The results of this study would also provide insights into the direction of future studies in the same field. The mechanism of interaction between MART-10 and PDAC can be further investigated, thus providing insights into the molecular pathways involved, and potentially revealing more therapeutic targets along the way. The combination of MART-10 with other anti-cancer drugs for PDAC, such as Taxol or ADH-1, can also be examined for potential synergistic effects between the drugs.

References

[1] Adamska, A., A. Domenichini, and M. Falasca, *Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies.* International Journal of Molecular Sciences, 2017. **18**(7): p. 1338.

[2] Ushio, J., et al., *Pancreatic Ductal Adenocarcinoma: Epidemiology and Risk Factors.* Diagnostics, 2021. **11**(3): p. 562.

[3] Orth, M., et al., *Pancreatic ductal adenocarcinoma: biological hallmarks, current status, and future perspectives of combined modality treatment approaches.* Radiation Oncology, 2019. **14**(1): p. 141.

[4] Sommariva, M. and N. Gagliano, *E-Cadherin in Pancreatic Ductal Adenocarcinoma: A Multifaceted Actor during EMT.* Cells, 2020. **9**(4): p. 1040.

[5] Takeichi, M., *Cadherin Cell Adhesion Receptors as a Morphogenetic Regulator.* Science, 1991. **251**(5000): p. 1451-1455.

[6] Takeichi, M., *Morphogenetic roles of classic cadherins.* Current Opinion in Cell Biology, 1995. **7**(5): p. 619-627.
[7] Mrozik, K.M., et al., *N-cadherin in cancer metastasis, its emerging role in hematological malignancies and potential as a therapeutic target in cancer.* BMC Cancer, 2018. **18**(1): p. 939.
[8] Shintani, Y., et al., *ADH-1 suppresses N-cadherin-dependent pancreatic cancer progression.* International Journal of Cancer, 2008. **122**(1): p. 71-77.
[9] Chiang, K.-C. and T. C Chen, *The anti-cancer actions of vitamin D.* Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 2013. **13**(1): p. 126-139.
[10] Chiang, K.-C., et al., *Hepatocellular carcinoma and vitamin D: A review.* Journal of Gastroenterology and Hepatology, 2011. **26**(11): p. 1597-1603.
[11] Deeb, K.K., D.L. Trump, and C.S. Johnson, *Vitamin D signalling pathways in cancer: potential for anticancer therapeutics.* Nature Reviews Cancer, 2007. **7**(9): p. 684-700.
[12] Garland, C.F., et al., *The Role of Vitamin D in Cancer Prevention.* American Journal of Public Health, 2006. **96**(2): p. 252-261.
[13] Mehta, R.G. and R.R. Mehta, *Vitamin D and cancer.* The Journal of Nutritional Biochemistry, 2002. **13**(5): p. 252-264.
[14] Perlman, K.L., et al., *1α,25-dihydroxy-19-nor-vitamin D3, a novel vitamin D-related compound with potential therapeutic activity.* Tetrahedron Letters, 1990. **31**(13): p. 1823-1824.
[15] Chiang, K.-C., et al., *MART-10, the vitamin D analog, is a potent drug to inhibit anaplastic thyroid cancer cell metastatic potential.* Cancer Letters, 2015. **369**(1): p. 76-85.
[16] Chiang, K.-C., et al., *MART-10, a less calcemic vitamin D analog, is more potent than 1α,25-dihydroxyvitamin D3 in inhibiting the metastatic potential of MCF-7 breast cancer cells in vitro.* The Journal of Steroid Biochemistry and Molecular Biology, 2014. **139**: p. 54-60.
[17] Chiang, K.-C., et al., *19-Nor-2α-(3-hydroxypropyl)-1α,25-dihydroxyvitamin D3 (MART-10) is a potent cell growth regulator with enhanced chemotherapeutic potency in liver cancer cells.* Steroids, 2011. **76**(13): p. 1513-1519.
[18] Yang, S.W., et al., *MART-10, a newly synthesized vitamin D analog, represses metastatic potential of head and neck squamous carcinoma cells.* Drug Des Devel Ther, 2016. **10**: p. 1995-2002.
[19] Ono, K., et al., *Efficient Synthesis of 2-Modified 1α,25-Dihydroxy-19-norvitamin D3 with Julia Olefination: High Potency in Induction of Differentiation on HL-60 Cells.* The Journal of Organic Chemistry, 2003. **68**(19): p. 7407-7415.
[20] Goumas, F.A., et al., *Inhibition of IL-6 signaling significantly reduces primary tumor growth and recurrencies in orthotopic xenograft models of pancreatic cancer.* International Journal of Cancer, 2015. **137**(5): p. 1035-1046.
[21] Nakajima, A., et al., *Inhibition of peroxisome proliferator-activated receptor γ activity suppresses pancreatic cancer cell motility.* Cancer Science, 2008. **99**(10): p. 1892-1900.
[22] Zimmerman, N.P., et al., *Cyclic AMP regulates the migration and invasion potential of human pancreatic cancer cells.* Molecular Carcinogenesis, 2015. **54**(3): p. 203-215.
[23] Wen, Z., et al., *High Expression of Interleukin-22 and Its Receptor Predicts Poor Prognosis in Pancreatic Ductal Adenocarcinoma.* Annals of Surgical Oncology, 2014. **21**(1): p. 125-132.
[24] Fu, Y., et al., *Downregulated miR-98-5p promotes PDAC proliferation and metastasis by reversely regulating MAP4K4.* Journal of Experimental & Clinical Cancer Research, 2018. **37**(1): p. 130.
[25] Aiello, N.M., A.D. Rhim, and B.Z. Stanger, *Orthotopic Injection of Pancreatic Cancer Cells.* Cold Spring Harb Protoc, 2016. **2016**(1): p. pdb.prot078360.
[26] Huynh, A.S., et al., *Development of an orthotopic human pancreatic cancer xenograft model using ultrasound guided injection of cells.* PLoS One, 2011. **6**(5): p. e20330.
[27] Pigula, M., et al., *Size-dependent Tumor Response to Photodynamic Therapy and Irinotecan Monotherapies Revealed by Longitudinal Ultrasound Monitoring in an Orthotopic Pancreatic Cancer Model.* Photochem Photobiol, 2019. **95**(1): p. 378-386.
[28] Zhou, K.-X., et al., *Increased Nuclear Transporter KPNA2 Contributes to Tumor Immune Evasion by Enhancing PD-L1 Expression in PDAC.* Journal of Immunology Research, 2021. **2021**: p. 6694392.