Comparison on the effect of MART-10 and calcitriol on the treatment of non-small cell lung cancer by determining which one is a more potent drug in treating non-small cell lung cancer

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Abstract

Non-small cell lung cancer is one of the most common types of lung cancer, with a high mortality rate worldwide. The current treatment methods have apparent side effects and inevitable drawbacks. A compelling new drug is badly in need of development. A previous study has reported that calcitriol and MART-10 can effectively block anaplastic thyroid cancer (ATC) spread. MART -10 is more potent than calcitriol in inhibiting migration and invasion of ATC. This study investigates the effect of NSCLC using MART-10 and calcitriol and finds out which is more potent, both in vitro and in vivo. The experiments will use known human non-small cell lung cancer cell lines (A549 cell line) and two vitamin D compounds: MART-10 and calcitriol and Xenograft mouse system. MTT measures cell proliferation. FACS measures cell apoptosis for AnnexinV and PI. Measure development in tumor size by mouse xengraft system. A positive control is taxol, and a negative control is PBS. There are three most possible results: (1) MART-10 and calcitriol can kill A549 lung cancer cells, and MART-10 reduce the number of A549 lung cancer cells at a lower concentration than with calcitriol. (2) MART-10 and calcitriol can kill A549 lung cancer cells, and calcitriol reduces the number of A549 lung cancer cells at a lower concentration than with MART-10. (3) MART-10 and calcitriol do not significantly kill A549 lung cancer cells. The result of the study will provide critical information for the future clinical trial of MART-10 and calcitriol in treating NSCLC. It would increase our understanding of the more effective treatment of NSCLC and improve human health. Future studies should focus on combinations of MART-10 or calcitriol with various anti-cancer chemotherapy drugs.

Keywords: MART-10, Calcitriol, non-small cell lung cancer, NSCLC, Cell proliferation, A549 cell

1. Introduction

Lung cancer is one of the most common and deadliest type of cancer in the world, it is the leading cause of cancer-related mortality in United States in recent years [1]. There were approximately 1.7 million people died from lung cancer in 2018 which is a pretty huge number [2]. There are two main types of lung cancer, small cell lung cancer and non-small cell lung cancer (NSCLC). Among all the subtypes of lung cancer, NSCLC accounts for about 80%-85% worldwide [2]. NSCLC has a poor prognosis as well as a typically low survival rate. The 60-month overall survival rate for NSCLC varies dramatically from 68% to 10% in patients with different stages [3]. Major risk factors of NSCLC include smoking, exposure to carcinogenic factors and ionizing radiation, environmental toxins, and genetic inheritance. Currently, the most common treatments are surgery, radiation therapy, cytotoxic chemotherapy and advanced target immunotherapy. However, these therapies are not effective enough to achieve complete cure of NSCLC because of their apparent side effects and drawbacks. An emerging effective treatment or drug is badly in need of development.

Calcitriol has the formula of 1α,25(OH)2D3 which is a man-made active form of vitamin D. It has the characteristics of an anti-cancer agent, and it has the properties of pro-differentiation, pro-apoptotic and anti-metastatic [4-7]. These properties can be applied in treating anaplastic thyroid cancer (ATC) to inhabit the migration and invasion of cancer cells and it has great potential in curing ATC. However, when 1α, 25(OH)2D3 is used clinically to treat cancer, the required anti-cancer concentration is often much higher than the physiological concentration and may result in hypercalcemia. In order to minimize this side effect, lots of new 1α, 25(OH)2D3 analogs have been synthesized. Among these analogs, there is one special type of vitamin D analog which is MART-10. It has the structure of 19-nor. (C19 methylene group is replaced by two hydrogen atoms) [8]. It also has a huge potential in treating ATC and shows less side effect on patients.

In the previous study, it has concluded that both calcitriol and MART-10 have the ability to block ATC spread

effectively using the experiment methods of western block and F-actin staining etc. Another conclusion that has already been made is: MART-10 is more potent than calcitriol in inhibiting the migration and invasion in ATC [9]. MART-10 has been proved to have the property of anti-cancer in several types of cancer including breast cancer, anaplastic thyroid cancer and pancreatic cancer. However, there were no research focus on investigating the effect of calcitriol and MART-10 on non-small cell lung cancer. There comes the question: since calcitriol and MART-10 show the property of anti-cancer and have great effect in treating many types of cancer, how will it perform in NSCLC, still acts as an anti-cancer agent or show no effect. What about their comparison, is MART-10 still more potent than calcitriol in inhibiting the migration and invasion in NSCLC?

Research question: Since MART-10 is more potent than calcitriol in inhibiting the migration and invasion in anaplastic thyroid cancer, would MART-10 reduce number of A549 lung cancer cells at a lower concentration than with calcitriol in the treatment of non-small cell lung cancer.

Hypothesis: Since MART-10 is more potent than calcitriol in inhibiting the migration and invasion in ATC, I predict that treatment with increasing amounts and for various durations with MART-10 will reduce number of A549 lung cancer cells at a lower concentration than with calcitriol. Measure cell proliferation by MTT. Measure cell apoptosis by FACS for AnnexinV and PI. Measure development in tumor size using a mouse xengraft system. The positive control is taxol which can surely decrease the number of lung cancer cells and the negative control is PBS which cannot decrease number of lung cancer cells.

therefore, in order to find out the effect of calcitriol and MART-10 on NSCLC and compare the abilities of killing A549 lung cancer cells in treatment of NSCLC of calcitriol and MART-10, a comparative study should be carried out. This paper investigates the abilities of killing A549 lung cancer cells of these two drugs in both in vitro and in vivo conditions and eventually hypothesizes that with increasing amounts and for various durations with MART-10 will reduce number of A549 lung cancer cells at a lower concentration than with calcitriol.

2. Material and methods:

2.1 *Cell lines*

This experiment will use A549 (non-small cell lung cancer cell line) that exhibit high and low expression of MART-10 and calcitriol respectively.

2.2 *In Vitro Cell Culture*

A549 cells will be cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS),100 μg/mL streptomycin (Sigma-Aldrich), and 100 U/ml penicillin, kept at 37 Celsius in a humidified environment with 5% CO_2 and 95% O_2. The cell media will be replaced every 48 hours, cells will be trypsinized and reduced once 90% confluence is obtained [10].

2.3 *Reagent*

20mg of MART-10 and 1α, 25(OH)2D3 (calcitriol) will be dissolved in 40 mL of physiological saline respectively and stored at 4°C before each use.

2.4 *MTT essay*

Cell proliferation will be determined by MTT assay. Cells will be seeded on 96-well culture plates at $5 \cdot 10^3$ cells per well after transfection in serum-free medium, such as DMEM, which contains 10% FCS. At indicated time points, 20 μl MTT reagent will be added to each well and the cells were incubated for additional 4 h at 37 °C. After that the medium will be removed, cells will be treated with taxol, PBS, MART-10 and calcitriol (0.1, 1, 10, 100, or 200 μg/ml). 10 min later, the purple crystals were dissolved. The absorbance will be measured at 490 nm using a microplate reader. MTT assays will be performed after 24,48, and 72 hours of treatment. The procedure will be performed in triplicates which are biological replicates [11].

2.5 *Cell Apoptosis Assay*

The cell apoptosis will be detected by FACS for Annexin V and PI.

Annexin-V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit will be used to detect apoptosis of A549 cells. A549 cells will be collected and then re-suspended in 100μL binding buffer. Annexin-V-and propidium iodide (PI) will be incubated with A549 cells in the dark for about 15min. The apoptosis will be analyzed by using FC-500 flow cytometer. Apoptotic cells may be identified from annexin V-negative live cells using microscopic and flow cytometric approaches based on their annexin V-affinity, caused by phosphatidylserine (PS) exposure at the plasma membrane's outer leaflet. A FACSort with a single Argon ion laser was utilized for flow cytometric analysis. The emission filters employed were 515-545 BP (green; FITC) and 600 LP, and the excitation wavelength was 488 nm (red; PI). A minimum of 10000 cells per sample were analyzed, and the results were saved in a list mode. Fluorescence bleed through was eliminated via electronic correction. All the treatment groups are compared with the control group which does not receive any treatment. Two-tailed P values were calculated through paired T-test. Concentration of MART-10 and calcitriol should be 0.1, 1, 10, 100, or 200 μg/ml. Cell

apoptosis assays will be performed after 24,48, and 72 hours of treatment. The procedure will be performed in triplicates which are biological replicates [12,13].

2.6 *Animal Experiment*

A549 cell lines will first be transplanted subcutaneously to heads and backs of 30 nude mice at 10×10^5 cells per xenograft. A week later, respective amount of PBS, Taxol, MART-10 or calcitriol (0.1, 1, 10, 100, or 200 μg/day) will be administered intraperitoneally to A549-transplanted mice every 24 hours for a consecutive of 21 days. The mice will be divided into 4 groups: MAR1-10, calcitriol, negative control with PBS injection, and positive control with taxol injection. After the 21-day cycle, tumors will be excised, immersed in 10% buffered formalin of neutral pH, dehydrated, and embedded in paraffin, for eventual operations of immunohistochemistry (IHC) and hematoxylin-eosin (HE) staining. The procedure will be performed in triplicates which are biological replicates [14] (Table 1).

Table 1: Possible observations

Possible observations	CR1	CR2	CR3	CR4	CR5	CR6	CR7	CR8
Cell apoptosis increases by FACS for Annexin V and PI	+	+	+	+	-	-	-	-
Cell proliferation decreases by MTT	+	+	-	-	+	+	-	-
Decrease of tumor size in Xenografted mice in vivo	+	-	+	-	+	-	+	-
Supporting Hypothesis?	YES	Partially	Partially	Partially	Partially	Partially	Partially	NO

2.7 *Statistical Analysis*

All the numerical data collected from MTT, FACS for AnnexinV and PI and
animal experiment will be recorded and analyzed using T-test. P value <0.05 was considered as a significant acceptable difference.

3. Results

Combination of Possible Results for comparison between MART-10 and calcitriol
Note. "+" represents a positive result as measurements change similarly to taxol and differently to PBS at a lower concentration for MART-10 than calcitriol. "-" represents a negative result measurement change similarly to PBS and differently to taxol at a lower concentration for MART-10 than calcitriol.

3.1 *Possible combination results 1*

Treating NSCLC cells with MART-10 1) Induces cell apoptosis at a lower concentration than calcitriol; 2) Inhibits cell proliferation at a lower concentration than calcitriol; 3) Inhibits the growth of tumor in xenografted mice and shrinks the tumor size.
In vitro, using A549 cell line, MTT decrease indicates deteriorating cell viability and cell proliferation, FACS for Annexin V and PI increase indicates a diminishing number of cells, tumor size in Xenografted mice in vivo decrease indicates the positive effect of inhibition of invasion and migration of NSCLC in animals.

3.2 *Possible combination results 2*

Treating NSCLC cells with MART-10 1) Induces cell apoptosis at a lower concentration than calcitriol; 2) Inhibits cell proliferation at a lower concentration than calcitriol; 3) Does not inhibit the growth of tumor in xenografted mice and the tumor size increases or stay the same.
In vitro, using A549 cell line, MTT decrease indicates deteriorating cell viability and cell proliferation, FACS for Annexin V and PI increase indicates a diminishing number of cells, tumor size in Xenografted mice in vivo increase or stay the same indicates the negative effect of inhibition of invasion and migration of NSCLC in animals.

3.3 *Possible combination results 3*

Treating NSCLC cells with MART-10 1) Induces cell apoptosis at a lower concentration than calcitriol; 2) Does not inhibit cell proliferation at a lower concentration than calcitriol; 3) Inhibits the growth of tumor in xenografted mice and shrinks the tumor size.
In vitro, using A549 cell line, MTT decrease indicates deteriorating cell viability and cell proliferation, FACS for Annexin V and PI decrease indicates an increasing number of cells or same number of cells, tumor size in Xenografted mice in vivo decrease indicates the positive effect of inhibition of invasion and migration of NSCLC in animals.

3.4. *Possible combination results 4*

Treating NSCLC cells with MART-10 1) Induces cell apoptosis at a lower concentration than calcitriol; 2) Does not inhibit cell proliferation at a lower concentration than calcitriol; 3) Does not inhibit the growth of tumor in xenografted mice and shrinks the tumor size.
In vitro, using A549 cell line, MTT decrease indicates deteriorating cell viability and cell proliferation, FACS for Annexin V and PI decrease indicates an increasing number of cells or same number of cells, tumor size in Xenografted mice in vivo increase or stay the same indicates the negative effect of inhibition of invasion and migration of NSCLC in animals.

3.5. *Possible combination results 5*

Treating NSCLC cells with MART-10 1) Does not induce cell apoptosis at a lower concentration than calcitriol; 2) Inhibits cell proliferation at a lower concentration than calcitriol; 3) Inhibits the growth of tumor in xenografted mice and shrinks the tumor size.
In vitro, using A549 cell line, MTT increase indicates increasing cell viability and cell proliferation, FACS for Annexin V and PI increase indicates a diminishing number of cells, tumor size in Xenografted mice in vivo decrease indicates the positive effect of inhibition of invasion and migration of NSCLC in animals.

3.6. *Possible combination results 6*

Treating NSCLC cells with MART-10 1) Does not induce cell apoptosis at a lower concentration than calcitriol; 2) Inhibits cell proliferation at a lower concentration than calcitriol; 3) Does not inhibit the growth of tumor in xenografted mice and shrinks the tumor size.
In vitro, using A549 cell line, MTT increase indicates increasing cell viability and cell proliferation, FACS for Annexin V and PI increase indicates a diminishing number of cells, tumor size in Xenografted mice in vivo increase indicates the negative effect of inhibition of invasion and migration of NSCLC in animals.

3.7. *Possible combination results 7*

Treating NSCLC cells with MART-10 1) Does not induces cell apoptosis at a lower concentration than calcitriol; 2) Does not inhibit cell proliferation at a lower concentration than calcitriol; 3) Inhibits the growth of tumor in xenografted mice and shrinks the tumor size.
In vitro, using A549 cell line, MTT increase indicates increasing cell viability and cell proliferation, FACS for Annexin V and PI decrease indicates an increasing number of cells or the same number of cells, tumor size in Xenografted mice in vivo decrease indicates the positive effect of inhibition of invasion and migration of NSCLC in animals.

3.8. *Possible combination results 8*

Treating NSCLC cells with MART-10 1) Does not induce cell apoptosis at a lower concentration than calcitriol; 2) Does not inhibit cell proliferation at a lower concentration than calcitriol; 3) Does not inhibit the growth of tumor in xenografted mice and shrinks the tumor size.
In vitro, using A549 cell line, MTT increase indicates increasing cell viability and cell proliferation, FACS for Annexin V and PI decrease indicates an increasing number of cells or the same number of cells, tumor size in Xenografted mice in vivo increase indicates the negative effect of inhibition of invasion and migration of NSCLC in animals.

4. Discussion

Previous studies had already demonstrated that both calcitriol and MART-10 have the ability to block ATC spread effectively and MART-10 is more potent than calcitriol in inhibiting the migration and invasion in ATC [9]. However, there hasn't been research conducted around the application and comparison of MART-10 and calcitriol to a variety of other types of cancers such as ATC. therefore, to test and compare the preclinical therapeutic effects of MART-10 and calcitriol on non-small cell lung cancer, this study applies these two treatments to A549 cell lines both in vitro and in vivo by various methods of experiment.
Possible combination results 1 fully supports my hypothesis. The result is consistent with previous research. Since MART-10 is more potent compared with calcitriol in the treatment of ATC, similar mechanism and effects should have in the treatment of NSCLC. MART-10 induces cell apoptosis, inhibits cell proliferation and inhibits growth of tumor at a lower concentration than calcitriol. A possible explanation may be MART-10 molecule has greater ability to enter through the cells and performs its function than calcitriol. Future investigation can be about potential side effects, medical delivery measures and combinations of MART-10 or calcitriol with various anti-cancer chemotherapy drugs such as taxol.
Possible combination results 2 partially supports my hypothesis. The result has similar results as previous research in the part of in vitro. This result contradicts the current understanding of the effect of MART-10. Since MART-10 has already shown to be a more potent drug than calcitriol in vitro, it is not expected to observe the opposite effect in vivo. The most possible explanation for this result would be that there is something wrong and errors occur 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 at the first place. Another explanation is that in vitro models cannot capture the complexity of body systems, so some adverse additional reaction may take place in animals such as enzyme may digest the drugs. This situation may also happen if cell apoptosis is not induced but cell proliferation is inhibited or cell proliferation is not inhibited but cell apoptosis is induced. A possible solution for this problem is to repeat the animal experiment with the same setup and extra attention. Future experiment can be more focused on in vivo side, such as more effective and reliable delivery method, varying concentrations and duration to find the most suitable condition for body systems.

Possible combination results 3 and 4 partially support my hypothesis. The results would contradict the hypothesis as they do not support MART-10 is a more potent drug than calcitriol in inhibiting cell proliferation in vitro. Possible combination results 3 and 4 both failed to inhibit cell proliferation at a lower concentration for MART-10 than calcitriol but succeed in inducing cell apoptosis. These results is different from the previous research's results. One possible explanation for such results would be that the A549 cell lines used innately have greater chemoresistance, therefore a lower concentration of MART-10 suitable in ATC treatment is less effective than calcitriol there. As a result, a larger dosage and higher concentration of MART-10 or a longer duration time could be applied in further experiment for a significant effect in cell proliferation to be seen. However, both these two results induced cell apoptosis at a lower concentration for MART-10 than calcitriol. This indicates that MART-10 are better at killing NSCLC cells than calcitriol at a lower concentration. This meant that MART-10 had a definite advantage in eliminating NSCLC cells, suggesting after further improvement, there will be a sizeable production of this drug if passing the biosafety test. There may be potential systematic errors in the experiment designs because of faulty testing equipment or a failed approach. To investigate further, it's a good choice to carry out MTT essay again. Future experiment could be varying concentrations and duration to find out a more suitable condition to let MART-10 inhibit cell proliferation.

Possible combination results 5 and 6 partially support my hypothesis. The results would contradict the hypothesis as they do not support MART-10 is a more potent drug than calcitriol in inducing cell apoptosis in vitro. Possible combination results 5 and 6 both failed to induce cell apoptosis at a lower concentration for MART-10 than calcitriol but succeed in inhibiting cell proliferation. These results is different from the previous research's results. This may be caused by the different mechanism. A loss of cell membrane integrity without going through cell apoptosis indicates that the MART-10 induce a different cancer cell mechanism in NSCLC cells at a lower concentration than calcitriol. Apoptosis inducer is in some other signaling pathways. Future experiment could be redoing the FACS essay again and varying concentrations and duration to find out most suitable condition to let MART-10 induce cell apoptosis at a lower concentration than calcitriol.

Possible combination results 7 partially supports my hypothesis. This result has something in common with the previous research result which is the tumor size decreases. However, although no cell apoptosis induced, no cell proliferation inhibited at a lower concentration for MART-10 than calcitriol, tumor size is still smaller compared with no treatment. A possible explanation is that the result of tumor size decrease is not a direct effect caused by MART-10 at a lower concentration than calcitriol. It is more likely to the result of body's immune systems. Future experiment could be carrying out the experiment again and focus on specific mechanism.

Possible combination results 8 fully opposes my hypothesis. This result contradicts with the previous research results. Not only there was no cell apoptosis induced, no cell proliferation inhibited at a lower concentration for MART-10 than calcitriol, but the size of NSCLC tumor also increases or doesn't change. This result implies the MART-10 is less potent on suppressing or eliminating NSCLC cells, and their potent ability to induces cell apoptosis and inhibit cell proliferation is limited to specific type of cancer due to differences in the mutation in a gene in various types of cancer. This indicates MART-10 has a great ability to be a more potent drug compared with calcitirol in treatment of ATC only, it has specific mechanism with ATC cells, for other types of cancer, it may not work.

If the measurement increases as concentration increases, it is a specific effective treatment, if the measurement does not change as concentration increases, the treatment is not specific which means not MART-10 or calcitriol causes the effect on NSCLC cells. If the effect overtime or measurement increases as duration increases, it is a specific effective treatment, if the effect overtime or measurement does not change as duration increases, it is not specific effect on NSCLC cells which means not MART-10 or calcitriol causes the effect on NSCLC cells.

5. Conclusion

In conclusion, this study investigates the effect of MART-10 and calcitriol on non-small cell lung cancer through in vitro methods, such as MTT, FACS for AnnexinV and PI

as well as in vivo method that studies Xenografted Mice. The results of this study will indicate whether or not MART-10 and calcitriol have the ability to kill NSCLC cells, and which one is a more potent drug in the treatment of NSCLC. Future studies could focus on investigating medical delivery measures for MART-10 and calcitriol in NSCLC as well as the combinations of MART-10 or calcitriol with various anti-cancer chemotherapy drugs such as taxol and examine if any astonishing result may be derived. The observed anti-cancer agent property would be able to provide a suitable indication for future clinical and pharmaceutical development against NSCLC.

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