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Research Paper

The effect of methylseleninic acid on paclitaxel efficacy in A2780 ovarian cancer cells ☆

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Abstract

Objective: The role of methylseleninic acid (MSeA), a selenium compound, has been documented in cancer chemoprevention. However, the therapeutic effect of MSeA in combination with paclitaxel, a chemotherapeutic agent used to treat ovarian cancer, is unknown. In this study, we investigated the effect of combination treatment of MSeA and paclitaxel against ovarian cancer cells. **Methods:**Ovarian cancer cells(A2780) were treated with different concentrations of MSeA, paclitaxel alone or in combination. The individual and combined concentrations of drugs that achieved certain cells growth/death were determined using a sulforhodamine B(SRB) assay. Drug effects on cell viability were further confirmed using floating cell count and trypan blue exclusion assay. The mean values ± standard deviation were calculated and compared between treatment groups using unpaired *t* test. **Results:** The concentration of paclitaxel alone that inhibited 50% of cell growth(IC_{50}) was 0.5 μ mol/L. This concentration increased to 1.2 μ mol/L when paclitaxel was given in sequential combination with MSeA. The number of dead cells after the combination treatment did not show a significance increase when compared with drug alone. **Conclusion:**Pretreatment with MSeA did not enhance the paclitaxel effect against A2780 ovarian cancer cells.

Keywords: methylseleninic acid; paclitaxel; ovarian cancer

INTRODUCTION

Ovarian cancer is a common cancer of the reproductive system in women, and is the fifth leading cause of cancer-related death among women. Among gynecological malignancies, ovarian cancer is the leading cause of death and the second most commonly diagnosed in the world[1]. In the USA in 2007 an estimated 22 430 newly diagnosed cases and 15 280 deaths could be attributed to this disease[2]. More than half of the deaths from ovarian cancer occur in women between 55 and 74 years of age, and approximately one quarter of ovarian cancer deaths occur in women between 35 and 54 years of age. Approximately 1 in 70 newborn girls will develop ovarian cancer during their lifetime[3]. The exact cause of ovarian cancer is unknown. Although it is documented to occur in females of all ages, ovarian cancer is a disease mostly affects postmenopausal women and prepubescent girls. To date, the standard treatment is cytoreductive surgery and platinum-taxane chemotherapy[4].

Paclitaxel is one of the most effective chemotherapeutic drugs used clinically to treat many solid tumors, such as ovarian, breast, prostate and non-small-cell lung cancer[5]. Paclitaxel is derived from the Pacific yew tree, Taxus brevifolia, and binds to β-tubulin protein in a deep hydrophobic cleft near the luminal surface of microtubules[6]. This agent is used for the treatment of many cancers due to its ability to target β-tubulin, thus blocking cell cycle progression in mitosis, and inducing apoptosis[7]. Paclitaxel clinical indications continue to be expanded for the treatment of other malignancies. However, its applicability and efficacy have been

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seriously limited by emerging drug resistance. Furthermore, the mortality rate of paclitaxel treated ovarian cancer patients remains high. Thus new approaches are needed to improve effectiveness of paclitaxel therapy.

Selenium(Se) is an essential non-metallic trace element that exists in both organic(selenocysteine and selenomethionine) and inorganic(selenite and selenate) forms[8-9]. Methylseleninic acid(MSeA) is a selenium-based compound and stable Se-containing reagent with a single methyl group. It has been documented that MSeA has potent anticancer activity and is an excellent compound to study the anticancer effects of Se *in vitro*[10]. The majority of investigations of the antitumor effects of Se have emphasized its cancer chemoprevention aspect. Various animal models and epidemiological studies have indicated that Se reduces cancer risk[11]. In the last several years, animal and human intervention trials have shown that a daily supplementation with Se-containing compounds reduces the risk of several malignancies[12-13]. There are many large prevention clinical trials that have focused on the ability of Se to down-regulate cell proliferation in prostate cancer[14-15]. Although Se-containing compounds are being evaluated extensively as chemopreventive agents, the use of Se-containing compounds in cancer therapy is a new approach. Recently when Se was used in therapy, it was documented that Se compounds such as MSeA and MSeC enhanced the efficacy of paclitaxel and docetaxel against prostate cancer, respectively. The enhanced therapeutic effect was associated with an increase in the level of caspase-dependent apoptosis and decrease in the level of the anti-apoptotic protein, survivin[16-17]. However, little is known regarding the use of Se-containing compounds(e.g. MSeA) in combination treatment with paclitaxel against ovarian cancer as proposed in this paper.

In this study we have evaluated the therapeutic efficacy of a combination treatment of MSeA and paclitaxel against ovarian cancer cells. Our hypothesis was that adding MSeA would improve paclitaxel activity against ovarian cancer, leading to less paclitaxel resistance in tumor cells. Thus, by adding MSeA, the ultimate goal was to improve the clinical outcome of the treatment of ovarian cancer with paclitaxel, achieving better response rate.

MATERIALS AND METHODS

Cell culture

The human ovarian carcinoma cell line A2780, was obtained from American Type Culture Collection (ATCC)(Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% glucose. Cells were grown at 37℃ in a humidified incubator containing 5% CO_2 and tested for mycoplasma contamination every 3 months with Mycoplasma plus PCR primer set.

Drugs and treatments

MSeA(CH_3SeO_2H) was obtained from PharmaSe, Inc (Lubbock, TX, USA). Paclitaxel($C_{47}H_{51}NO_{14}$) was purchased from Sigma-Aldrich(St. Louis, MO, USA). Four experimental groups of A2780 cells were studied using a cell counting and a trypan blue assay. These treatment groups were:(1)untreated control; (2)MSeA, 24 h at a concentration that inhibited growth of cells by 20%(IC_{20}=1.5 μ mol/L); (3)paclitaxel, 2h at a concentration that inhibited growth of cells by 50% (IC_{50}= 0.5 μmol/L); (4)sequential combination treatment of MSeA and paclitaxel(MSeA/paclitaxel) applied with the same individual drug concentration and treatment time, with 22 h MSeA exposure proceeding paclitaxel treatment.

Sulforhodamine B assay

Cells were seeded in flat-bottomed 96-well plates in 100 μl of culture medium. Twenty four hours after the initial seeding, exponentially growing cells were treated with different concentrations of MSeA alone for 24 h, paclitaxel alone for 2 h, and sequential combination of MSeA(IC_{20}) and different concentrations of paclitaxel. Cells were incubated in a drug-free medium for 5 doubling times after drug exposure. The culture medium was removed and cells were fixed with 10% trichloroacetic acid, washed with distilled water and stained with sulforhodamine B(100 μl/well) for 15 minutes. The excess dye was removed by rinsing the plates with distilled water. Unbound dye was removed with 1% acetic acid and protein-bound dye was extracted with 150 μl Tris base(10 mmol/L, pH 10.5). The absorbance was measured at 570 nm with an automated Bio-Kinetics reader model EL 340, Bio-Tek Instruments (Winooski, VT, USA).

Cells counting

Twenty four hours after seeding, cells were treated with MSeA alone, paclitaxel alone and a sequential combination of MSeA/paclitaxel using the same drug concentrations and treatments groups as mentioned above. After drug treatments, flasks were washed and cells were cultured in drug free medium for 3 and 5 days. Adherent cells were detached using 0.05% trypsin and veresene and counted using Coulter counter model Z2, Beckman Coulter Inc.(Fullerton, CA, USA). Both floating cells and adherent cells were counted in all groups, and the total number of cells were calculated and compared in all treatment groups.

Trypan blue exclusion assay

The cytotoxic effect of drugs alone or in sequential combination was evaluated using a trypan blue exclusion assay. After treatments, cells were washed with PBS and stained with 0.2% trypan blue dye. Uptake/exclusion of trypan blue dye was assayed 3 and 5 days after drug treatment. A collection of supernatant and adherent cells were obtained by trypsin treatment, as above and the mixture was incubated in 0.2% trypan blue dye for 5 minutes, pipetted onto a hematocytometer and manually counted under a microscope. The cells in every treatment groups were counted in the same selected counting chamber squares. The percentages of the control cells that took up trypan blue were calculated.

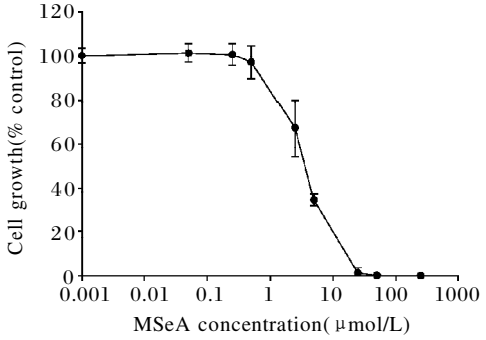
Statistical analysis

Data are presented as $\bar{x} \pm s$. Treatment groups were compared using unpaired *t* test. $P < 0.05$ is considered statistically significant.

RESULTS

Dose response curve of MSeA

The concentration dependence of the MSeA inhibition of cell growth was assessed using a SRB assay 5 days after 24 h treatment. Figure 1 shows the MSeA cytotoxicity curve of triplicate experiments. The MSeA concentration that inhibits 20% of cell growth(IC_{20}) was approximately 1.5 μmol/L. This concentration was used in combination treatment experiments with paclitaxel. The MSeA concentrations that inhibited 50%(IC_{50}) and 90%(IC_{90}) cell growth were approximately 3.5 μmol/L and 14 μmol/L, respectively(***Fig. 1***).

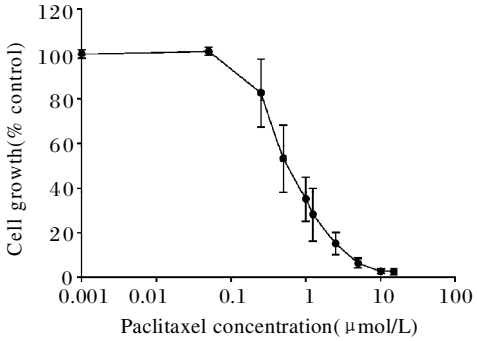


Cells were treated with different concentrations of MSeA for 24 h. MSeA concentrations that inhibited 20%, 50% and 90% cell growth were 1.5 μmol/L, 3.5 μmol/L and 14 μmol/L, respectively.

Fig. 1 Dose response curve of MseA

Dose response curve of paclitaxel

The effect of various concentrations of 2 h paclitaxel treatment on cell growth was assessed using a SRB assay after 5 days of culture. ***Fig. 2*** shows the cytotoxicity curve of triplicate experiments. The paclitaxel concentration that inhibits 50% of cell growth(IC_{50}) was approximately 0.5 μmol/L. This concentration was used alone or in combination treatment experiments with MSeA to determine the effects on cell viability. The paclitaxel concentration that inhibits 90% of cell growth (IC_{90}) was approximately 1.5 μmol/L.

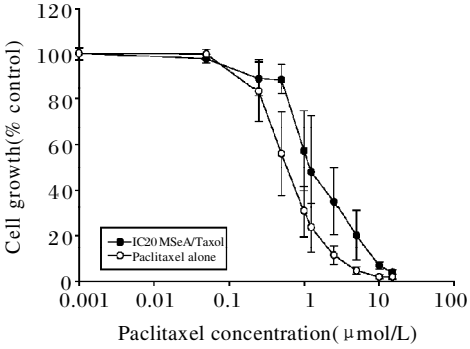


Cells were treated with different concentrations of paclitaxel for 2h. Paclitaxel concentrations that inhibited 50% and 90% of cell growth were 0.5 μmol/L and 1.5 μmol/L, respectively.

Fig. 2 Dose response curve of paclitaxel

Dose response curve of drug combination treatment

To determine whether pretreatment with MSeA enhanced paclitaxel inhibition of cell growth, cells were treated with paclitaxel alone or following treatment with MSeA. Cells were treated with MSeA IC_{20}(1.5 μmol/L) for 22 h followed by the addition of various concentrations of paclitaxel for 2 h. Cells growth was evaluated 5 days after the combination treatment of MSeA and paclitaxel. The MSeA did not summate with, or potentiate the cell growth inhibition caused by paclitaxel. In fact, the dose-response curve of the combination treatment was shifted significantly to the right of that of paclitaxel alone(***Fig. 3***). The IC_{50} of paclitaxel alone was approximately 0.5 μmol/L, but IC_{50} of paclitaxel in combination with MSeA was approximately 1.2 μmol/L.

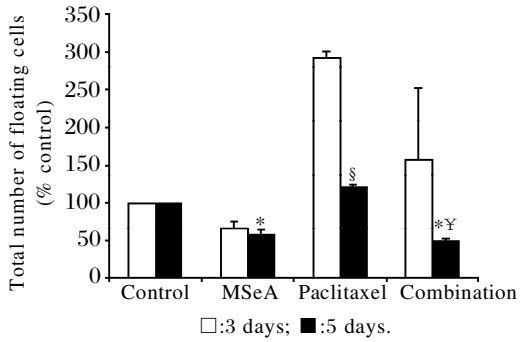


Cells were treated with sequential combination of MSeA(IC_{20}, 1.5 μmol/L) for 22 h followed by different concentrations of paclitaxel for 2 h. The concentration of the combined treatment of MSeA/paclitaxel that inhibited 50%(IC_{50}) of cell growth was 1.2 μmol/L.

Fig. 3 Dose response of combination treatment

Effects on cell viability

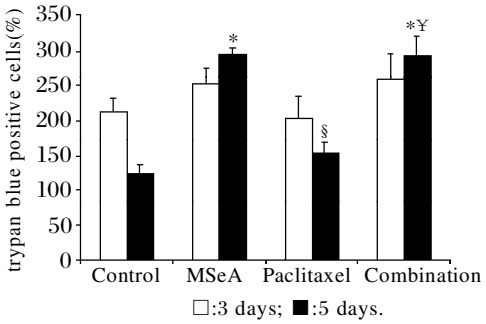
After drug treatment, cells were harvested in drug free medium at 3 and 5 days and then counted. When cells were counted on day 3 and compared to untreated controls, the percentage of floating cells that indicate dead cells were 67%, 293% and 158% after treatment with MSeA, paclitaxel and combination of MSeA/paclitaxel, respectively(***Fig. 4***). When treatment groups were compared, the only significant changes in floating cells were after paclitaxel alone. The floating cells significantly increased after treatment with paclitaxel alone when compared to untreated control($P = 0.005$) and MSeA alone($P = 0.002$). No other treatment group comparisons were statistically significant. When cells were counted on day 5 and compared to untreated control, the percentage of floating cells were 52%, 120% and 48% after treatment with MSeA, paclitaxel and combination of MSeA/paclitaxel, respectively(***Fig. 4***). When treatment groups were compared to untreated control, the decrease in floating cells was significant after treatment with MSeA($P = 0.02$) and combination of MSeA/paclitaxel($P = 0.003$). The level of floating cells were significantly decreased($P = 0.001$) after combination treatment when compared with paclitaxel alone and significantly increased after paclitaxel($P = 0.003$) when compared to MSeA alone. No other treatment group comparisons were statistically significant.



*denotes significant changes($P < 0.05$) when treatment groups are compared to untreated controls. § denotes significant changes($P < 0.05$) when treatment groups are compared to MSeA alone. ¥ denotes significant changes($P < 0.05$) when treatment groups are compared to paclitaxel alone. At day 5 after treatment, paclitaxel alone significantly increased the number of floating cells, whereas combining MSeA and paclitaxel did not.

Fig. 4 Number of floating cells in the various study groups

To confirm these results, a trypan blue exclusion assay was performed on the same treatment groups. On day 3 after treatments, the percentages of cells that took up trypan blue were 50%, 41% and 52% after treatment with MSeA, paclitaxel and a combination of MSeA/paclitaxel, respectively(***Fig. 5***). When treatment groups were compared, trypan blue uptake(indicating dead cells) was not significantly increased on day 3 after treatment(***Fig. 5***). No other treatment group comparisons were statistically significant. On day 5 after treatments, the percentages of cells that took up trypan blue were 59%, 31% and 59% after treatment with MSeA, paclitaxel and combination of MSeA/paclitaxel, respectively(***Fig. 5***). When treatment groups were compared to untreated control, the increase in trypan blue uptake was significant after treatment with MSeA ($P = 0.00001$) and combination of MSeA/paclitaxel ($P = 0.0009$). The level of trypan blue stained cells were significantly increased($P = 0.002$) after combination treatment when compared with paclitaxel alone and significantly decreased after paclitaxel($P = 0.00002$) when compared to MSeA alone. No other treatment group comparisons were statistically significant.



*denotes significant changes($P < 0.05$) when treatment groups are compared to untreated controls. § denotes significant changes($P < 0.05$) when treatment groups are compared to MSeA alone. ¥ denotes significant changes($P < 0.05$) when treatment groups are compared to paclitaxel alone. The combination treatment of MSeA and paclitaxel did not significantly increased trypan blue stained cells when compared to each drug alone on day 3. The level of trypan blue stained cells were significantly increased($P = 0.002$) after combination treatment when compared with paclitaxel alone on day 5.

Fig. 5 Cell viability assessed by trypan blue dye uptake

DISCUSSION

Paclitaxel is an effective chemotherapeutic drug for the treatment of different cancers. However, development of drug resistance and dose-limiting toxicity has raised major clinical challenges[18]. It has been documented that organic and inorganic Se compounds inhibit cell growth in multiple human cancer cell lines through different pathways[19-20]. The aim of this study was to investigate the role of MSeA as a therapeutic enhancer of paclitaxel efficacy against ovarian cancer.

The potential role for Se in anticancer activity is an area that has shown significant promise in preclinical and clinical trials. In this study we investigated the effect of MSeA in combination with paclitaxel against ovarian cancer. Sequential combined treatment with MSeA/paclitaxel was chosen based on previously

published data showing that pretreatment with Se containing compounds is essential for the enhancement of various chemotherapeutic agents against various cancers[16,21]. The most striking observation from this study was that MSeA did not enhance paclitaxel efficacy against the ovarian cancer A2780 cell line. The paclitaxel concentration that inhibits 50% of cell growth (IC_{50}) was 0.5 μmol/L(***Fig. 2***). When compared to paclitaxel alone, the dose of paclitaxel in combination with MSeA that equally inhibited cell growth was 2.4 fold higher(1.2 μmol/L)(***Fig. 3***). These data suggest that MSeA actually protected A2780 cancer cells from paclitaxel toxicity. However, one of the limitations of the SRB assay is the inability to distinguish between a cytostatic effect(cell growth arrest) and a cytotoxic effect (cell killing). Thus, the drug combination effect was further investigated to determine the viability of cells after the sequential combination treatment using more specific assays, such as cells counting and a trypan blue uptake/exclusion assay. The floating or detached cells that are indicative of cell death were calculated in the 4 treatment groups at 3 and 5 days after treatments. The combination groups had fewer floating cells than the groups treated with paclitaxel alone(***Fig. 4***). The trypan blue exclusion assay did not show significant differences in the number of cells taking up the dye(dead cells) when cells treated with both MSeA and paclitaxel were compared with those receiving paclitaxel alone 3 days after treatment. However, unlike the floating cell assay, by 5 days after treatment with the drug combination there was a significantly greater number of cells taking up the dye than with paclitaxel alone(***Fig. 5***). Overall, these data indicated that pretreatment with MSeA did not result in any additive or synergistic stasis or cell killing activity by paclitaxel.

p53 is a transcription factor encoded by the TP53 gene. p53 is important in multicellular organisms, where it regulates the cell cycle and functions as a tumor suppressor gene. Thus, p53 is involved in preventing cancer and conserving stability by preventing genome mutations[22]. In addition, p53 is a vital marker for differentiation of tumor cells from normal cells. Defective p53 functions are thought to contribute to the development, progression, prognosis and therapeutic outcome of human cancers[23]. p53 gene alterations are the most widespread genetic alterations seen in human cancers, with as many as 70% of tumors having a mutant p53 phenotype[24], and p53 is a marker of chemosensitivity of tumor cells *in vitro*[25].

In one study, Se selectively protected genetically normal cells from DNA-damaging agents, while simultaneously offering no detectable protection to tumor cells that either completely lacked p53 or possessed only mutant p53[26]. After DNA damage, p53 levels increase and mediate a cellular response manifested in cell cycle G1 arrest, via transcriptional activation of p21 gene[27]. In this study, pretreatment with MSeA did not show any additive or synergistic cell killing activity. A2780 ovarian cancer is a wild-type p53-expressing cell line, and it may not have been ideally suited for Se treatment. Pretreatment with MSeA may have changed the proportion of tumor cells in different stages of the cell cycle. Thus more cells may have been arrested in G1 or S phase and fewer cells in G2 phase. Paclitaxel is a G2M specific drug which binds and stabilizes microtubules resulting in cell death due to division arrest. Therefore, there may have been fewer cells in G2M for paclitaxel to target and kill. Future studies will evaluate the effect of MSeA on cell cycle arrest to prove whether this hypothesis is correct.

In summary, sequentially combining MSeA with paclitaxel resulted in less suppression of cell growth/death of A2780 ovarian cancer cells than with paclitaxel alone. It is hypothesized that because the A2780 cell line expresses wild-type p53, MSeA may have reduced the proportion of cells arrested at a phase where they would be susceptible to being killed by paclitaxel. Future studies are planned to evaluate the effect of combination treatment on the cell cycle to test this hypothesis.

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