D-α-tocopherol polyethylene glycol (1000) succinate-containing microemulsion enhances the anticancer effect of cisplatin in human lung epidermoid carcinoma cells

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Received: 03 May 2023 / Revised: 22 May 2023 / Accepted: 22 May 2023

ABSTRACT: Most of the cancer cases are detected in advanced stage when surgical intervention is not possible, thus chemotherapy is the only treatment option. In the majority of the cases, chemotherapy has severe side effects and cancer cells rapidly develop resistance to the treatment. Therefore, milder treatment options for chemotherapy patients are needed. The aim of this study was to investigate the potential of a vitamin E derivative, D-α-tocopherol polyethylene glycol (1000) succinate, in a microemulsion formulation to increase the anticancer activity of cisplatin. The vitamin E derivative was formulated as microemulsions with particle sizes between 100 and 150 nm, and zeta potential values between +4.1 and +5.8 mV. The IC50 value of the selected microemulsion was determined in human lung epidermoid carcinoma (Calu1) and adenocarcinoma (A549) cells. The microemulsion components had no significant effect on the cell viability. Co-treatment of cisplatin and the microemulsion increased the level of early mediator caspases, caspase-8 and caspase-9, and the effector caspase-3 in the cancer cells. Simultaneous application of the microemulsion with cisplatin enhanced the anticancer effect of the chemotherapy drug. The novel microemulsion has the potential to reduce the required dose of cisplatin for effective cancer treatments.

KEYWORDS: microemulsions; TPGS; chemotherapy; apoptosis; cisplatin.

1. INTRODUCTION

Cancer has become a major global health concern in the past several decades. Main treatment options for cancer therapy are surgical dissection for removal of the tumor tissue [1], chemotherapeutic [2], hyperthermia [3] and hormone therapy [4] that contribute to the killing and suppressing the invasiveness of cancer cells. Surgical resection is the main treatment for most of the cases but it has the potential to trigger metastasis [1]. For this reason, chemotherapy and/or radiotherapy are applied after surgery to suppress the recurrence of the disease. However, these are subject to resistance development by cancer cells rendering the treatments less efficient [5]. Chemoresistance, is a major problem of chemotherapy. It is a phenomenon in which cancer cells develop resistance to the drug and become less susceptible to the administered dose [6]. This leads to inefficient therapy and necessitates increased dose treatment. In the last two decades, very potent novel techniques such as immunotherapy and targeted therapies [7,8] and gene-/cell-based therapies [9,10] have been developed for managing and treating cancer. However, given the fact that even conventional chemotherapeutics are still quite expensive, only limited number of patients can receive these innovative advanced therapies [11]. For this reason, chemotherapy remains primary option for the majority of patients. Lung cancer is the most common cause for cancer-related deaths worldwide [12]. Cisplatin administration is one of the options for lung cancer chemotherapy. However, it has severe side effects due to formation of non-specific crosslinks in healthy cells’ DNA. Also, cancer cells develop resistance to cisplatin which reduces its efficacy [12,13]. It has been demonstrated that decreased apoptotic activity promotes cisplatin resistance in non-small cell lung cancer cells [14].

Vitamin E derivatives have been explored as potential drug carrier systems due to their biocompatibility, low toxicity, and ability to enhance drug solubility and bioavailability. Some of the commonly used vitamin E derivatives in drug delivery systems include tocopherol, tocotrienol, and tocopheryl polyethylene glycol (1000) succinate (TPGS) [15]. TPGS is a water-soluble amphiphilic derivative

How to cite this article: Kotmakçı M, Kantarci AG, Bozok Çetintaş V. D-α-tocopherol polyethylene glycol (1000) succinate-containing microemulsion enhances the anticancer effect of cisplatin in human lung epidermoid carcinoma cells. J Res Pharm. 2023; 27(6): 2389-2398.
Kotmakçı et al. D-α-tocopherol PEG1000 succinate microemulsion enhances the anticancer effect of cisplatin

Journal of Research in Pharmacy

Research Article

http://dx.doi.org/10.29228/jrp.526
J Res Pharm 2023; 27(6): 2389-2398

of vitamin E that has been extensively studied as a drug carrier system [15,16]. It has been shown to improve the solubility and bioavailability of poorly soluble drugs, such as paclitaxel, docetaxel, and curcumin [17,18].

Recently, it was reported that TPGS has potent anticancer activity by inducing reactive oxygen species production in hepatocellular carcinoma and breast cancer cells leading to increased apoptosis rate [16,19,20]. Effective inhibition of the growth of in vivo tumor model by TPGS administration was also reported [19,20]. Besides the direct anticancer activity, TPGS is able to enhance the efficacy of other chemotherapeutics by inhibiting the ATP-binding cassette sub-family B member 1 (ABCB1) P-glycoprotein which acts as a drug efflux [21,22]. In hepatocellular carcinoma cells, it has been demonstrated that TPGS micelles enhance the anticancer efficacy of cisplatin and show reduced renal injury [23].

Microemulsions are ternary systems composed of oil, surfactant and water phases. They form by simple mixing of all components at specific ratio that ensures thermodynamic equilibrium and self assembly of small droplets either of water-in-oil (w/o) or oil-in-water (o/w) type [24]. The ease of preparation, long-term thermodynamic stability, ability to solubilize both hydrophilic and hydrophobic molecules and their biocompatibility render microemulsions attractive candidates for delivery of a variety of drugs.

The aim of this study was to investigate the potential of vitamin E derivatives-loaded microemulsions to increase the anticancer efficacy of the chemotherapeutic drug cisplatin in lung cancer cells for more effective treatment.

2. RESULTS AND DISCUSSIONS

The pseudoternary phase diagram obtained by titration of oil/surfactant mixtures (10:90, 20:80, 30:70, 40:60 and 50:50 w/w) is presented in Figure 1. As seen in the figure, the mixtures yield transparent single phase systems at the water-rich corner of the diagram. Mainly oil-in-water type microemulsion are seen in this region. One formulation comprising 5% oil (consisting of oleic acid:vitamin E acetate [OA/VE 1:1 w/w]), 15% surfactant/co-surfactant (S/CoS) consisting of Cremophor RH-40/Spa 80/propylene glycol (1.5:0.5:2 w/w) and 80% water was selected from the microemulsion area to incorporate TPGS at different ratios.

Figure 1. Pseudoternary phase diagram obtained as a result of water titration of OA/VE:S/CoS mixtures. Black dot represents the microemulsion selected for further investigation (5% oil, 15% S/CoS and 80% water).

The physicochemical properties of microemulsion prepared by incorporating 0%, 10%, 20% or 30% of TPGS (w/w) in the S/CoS phase are presented in Figure 2. The original microemulsion without TPGS has droplet size of 134 nm and zeta potential of +5.6 mV. Addition of the amphiphilic molecule, TPGS to the formulation decreased the particle size to 102 and 104 nm at 10% and 20%, respectively. Further increase of TPGS content increased the droplet size to 152 nm. All formulations had PDI close to, or below 0.2. This means all formulations had monodisperse size distribution. No meaningful difference was observed between the zeta
potentials (ranging between +4.1 and +5.8 mV), nor between pH values (ranging between 5 and 5.5) of formulations. The formulation having the smallest particle size of 102 nm (M2, see Table 2) was selected for further investigation in cell culture experiments.

Figure 2. Physicochemical properties of the microemulsions containing TPGS. a- droplet size and polydispersity values, b- zeta potential and pH values.

The selected formulation was evaluated for its antiproliferative effect on A549 and Calu1 lung cancer cell lines to determine the IC₅₀ value. Initially, high doses between 0.5- to 2.5 mM concentration were screened. However, significant drop in cell proliferation was observed within the first 6h after the treatment (Figure 3a and 4a). Subsequently, lower concentrations between 0.1- to 150 µM were tested (Figure 3b and 4b). At this dosing, gradual decrease in cell proliferation was observed which allowed for calculation of the IC₅₀ values (Figure 3c and 4c). According to these results, based on the TPGS content, the microemulsion had IC₅₀ of 50 µM and 31 µM for A549 and Calu1 cells, respectively. These values were taken as reference for combination treatments together with cisplatin.

The effect of microemulsion components on the cell viability is presented in Figure 5a. Only TPGS, when applied alone showed significant toxicity both on A549 and Calu1 cells. It is interesting because the TPGS dose corresponds to the IC₅₀ in the microemulsion (Figure 3 and 4). This is in correlation with our previous findings that amphiphilic ingredients of microemulsions and solid lipid nanoparticles can display higher nonspecific toxicity in free micellar form due to easy interaction with and disruption of cell membranes [14,25]. This can be explained by their amphiphilic property which leads to fast diffusion between phospholipid layers of the cell membrane. All other microemulsion ingredients showed acceptable cytotoxicity with viability close to or above 80%.

The additive effect of vitamin E containing microemulsion on the anticancer activity of the chemotherapeutic drug cisplatin is shown in Figure 5b. In A549 cells, the effect was not obvious when doses lower than IC₅₀ are applied. At the doses of ≥IC₅₀, the viability of cancer cells decreased significantly as compared to cisplatin alone. On the other hand, Calu1 cells were more susceptible to the combination treatment than A549 cells. Even at a dose of 0.5x IC₅₀ significantly higher toxicity as compared to cisplatin alone was observed. This means that Calu 1 epidermoid carcinoma cells are more susceptible to the synergistic effect of vitamin E microemulsion and cisplatin combination than A549 adenocarcinoma cells. This observation can be explained by the increased caspase activity in all combination doses in Calu1 cells whereas in A549 cells, the caspase levels are not elevated at doses below IC₅₀ (Table 1). It is known that caspase-8 is an upstream mediator for intrinsic apoptosis signaling while caspase-9 is an upstream mediator for extrinsic apoptosis signaling. When these are activated, they trigger effector caspases such as caspase-3 and -7 which are necessary for the termination of the apoptotic cell death [26]. In the Table 1, it is seen that microemulsion treatment alone increases the caspase levels to higher extent in Calu1 cells than in A549 cells. Also, in Calu1 cells the effector caspase-3 activation was increased in all treatment doses, which explains the observation of the higher drop in cell viability as compared to A549 cells.
**Figure 3.** Effect of vitamin E microemulsion treatment on the proliferation of A549 cells determined by real-time impedance measurements. a- proliferation curves after high dose microemulsion treatment (0.5-2.5 mM), b- proliferation curves after low dose microemulsion treatment (0.1-150 µM), c- Log normalized dose-response plot for IC_{50} calculation.
Figure 4. Effect of vitamin E microemulsion treatment on the proliferation of Calu1 cells determined by real-time impedance measurements. a- proliferation curves after high dose microemulsion treatment (0.5-2.5 mM), b- proliferation curves after low dose microemulsion treatment (0.1-150 µM), c- Log normalized dose-response plot for IC\(_{50}\) calculation.
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Journal of Research in Pharmacy

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http://dx.doi.org/10.29228/jrp.526

J Res Pharm 2023; 27(6): 2389-2398

Figure 5. Cytotoxicity of individual microemulsion components on A549 and Calu1 cells (n=3) (a), and anticancer activity of cisplatin alone and in combination with vitamin E microemulsion on A549 and Calu1 cells (n=4) (b). The concentration for cytotoxicity of individual components was set equal to that in the IC_{50} dose. The doses for anticancer activity were set at 0.5x, 0.75x, 1x, 1.5x and 2x of the IC_{50}.

Table 1. Fold change in apoptosis markers in A549 and Calu1 cells after treatment with cisplatin alone, microemulsion alone or cisplatin and microemulsion in combination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A549 cells</th>
<th></th>
<th>Calu1 cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caspase-3</td>
<td>Caspase-8</td>
<td>Caspase-9</td>
<td>Caspase-3</td>
</tr>
<tr>
<td>UT</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cis</td>
<td>1.15</td>
<td>1.25</td>
<td>1.26</td>
<td>1.12</td>
</tr>
<tr>
<td>ME</td>
<td>1.70</td>
<td>1.55</td>
<td>1.55</td>
<td>1.93</td>
</tr>
<tr>
<td>Cis+ME (0.5xIC_{50})</td>
<td>1.10</td>
<td>0.86</td>
<td>0.89</td>
<td>2.88</td>
</tr>
<tr>
<td>Cis+ME (0.75xIC_{50})</td>
<td>1.14</td>
<td>0.90</td>
<td>1.00</td>
<td>2.80</td>
</tr>
<tr>
<td>Cis+ME (1xIC_{50})</td>
<td>2.80</td>
<td>2.58</td>
<td>2.57</td>
<td>1.90</td>
</tr>
</tbody>
</table>

Abbreviations: UT-untreated control, Cis- cisplatin-treated cells, ME- microemulsion-treated cells, Cis+ME(0.5xIC_{50}), Cis+ME(0.75xIC_{50}) and Cis+ME(1xIC_{50})- combination treatment of cisplatin and vitamin E microemulsion at 50%, 75% and 100% of the IC_{50} dose, respectively.
4. CONCLUSION

The developed novel vitamin E microemulsion significantly increased the anticancer effect of cisplatin in epidermoid carcinoma cells by increasing the level of upstream and effector caspases. These results suggest that vitamin E microemulsion might be useful to reduce the required cisplatin dose and therefore to reduce the potential toxic side effects. Further research is needed to observe the distinct effect of different vitamin E analogues formulated as microemulsions both in vitro and in vivo.

5. MATERIALS AND METHODS

DL-α-tocopherol acetate (VE) was from Applichem (Germany), oleic acid, α-tocopherol polyethylene glycol (1000) succinate (TPGS), Cremophor RH-40 (CRH) and Span® 80 (S80) were from Sigma-Aldrich (Germany), Ham's F12 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Penicillin/Streptomycine solution were from Biological Industries (Israel). Caspase 3, Caspase 8 and Caspase 3 colorimetric assay kits were obtained from BioVision (Waltham, MA). All chemicals and solvents were reagent grade and used as received without further purification. Ultrapure water was used in all experiments unless otherwise specified.

5.1. Preparation of the microemulsions

The microemulsion formation area of the system comprising oleic acid/vitamin E acetate (1:1 w/w) (the oil phase), Cremophor RH-40/Span80 [3:1 w/w] / propylene glycol (1:1 w/w) (the surfactant/CoSurfactant, S/CoS phase) and ultrapure water was evaluated by ternary phase diagram titration. Microemulsion formation area was plotted using a software developed in the computer center at the Ege University Faculty of Pharmacy as described previously [27]. One formulation was selected from the microemulsion area and TPGS was included in the S/CoS mixture at 0%, 10%, 20% or 30% ratio. The composition of the microemulsions are presented in Table 2.

Table 2. Composition of the prepared microemulsions.

<table>
<thead>
<tr>
<th>No</th>
<th>OA:VE (1:1) (g)</th>
<th>S/CoS (g)</th>
<th>H₂O (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0% TPGS</td>
<td>10% TPGS</td>
<td>20% TPGS</td>
</tr>
<tr>
<td>M1</td>
<td>0.2</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>0.2</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>M3</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M4</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

5.2. Characterization of the microemulsions

Droplet size of the microemulsions was measured by dynamic light scattering (DLS) using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Briefly, samples were transferred to micro-volume polystyrene spectroscopic cuvettes and the particle size and polydispersity index were measured at 25°C without further dilution at 173° back scattering mode. The same device, run at electrophoretic light scattering mode was used to measure the zeta potential of the microemulsions. Samples were filled into zeta cuvettes and measurements were performed at 25°C at the automatic counting mode without further dilution. pH of microemulsions were measured using Sevengo Duo pH/Conductivity meter (Mettler Toledo, Switzerland) at room temperature.

5.3. Cell culture

Human non-small cell lung cancer (NSCLC): A549 adenocarcinoma (CCL-185™) and Calu1 epidermoid carcinoma (HTB-54™) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). A549 cells were cultured in Ham's F12 medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Calu1 cells were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Both cell lines were maintained under humidified atmosphere of 5% CO₂ and 95% air at 37°C.
5.3.1. IC₅₀ values of cisplatin and vitamin E microemulsion

We previously demonstrated that cisplatin has IC₅₀ value of 30 µM and 13.68 µM for A549 and Calu1 cell lines, respectively [14]. To investigate the IC₅₀ of the microemulsion, 24 h before the treatment, cells were seeded at a density of 1.5x10⁶ cells/well in a 96-well E-plate (Roche Applied Science). The final volume was made up to 200 µL, the plate was mounted to the xCELLigence® Real-Time Cell Analyser (Roche Applied Science) and incubated under humidified atmosphere of 5% CO₂ and 95% air at 37°C for 24 h. After the incubation, 150 µL of the medium was removed and 150 µL of fresh full medium containing dilutions of the selected VE-microemulsion were added to wells. Application of the microemulsion was performed at high doses (ranging between 0.5 – 2.5 mM of TPGS) and low doses (ranging between 0.1 – 150 µM of TPGS) in separate setups. Cells were incubated at 37°C for at least 72 h and the impedance values were recorded every 15 min. Data analysis and IC₅₀ calculation was performed by the software included in the xCELLigence instrument.

5.3.2. Cytotoxicity of microemulsion components on the cells and anticancer effect of vitamin E microemulsions in combination with cisplatin

To examine whether the microemulsion components have cytotoxic effect on the cells, oleic acid, vitamin E acetate, Span 80, TPGS, Cremoprop RH-40 and propylene glycol were dissolved separately in the ethanol. Further dilutions were prepared in the culture medium before application to the cells. Cells were seeded into 96-well cell culture plates at a density of 5x10⁵ cells/well and allowed to attach for 24 h. After the incubation the medium was replaced with fresh medium containing the microemulsion components. The doses of all components were calculated according to the concentrations found in the IC₅₀ dose of the microemulsion. The exact doses for A549 cells are as follows: OA and VE: 126.1 µg/mL, S80: 85.1 µg/mL, CRH: 255.4 µg/mL, PG: 340.5 µg/mL and TPGS: 75.7 µg/mL. The exact doses for Calu1 cells are as follows: OA and VE: 78.1 µg/mL, S80: 52.7 µg/mL, CRH: 158.2 µg/mL, PG: 210.9 µg/mL and TPGS: 46.9 µg/mL. Cells were incubated for 48 h and cell viability was determined by WST-1 cell proliferation reagent (Roche Applied Science), according to the manufacturer’s instructions. The relative amount of the formed soluble formazan was measured using a microplate reader (Thermo, USA) at 450 nm.

The anticancer effect of the combined treatment was assessed at 2x, 1.5x, 1x, 0.75x and 0.5x times the IC₅₀ dose of cisplatin and the ME. Viability was assessed by WST-1 cell proliferation reagent as described above. In both experiments untreated cells were used as 100% viability control and all treatment results were normalized to this group.

5.3.3 Measurement of apoptotic markers

To determine the change in apoptosis markers levels, cells were seeded into 25 cm² cell culture flasks at density of 1x10⁵ cells/flask and incubated for 24 h to allow cells to attach. After the incubation, the media were exchanged with fresh media containing cisplatin alone at IC₅₀ dose, microemulsion alone at IC₅₀ dose, or cisplatin + microemulsion at 1x, 0.75x and 0.5x times the IC₅₀ dose. Treated cells were incubated for 48 h at 37°C. After the incubation, cells were harvested by trypsinization, and 10⁶ cells were lysed with the lysis solution provided with the colorimetric caspase assay kits. 50 µL of the lysates were transferred into separate wells of a 96-well plate and specific substrates of either caspase-3, caspase-8 or caspase-9 were added and incubated at 37°C for 1 h. After the incubation, the absorbance of the reaction medium was measured at 405 nm by a multiplate reader (Thermo, USA). Untreated cells were used to determine the basal level of the tested apoptotic markers. The results of the assay are reported as the fold change normalized to untreated group.

5.4. Statistical analysis

Data are presented as mean ±SD of at least triplicate measurements. Statistical significance of the difference between separate treatment groups was assessed by one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni multiple comparisons test. The difference was considered statistically significant when P<0.05. GraphPad Prism 5.0 software (GraphPad software, Inc.) was used for analyses.
Acknowledgements: The authors would like to thank Ms. Öykü Özdemir and Mr. Can Ekicioğlu for their assistance in the project. This research received no external funding.


Conflict of interest statement: Part of this research was presented at TÜBİTAK’s 46th Secondary Education Research Projects Regional Competition.

REFERENCES


http://dx.doi.org/10.29228/jrp.526
J Res Pharm 2023; 27(6): 2389-2398


