Cancer Cell International

RESEARCH

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Synergistic antitumor effect of liposomal-based formulations of olaparib and topotecan in primary epithelial ovarian cancer cells

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Abstract

Background Olaparib is a PARP inhibitor inducing synthetic lethality in tumors with deficient homologous recombination (HRD) caused by BRCA1/2 mutations. The FDA has approved monotherapy for first-line platinum-sensitive, recurrent high-grade epithelial ovarian cancer. Combination therapy alongside DNA-damaging therapeutics is a promising solution to overcome the limited efficacy in patients with HRD. The present study was designed to develop topotecan- and olaparib-loaded liposomes (TLL and OLL) and assess the effectiveness of their combination in patient-derived ovarian cancer samples.

Methods We used HEOC, four clear-cell tumors (EOC 1–4), malignant ascites, and an OCI-E1p endometrioid primary ovarian cancer cell line and performed NGS analysis of BRCA1/2 mutation status. Antiproliferative activity was determined with the MTT assay. The Chou-Talalay algorithm was used to investigate the in vitro pharmacodynamic interactions of TLLs and OLLs.

Results The OLL showed significantly higher efficacy in all ovarian cancer types with wild-type BRCA1/2 than a conventional formulation, suggesting potential for increased in vivo efficacy. The TLL revealed substantially higher toxicity to EOC 1, EOC 3, ascites and lower toxicity to HEOC than the standard formulation, suggesting better therapeutic efficacy and safety profile. The combination of studied compounds showed a higher reduction in cell viability than drugs used individually, demonstrating a synergistic antitumor effect at most of the selected concentrations.

Conclusions The concentration-dependent response of different ovarian cancer cell types to combination therapy confirms the need for in vitro optimization to maximize drug cytotoxicity. The OLL and TLL combination is a promising formulation for further animal studies, especially for eliminating epithelial ovarian cancer with wild-type BRCA1/2.

Keywords Epithelial ovarian cancer, BRCA1/2, PARPi, Liposomes, Drug combination

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Introduction

Ovarian cancer is the fifth leading cause of cancer-related death among women in the US and remains the most lethal gynecological cancer in the Western World [1]. Most ovarian tumors (~ 90%) are of epithelial origin, with the prevalence of serous carcinoma, while a small portion originates from sex cord-stromal tissues or germ cells [2, 3]. Ovarian cancer is mainly detected in advanced stages because there are no symptoms in the early phases of the disease. Symptoms that already appear are nonspecific and include, for example, abdominal pain, early satiety, or changes in bowel habits [4, 5]. According to the International Federation of Gynecology and Obstetrics (FIGO) system, most serous carcinomas are still diagnosed at advanced stage III or IV, where 5 year survival rates are only 42% and 26%, respectively [6–8]. Overall survival has not substantially improved over the past decades. Epithelial ovarian cancer (EOC) encompasses a heterogeneous group of malignancies with five major histotypes: high-grade serous carcinoma (HGSC), clear cell carcinoma (CCC), endometrioid carcinoma (EC), mucinous carcinoma (MC) and low-grade serous (LGSC). They vary in etiology, pathogenesis, molecular alterations, risk factors, prognosis, and numerous other characteristics. Additionally, different histological and molecular subtypes involve different cells of origin and varying patterns of progression and response to therapy [9]. Genetic susceptibility is manifested by rare inherited mutations with high to moderate penetrance. The most notable example is the rare high-penetrant mutations in the BRCA1 and BRCA2 genes. These factors significantly increase the disease's lifetime risk and account for most hereditary cases and 10%–15% of all cases. Mutations in BRCA genes cause hereditary breast and ovarian cancer syndrome (HBOC) [10, 11]. It was reported that cells deficient in BRCA1/BRCA2-dependent homologous recombination are sensitive to poly (ADP-ribose) polymerase inhibitors (PARPi), such as the FDA-approved olaparib (Lynparza, AstraZeneca) [12, 13]. The antitumor effect of such drugs results from the simultaneous disruption of both genes, while disruption of either gene alone is not lethal for cancer cells. This genetic concept of gene interaction is called synthetic lethality [14]. BRCA1 and BRCA2 regulate cellular processes, e.g., transcription, the cell cycle, and the DNA damage response (DDR), with a particularly significant role in the mechanism of DNA double-strand break repair and homologous recombination [15].

For decades, the standard of care for first-line treatment of advanced ovarian cancer consisted of cytoreductive surgery followed by platinum- and taxane-based chemotherapy. Unfortunately, most patients relapse due to residues of micrometastases and the induction of drug resistance mechanisms. The incorporation of PARPi has transformed the treatment of patients with HGSC, EC, primary peritoneal, and fallopian tube cancers [16–18]. The FDA and EMA have approved olaparib as a first-line treatment for primary and recurrent platinum-sensitive ovarian tumors. However, a majority eventually develop resistance to PARPis, including those with primary resistance [13, 17]. While PARPi treatment exerts limited efficacy in HR-proficient patients, combination therapy alongside DNA-damaging therapeutics is a promising solution [19]. Lin et al. demonstrated another proof-of-principle approach with the combination of olaparib, triapine, the DNA synthesis inhibitor, and cediranib (selective inhibitor of vascular endothelial growth factor receptor kinases) on BRCA wild-type EOC using xenograft mouse models. Triapine is a small molecule inhibitor of ribonucleotide reductase (RNR), which blocks de novo dNTP production. Data shows suppression of EOC growth and significant prolongation of the survival time of mice, with efficacy greater than any single drugs and double conjunction [20]. Drug combinations and targeted therapies have been widely used and have become the leading choice to overcome drug resistance and minimize off-target toxicity [21–25].

We suggest using a liposomal formulation of olaparib and topotecan to maximize the treatment effect. Liposomes are spherical phospholipid vesicles comprising one or more lipid bilayers with an internal aqueous cavity, creating hydrophilic and hydrophobic hollows [26]. These properties make them ideal nanosized drug delivery systems transporting hydrophilic and lipophilic drugs and compounds. We used neutral liposomes, which are non-toxic, biodegradable, biocompatible, and nonimmunogenic [27–29]. The objective of this study was to develop liposome formulations of olaparib and topotecan and evaluate their anticancer efficacy against primary EOC cells. As an experimental model, we used four samples of clear-cell tumors (EOC 1–4), malignant ascites (Ascites) from a patient with HGSC, an OCI-E1p endometrioid primary ovarian cancer cell line, and a sample of healthy epithelial ovarian tissue (HEOC), as the control to confirm the specificity of action. Ovarian carcinomas as a group of heterogeneous neoplasms, classified according to cell type. The most common and aggressive subtype of ovarian cancer is HGSC. The ascites, an abnormal accumulation of fluid in the peritoneum occurs in more than one-third of patients at initial diagnosis and in almost all cases of relapse as a commodity. Malignant ascites contribute to metastasis and therapy resistance and portend the poorest outcomes for cancer patients [30]. CCC represents approximately 10% of ovarian carcinomas with a strong association with endometriosis. Patients are typically younger and diagnosed at earlier stages than those with the HGSC. Advanced or recurrent

cases experience poor outcomes mostly due to intrinsic chemoresistance. Endometrioid tumors occur with similar frequency to CCC, and the vast majority are malignant and invasive [9]. Alternative treatments, including combination therapy, represent the most promising strategies seeking to improve oncological outcomes and quality of life for patients with these aggressive diseases.

Methods

Chemicals

All lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Topotecan hydrochloride and olaparib were obtained from Selleck Chemicals LLC (Houston, TX, USA). L-α-phosphatidylcholine 95% (EPC) and cholesterol (chol; ovine wool, > 98%) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

Preparation of liposomes

Liposomes were prepared according to previously described methods with modifications [31]. Topotecan-loaded liposomes (TLLs) and corresponding non-loaded (NL) formulations were prepared by thin lipid hydration with active drug loading by an ammonium sulfate gradient. EPC and cholesterol were dissolved in chloroform, and stock solutions were mixed to obtain a molar ratio of 55:45. The organic solvent was evaporated at 40 °C to form a thin lipid film, which was rehydrated with 250 mM ammonium sulfate at 45 °C. Liposomes were extruded gradually using polycarbonate membranes with pore sizes of 200 nm (5 times) and 100 nm (6 times) at room temperature using an extruder device from Northern Lipids Inc. (Vancouver, BC, Canada). To obtain the ammonium sulfate gradient, three consecutive dialysis exchanges using Spectra-Por® Float-A-Lyzer®, with a molecular weight cutoff (MWCO) of 100 kDa (Spectrum Laboratories, Inc., Piscataway, NJ, USA), against 10% sucrose were used. An aqueous solution of topotecan (6 mg/mL) was added to the liposome dispersion after the creation of an ammonium sulfate gradient during vigorous shaking on an orbital incubation shaker Gyromax™ 703 (Amerex Instruments Inc., Concord, CA, USA) at 250 rpm for 1 h at 45 °C. The non-entrapped drug was removed by three consecutive dialysis exchanges against 10% sucrose (1 step), followed by 0.9% NaCl (2 steps).

Olaparib-loaded liposomes (OLLs) and corresponding non-loaded formulations (NLs) were prepared by thin lipid hydration with passive drug loading during hydration. Olaparib, EPC, and cholesterol were dissolved in chloroform, and stock solutions were mixed to obtain a molar ratio of 10:80:10. The organic solvent was evaporated at 40 °C to form a thin lipid film, which was rehydrated with 0.9% NaCl at 45 °C. Liposomes were extruded gradually using polycarbonate membranes with pore sizes of 200 nm (5 times) and 100 nm (6 times) at room temperature using an extruder device from Northern Lipids Inc. (Vancouver, BC, Canada). Three consecutive dialysis exchanges removed the non-entrapped drug against 0.9% NaCl.

Topotecan- and olaparib-loaded liposomes were freshly prepared before in vitro experiments.

Liposome characterization

Drug encapsulation

Liposomes were diluted ten times in methanol, and drug concentrations were determined by chromatographic methods. The encapsulation efficacy (EE, %) was calculated according to Eq. 1

$$EE\ (\%) = (CM/Ci) \times 100 \quad (1)$$

CM is the measured concentration of the drug-loaded into liposomes, and Ci is the initial concentration of the drug during liposome preparation.

Size and zeta potential measurements

Liposome size and zeta potential were measured at 37 °C using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) by dynamic light scattering (DLS) and laser Doppler velocimetry, respectively. Measurements were carried out in disposable folded capillary cells (zeta potential) or standard polystyrene cuvettes (size). Measurements were repeated three times. Before the measurements, liposome samples were diluted ten times in deionized water (zeta potential) or 0.9% NaCl (size analysis).

Stability of liposomes

Liposomes were stored in 0.9% NaCl at 4 °C. Upon preparation and on days 7, 14, and 21 following the preparation, 1 ml of liposomal sample was dialyzed against 0.9% NaCl for 24 h using Spectra-Por® Float-A-Lyzer®, with a molecular weight cut-off (MWCO) of 100 kDa (Spectrum Laboratories, Inc., Piscataway, NJ, USA), and subsequently, drug loading, particle size and PDI were analyzed following the procedures described above.

In vitro release of drug from liposomes

The release of topotecan and olaparib from liposomes was analyzed by the dialysis method in PBS pH 7.4, which was used as a release medium. One mL of the liposomal formulation was added to dialysis bags (15 kDa MWCO, Spectra/Por, Spectrum Laboratories, Inc., Piscataway, NJ, USA) and immersed in the release medium in 50 mL tubes to provide sink conditions. The tubes were protected from light and shaken at 150 rpm and 37 °C using an orbital incubation shaker Gyromax™ 703 (Amerex Instruments Inc., Concord, CA, USA). Samples (0.7 mL)

were withdrawn from the release medium at predetermined time points and replaced with fresh medium. All measurements were repeated three times. The release kinetics were presented as a ratio of drug released/drug added to dialysis bags against time. The kinetics of drug release from liposomes were determined using DDSolver software by fitting the obtained results to different kinetic models: Higuchi, Krosmeyer-Peppas, and Peppas-Sahlin [32].

High-performance liquid chromatography (HPLC)

Samples were analyzed by HPLC with a UV/vis detector using an XTerra C18 Column, 125 Å, 5 µm, 4.6 mm × 150 mm (Waters Corporation, Milford, MA, USA) operated at 25 °C. The mobile phase consisted of 0.1% TFA in acetonitrile/water 20:80 (v:v) for topotecan analysis and acetonitrile–water 30:70 (v:v) for olaparib, and the flow rate was set to 1 mL/min. The signal was detected at 380 and 230 nm for topotecan and olaparib, respectively. The chromatographic apparatus consisted of a Model 1525 pump (Waters Corporation, Milford, MA, USA), a Model 717 Plus auto-injector (Waters Corporation), and a Model 2487 dual-wavelength UV/vis detector (Waters Corporation).

Cell culture

Five human primary ovarian tumor samples (EOC 1–4, Ascites), a primary ovarian cancer cell line (OCI-E1p), and one normal ovarian tissue sample (HEOC) were used in this study. Dr. Tan Ince from Weill Cornell Medicine kindly provided the deidentified patient-derived OCI-E1p ovarian cancer cells [33]. The discarded anonymous pathological material, which was deidentified and banked, was obtained at the Tumor Retrieval Core Facility at the Cancer Institute of New Jersey. Primary epithelial ovarian cells isolated from a discarded tumor or healthy tissue were cultured in RPMI-1640 (Life Technologies, NY, USA) supplemented with 0.01 mg/ml bovine insulin (Thermo Fisher, NY, US), 10% (v/v) fetal bovine serum (Thermo Fisher, NY, US), and 1% penicillin/streptomycin (Thermo Fisher, NY, US). All tumor samples were grown at 37 °C in an atmosphere containing 5% (v/v) CO_2 at 95% (v/v) relative humidity. The OCI-E1p was maintained on Falcon® Primaria cell culture flasks in OCMIe media (US Biological, MA, USA) supplemented with 10% (v/v) FBS (Sigma–Aldrich, Germany) at a 5% (v/v) O_2 atmosphere at 95% (v/v) relative humidity.

Cell viability assays

The cytotoxicity of the studied compounds and their liposomal formulations was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The assay was performed as previously described [34]. Cells were plated at a density of 5×10^3 in 90 µL of total medium dedicated to the cell line in sterile 96-well plates and cultured overnight. The cells were treated with various concentrations of topotecan (0,001–25 µM), olaparib (0,0016–1,6 mM), and 0.25% DMSO, which served as a control group. Both drugs were dissolved in DMSO at a final solvent concentration of 0.25% (v/v), which did not affect cell viability. Cells were exposed to the drugs or their combination for 72 h, and MTT (Sigma–Aldrich, Germany) solution (5.0 mg/mL) was added to each well. The cells were incubated at 37 °C for 4 h, followed by 100 µL of solubilization buffer (10% SDS in 0.01 M HCl) addition. Finally, the absorbance at 570 nm was measured with a reference wavelength of 690 nm, and the background absorbance of 690 nm was subtracted from the 570 nm measurement. Three separate experiments were performed, with two repeats for each concentration. Relative cell viability was determined using the following formula: %viability = (mean of A570-A690 of an experimental group)/(mean of A570-A690 of the DMSO group) × 100%. The IC_{50} (the concentration of a drug needed to inhibit cellular metabolism by 50%) values were obtained using the nonlinear regression curve fit performed by CompuSyn v1.0 software (ComboSyn Inc., NJ, US) and are presented as the estimate ± SEM [34].

In vitro drug combination analysis

The cells were treated with either a single or a combination of the liposomal drugs in a constant ratio combination (based on their IC_{50} values). After 72 h of incubation with drugs, cell viability was assessed by the MTT assay. The experiments with a single drug and its combinations were conducted under the same experimental conditions to avoid variability caused by personnel changes and agent or target inconsistency. For drug combination experiments, the results were analyzed based on a cell viability assay for synergistic, additive, or antagonistic effects using the combination index (CI) method developed by Chou and Talalay by using Compusyn v1.0 software (ComboSyn, Inc., NJ, US). Combination indices, CI < 1, CI 1, and CI > 1 indicate synergism, additive effects, and antagonism, respectively. The liposomal formulation's inhibitory properties were visualized using SynergyFinder 3.0 (https://synergyfinder.fimm.fi), a free web application for multidrug combination response analysis. The input data included cell response to the single drug and combination treatment as %viability.

DNA extraction and library construction

DNA was extracted from cultured tumor and OCI-E1p cell line samples using a Blood & Cell Culture DNA Kit (Qiagen Inc., MD, USA) according to the manufacturer's

protocol. After extraction, all DNA samples were stored at −20 °C. Before the analysis, the DNA quality and concentration were determined photometrically (OD260/OD280, 1.8 to 2.0). The 20 ng of DNA was used as input in the CleanPlex® BRCA1 & BRCA2 Panel v3 (Paragon Genomics Inc., CA, US). The panel targets all exonic regions and 20 bp of flanking intronic sequences of BRCA1 and BRCA2.

BRCA1 and BRCA2 sequencing and data analysis

BRCA1/2 are tumor suppressor genes, so there are no hotspot mutations. A wide spectrum of pathogenic alternations can be found widely distributed throughout the coding sequence and splice site regions of both genes. Therefore, for the analysis, it is necessary to sequence the entire coding region as well as exon/intron junctions. Moreover, these loss-of-function variants are heterogeneous, including single nucleotide variants (SNVs), small insertions and deletions (indels), and copy number variants (CNVs).

The sequencing reaction was carried out using the Illumina MiSeq System (Illumina, CA, US). Only alternations meeting quality criteria, such as allele frequency ≥ 5% and coverage of at least 500×, were included to obtain reliable results. Classification and interpretation involved the detected filtered and unfiltered BRCA1/2 variants. The variant annotation provided by the BaseSpace Variant Interpreter, version 2.9.1.15. software (Illumina, CA, US) was manually reviewed according to the online databases ClinVar, dbSNP, and Cosmic. For this study, the annotation of pathogenicity of the detected variants was determined according to the ClinVar classification in: "benign," "likely benign," "uncertain significance," "conflicting interpretations of pathogenicity, "likely pathogenic," and "pathogenic." Sequencing variant nomenclature was carried out in concordance with the Human Genome Variation Society (HGVS) guidelines to achieve a consistent approach to naming all variants.

Statistical analysis

The obtained data are expressed as the mean ± SD of at least three independent experiments and in the case of IC_{50}, as an estimated value ± SEM. Differences were assessed for statistical significance using repeated-measures ANOVA. The threshold for significance was defined as $p \leq 0.05$; the symbol (*) was used for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$. The IC_{50} data were analyzed using a one-way analysis of variance (ANOVA), and statistical significance was determined using Duncan's post hoc test (p-value < 0.05). Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., CA, USA).

Results

Liposome characterization and the assessment of stability and in vitro release of drug from liposomes

We prepared topotecan- and olaparib-loaded liposomes (TLL and OLL) composed of EPCs and cholesterol for preliminary evaluation in a 2D model of patient-derived ovarian cancer. The characteristics of TLL and OLL are presented in Table 1. The z-average size of TLL (113.9 nm) was slightly larger than that of OLL (100.8 nm), with an identical polydispersity index (PDI) of 0.056 for both liposomal formulations, indicating a homogeneous size distribution as presented in Table 1 and Fig. 1a, b (day 0). Much higher liposome loading of topotecan hydrochloride in comparison to olaparib was achieved, as presented in Fig. 1d, which is related to the applied loading method of each active substance (the active loading method was used to load topotecan vs. passive loading in the case of olaparib). Notably, the pKa values of olaparib suggest the feasibility of applying an active loading method to encapsulate the drug substance into liposomes, which may result in higher drug loading).

The relatively simple composition of TLL and OLL used in the preliminary studies, which encompasses EPC and cholesterol, affects the long-term stability of liposomes. As demonstrated in Table 1, both TLL and OLL are characterized by low zeta potential, which seems not to influence TLL and OLL liposomes size (Fig. 1a) and size distribution (Fig. 1b) during 2–3 week storage. The leak of topotecan from TLL corresponds to a decrease in zeta potential, while the zeta potential of OLL stays constant over time (Fig. 1c). In contrast, as shown in Fig. 1d, the topotecan and olaparib loading in TLL and OLL decreased after ca. 14 days, indicating that the liposomal membrane is not stiff enough to retain drug substances longer. Considering the long-term stability features of TLL and OLL, liposomes were freshly prepared before in vitro studies. The above

Table 1 Size, size distribution (expressed as z-average and polydispersity index (PDI), respectively), zeta potential and encapsulation efficiency (EE) of studied topotecan- (TLL), olaparib-loaded liposomes (OLL) and corresponding non-loaded liposomes, NLL-1 and NLL-2, respectively (na – not applicable)

Formulation	Z-average (nm)	PDI	Zeta potential (mV)	EE (%)
TLL	113.9 ± 1.0	0.056	−6.3 ± 0.5	68.3 ± 1.2
OLL	100.8 ± 0.2	0.056	−3.3 ± 0.8	20.8 ± 1.3
NLL-1	105.3 ± 0.4	0.064	−9.0 ± 0.5	Na
NLL-2	123.1 ± 0.9	0.032	−10.2 ± 0.2	Na

Particle size (Z-average), size distribution (PDI), zeta potential, and encapsulation efficiency (EE) of formulated topotecan – and olaparib-loaded liposomes

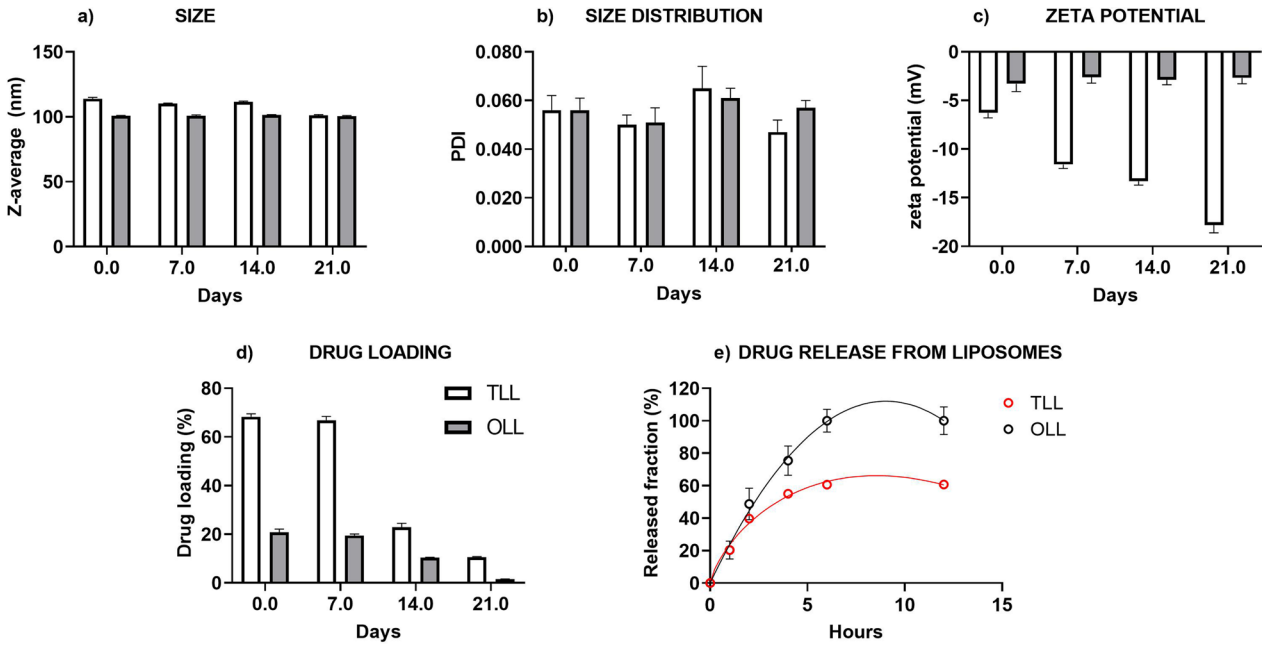

Fig. 1 Topotecan- (TLL) and olaparib-loaded (OLL) liposomes characteristics involving **a** size expressed by z-average, **b** size distribution presented as polydispersity index, PDI, **c** zeta potential, and **d** drug loading expressed as a percentage of encapsulation efficacy after preparation of overtime after preparation (day 0) and 7, 14, 21 days following drug encapsulation); **e** in vitro drug release profiles of topotecan and olaparib from TLL and OLL—open circles represent experimentally determined values, lines – correspond to data predicted from the Peppas-Sahlin mathematical model. Data are given as the mean value ± SD from three independent experiments

results also indicate the need to optimize the composition of the TLL and OLL further to provide improved long-term stability.

The in vitro release profiles of topotecan and olaparib from TLL and OLL are shown in Fig. 1e 50% of topotecan and olaparib were released from TLL and OLL within 4.7 and 2.4 h, respectively. Approximately 60% of topotecan was released from TLL during the experiment, while ca. 100% of olaparib was released from OLL. After six hours, the release profiles of both drugs reached a plateau, and drug substances were continuously released for another six hours. According to the data presented in Table 1S (please see the supplemental data), the experimental in vitro release data best fit the Peppas-Sahlin model, for which the highest r2 and the lowest Akaike information criterion (AIC) parameters were obtained (Table 1S). That briefly means that the release of topotecan and olaparib from the studied liposomes is governed by the contribution of two mechanisms, Fickian diffusion and liposome relaxation [35].

Comparison of cytotoxic activity of topotecan-loaded liposomes (TLL) or olaparib-loaded liposomes (OLL) with standard formulations.

We used four samples of clear-cell ovarian tumors (EOC 1–4), malignant ascites (Ascites), an OCI-E1p endometrioid primary ovarian cancer cell line, and as the control, a sample of HEOC. The BRCA1/2 mutation status analysis was performed using NGS (next-generation sequencing) for all primary cell lines listed above. We did not identify any defects classified as pathogenic (please see Additional file 1). We found two uncertain significance variants (VUS) throughout all samples (Table 2). In ClinVar, if differences in interpretation among submitters are observed, the genetic alteration is classified as "conflicting interpretations of pathogenicity." Due to the lack of convincing evidence for the significance of the detected

Table 2 The distribution of variants with conflicting interpretations of pathogenicity

Localization	Genetic variation	Region	dbSNP	HEOC	EOC1	EOC2	EOC3	EOC4	OCI-Elp	Ascites
Chrl3 (BRCA2)	C1114A > G	Coding	rs144848	–	–	+	+	–	+	–
Chrl7 (BRCA1)	C2612C > A	Coding	rs799917	–	+	–	+	+	+	+

variants and based on previous assessments, we assume that the status for BRCA1/2 genes in tested samples is wild type (WT).

The cytotoxic effects of the studied drugs and their liposomal formulations were assessed after 72 h of treatment within the concentration range of 0.001–25 μM for topotecan or 0.0016–1.6 mM for olaparib using an MTT assay. Treatment with standard formulations resulted in a dose-dependent reduction in the viability of the studied cell lines (Fig. 2). Interestingly, despite using tumor cells derived from the same organ, we obtained a wide range of IC_{50} values (half maximal inhibitory concentration) for both drugs in the standard formulation (Table 3).

In HEOC, treatment with TLL revealed a decrease in the toxic effect, and determination of the IC_{50} using the given concentration range was impossible (Fig. 2AI). In the case of OLL, a similar cytotoxic potential (Fig. 2AII) (IC_{50} = 1.22 mM) was noted compared to the standard formulation (IC_{50} 1.13 mM) (Table 3). The weakest antitumor effect of topotecan was reported for EOC1, one of the clear-cell ovarian tumor samples (IC_{50} 22.6 μM). In this experimental model, a liposomal formulation increased topotecan activity and decreased the IC_{50} value by half (IC_{50} 10.59 μM) (Fig. 2BI). Additionally, the liposomal formulation of olaparib caused a three-fold increase in cytotoxic potential (IC_{50} 0.46 mM) compared to the standard formulation (IC_{50} 1.70 mM) (Table 3). In the other three tissue samples of the same subtype of ovarian cancer, TLL treatment showed no significant increase in antitumor activity compared to topotecan (Fig. 2CI, DI, and EI). In the case of olaparib, the liposomal formulation increased its cytotoxic potential, causing a reduction in IC_{50} values compared to the conventional formulation (Table 3). The most prominent decrease was noted in EOC2 from 0.78 mM for the standard formulation to 0.09 mM for OLL (Fig. 2CII). In ECO 3 cells, the noted shift was from 0.16 mM for olaparib to 0.08 mM for OLL (Fig. 2DII), and in the ECO 4 tissue sample from 0.85 mM to 0.34 mM in the same configuration (Fig. 2EII).

The lowest IC_{50} for topotecan (0.18 μM), and thus the highest activity, was estimated after incubation of the OCI-E1p endometrioid primary ovarian cancer cell line (Table 3). Treatment with the liposomal formulation showed a substantial increase in cytotoxic potential in this cell line (IC_{50} 0.04 μM) (Fig. 2FI). As shown in Fig. 2FII, OLL also revealed a higher IC_{50} value of 0.21 mM compared to olaparib with a 1.06 mM concentration. As the present study has shown (Table 3), the highest increase in the antitumor activity of liposomal formulation was revealed for the Ascites cell sample (Fig. 2GI). The IC_{50} for the standard formulation of topotecan was estimated to be 0.34 μM, and treatment with TLL resulted in a 0.03 μM value in this experimental model. Additionally, the Ascites cells revealed high susceptibility to olaparib, with an IC_{50} value of 0.54 mM and 0.24 mM for OLL (Fig. 2GII). Consequently, the liposomal formulation increased drug activity in six of the seven tested cell models.

The combination of topotecan-loaded liposomes (TLLs) and olaparib-loaded liposomes (OLLs) demonstrates synergistic effects in primary EOC cells.

Topotecan is a water-soluble derivative of camptothecin and acts as a topoisomerase I (topo I) inhibitor. Topo I, an abundant nuclear enzyme, facilitates the unwinding of supercoiled DNA. This inhibition process, known as 'poisoning,' involves the agent binding to the interface between the enzyme and cleaved DNA. The resulting stabilization of the covalent complex turns the normal enzyme into an agent, causing DNA damage [36, 37]. The initial lesions must be recognized and repaired to prevent DNA DSB formation. PARP1 is one of the key molecules involved in this process [38].

To investigate the in vitro pharmacodynamic interactions of combining topotecan-loaded liposomes and olaparib-loaded liposomes, we used a combination index (CI). The Chou-Talalay method for drug combination relies on the median-effect equation, forming the theoretical basis for the CI equation. This algorithm allows the quantitative determination of drug interactions, classifying them as synergism ($CI < 1$), addition (CI 1), and antagonistic effects ($CI > 1$) [39]. Cell viability data was analyzed using the CompuSyn software to determine the type of relationship between tested drugs.

The present study indicates that the interaction between topotecan and olaparib is moderate to highly synergistic, with CI values ranging from 0.12 to 0.95 (Fig. 3). The range of studied concentrations was chosen based on estimated IC_{50} values. OLL was used in the same 12.5–400 μM concentration range to treat all six studied ovarian cancer cell samples. In the combination treatment of EOC 1 (IC_{50} 10.59 μM) and EOC 2 (IC_{50} 20.04 μM), we used TLL at a range of 0.5–20 μM and for the incubation of EOC 3 (IC_{50} 3.04 μM) and EOC 4 (IC_{50} 17.83 μM) at 0.25–10 μM. The OCI-E1p (IC_{50} 0.04 μM) and Ascites (IC_{50} 0.03 μM) cell lines were more susceptible to TLL, so the concentrations used for combinations were 4–125 nM and 2–62.5 nM, respectively.

In EOC 1 and EOC 2, the mixture of the highest-used concentrations of both drugs (400 μM OLL and 20 μM TLL) revealed a solid synergistic effect (CI 0.24, $p \leq 0.01$ and CI 0.47, $p \leq 0.01$, respectively) (Fig. 3A, B). The results indicate the highest DRI for TLL in this setup, 19.1 and 50.5, respectively. The DRI determines how many-fold dose reduction is possible for each drug

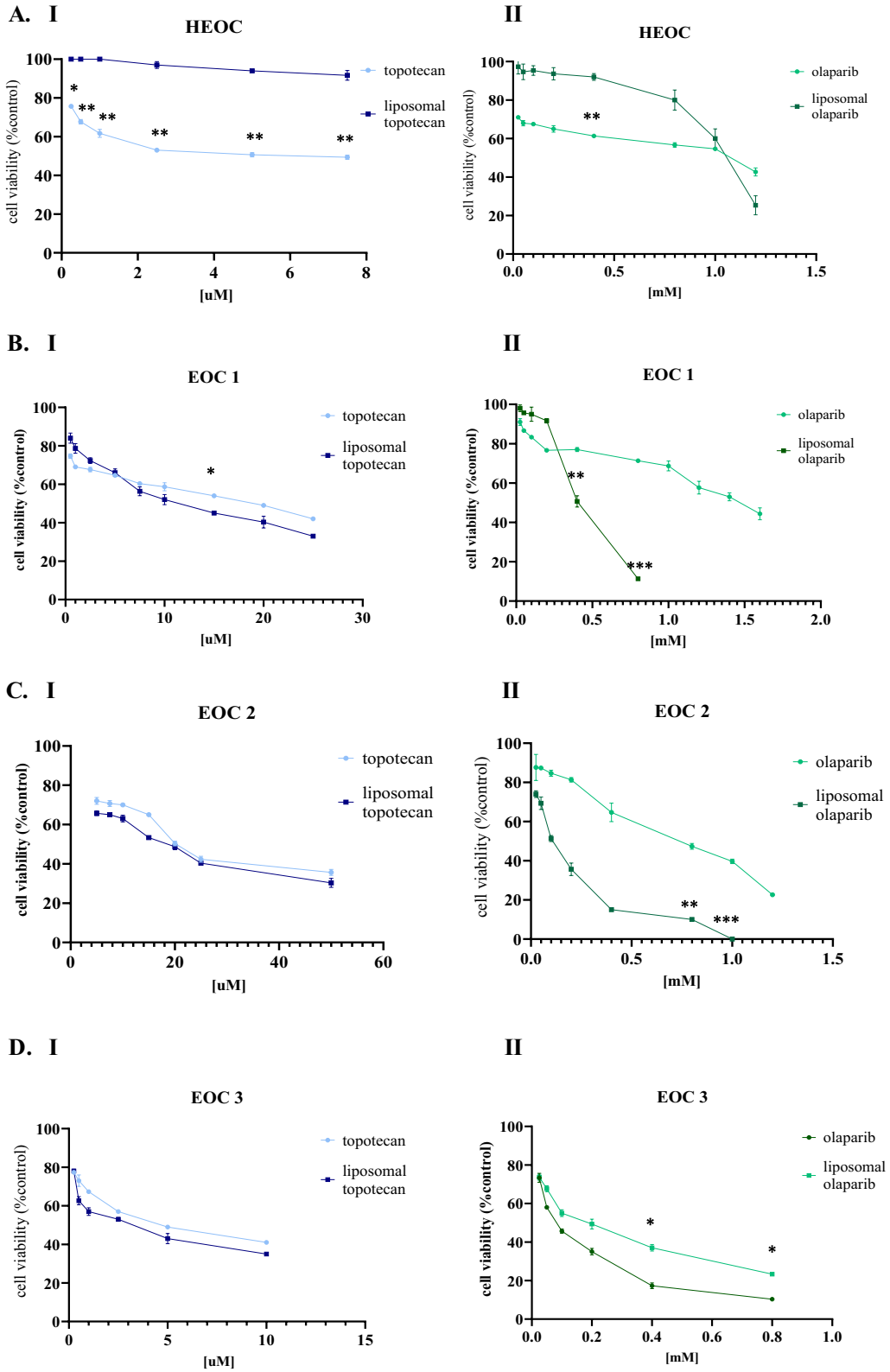

Fig. 2 The cell viability assessment after 72 h treatment. The graphs show the influence of the dose of topotecan and liposomal-based topotecan (I), or olaparib, and liposomal-based olaparib (II) **A** HEOC, **B** EOC 1, **C** EOC 2, **D** EOC 3, **E** EOC 4, **F** Ascites, **G** OCI-E1p. Data are shown as the mean ± SD, n = 3. The threshold for significance was defined as $p \leq 0.05$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

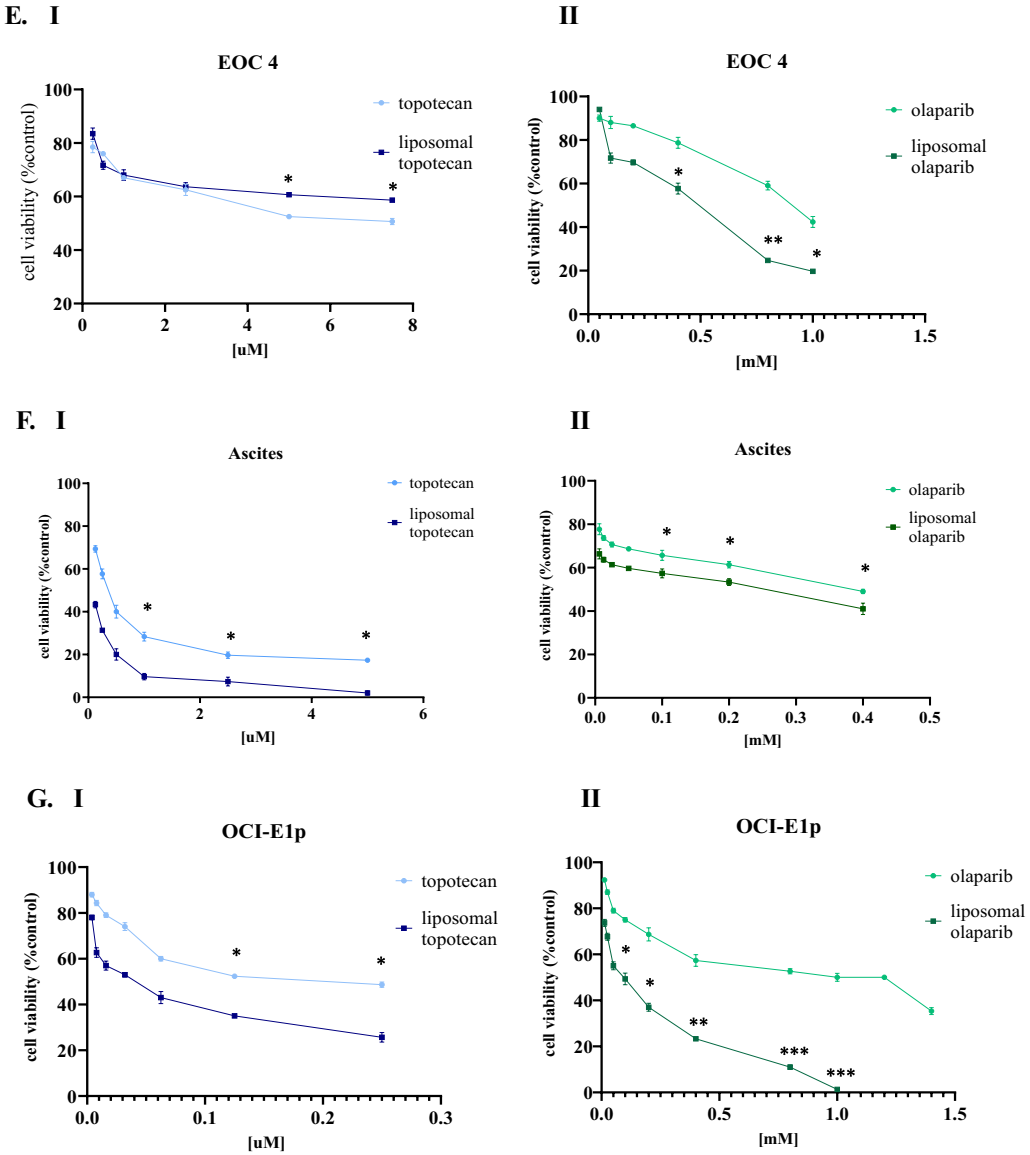

Fig. 2 continued

Table 3 In vitro cytotoxic activity (IC_{50}) of liposomal and standard formulations of topotecan or olaparib

cell type	IC_{50}			
	topotecan [μM]	liposomal topotecan [μM]	olaparib [mM]	liposomal olaparib [mM]
HEOC	4.99 ± 0.35	–	1.13 ± 0.18	1.22 ± 0.48
EOC 1	22.60 ± 0.31	10.59 ± 0.57	1.70 ± 0.53	0.46 ± 1.37
EOC 2	20.94 ± 0.55	20.04 ± 0.48	0.78 ± 0.72	0.09 ± 0.96
EOC 3	4.61 ± 0.43	3.04 ± 0.28	0.16 ± 0.67	0.08 ± 0.88
EOC 4	7.90 ± 0.34	17.83 ± 0.24	0.85 ± 1.44	0.34 ± 1.45
OCI-E1p	0.18 ± 0.54	0.04 ± 0.37	1.06 ± 0.45	0.21 ± 0.50
Ascites	0.34 ± 0.76	0.03 ± 0.44	0.54 ± 0.28	0.24 ± 0.20

in synergistic combinations. Dose reduction leads to reduced toxicity while maintaining the desired efficacy. Interestingly, in EOC 2, five of the six used combinations demonstrated a synergistic effect (Fig. 3B), while in EOC 3 only the highest-used combinations of 400 μM OLL and 10 μM TLL revealed CI = 0.38, $p \leq 0.01$ (Fig. 3C). Moreover, EOC 4 responded only to the conjunction of 50 μM OLL and 1 μM TLL with a statistically significant effect of CI 0.59, $p \leq 0.05$ (Fig. 3D). The endometrioid cancer cell line, OCI-E1p, responded with a synergistic outcome (CI 0.71, $p \leq 0.05$) to an even lower concentration configuration (12.5 μM OLL and 4 nM TLL) (Fig. 3E). The DRI for topotecan-loaded liposomes is 16.1. The most substantial

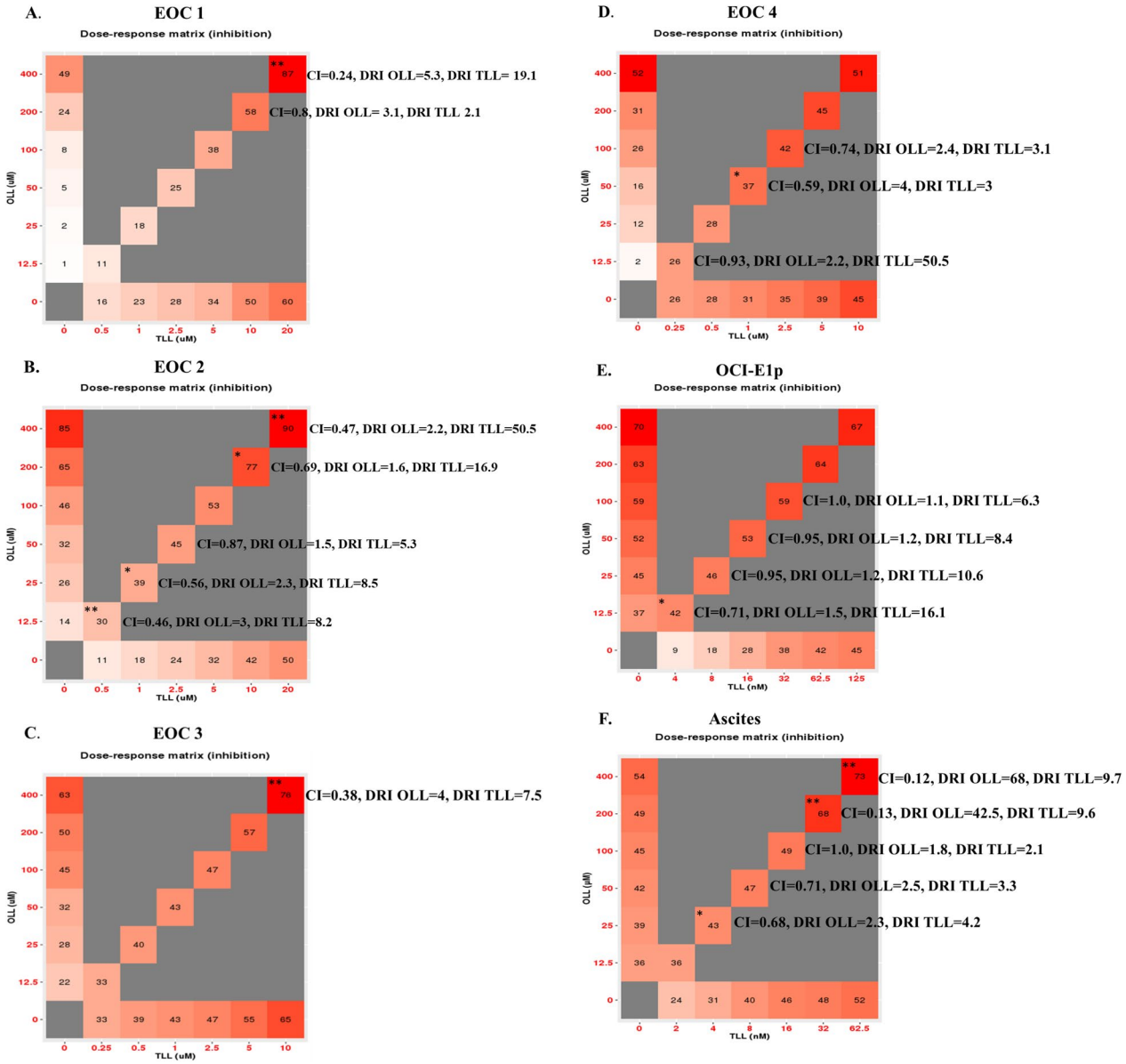

Fig. 3 Dose–response matrixes of proliferation inhibition after 72 h single or combination treatment with TLLs and OLLs. Data are shown as the % of proliferation decrease, combination index (CI), and dose reduction index (DRI) in **A** EOC 1, **B** EOC 2, **C** EOC 3, **D** EOC 4, **E** OCI-E1p, **F** Ascites. CI < 1 indicates a synergistic effect, and CI = 1 indicates an additive effect. The inhibitory properties were visualized using SynergyFinder 3.0 (https://synergyfinder.fimm.fi). *p ≤ 0.05, **p ≤ 0.01

synergistic effect of the TLL and OLL combination was noted in Ascites after incubation with 62.5 nM and 400 μM, respectively. The CI for that mixture reached 0.12 ($p \leq 0.01$); the dose-reduction index (DRI) for OLL was 68, and for TLL was 9.7 (Fig. 3F). The 31.25 nM TLL and 200 μM OLL combination resulted in a CI of 0.13 ($p \leq 0.01$); an OLL DRI of 42.5, and an estimated TLL DRI of 9.6. The liposomal formulation of topotecan revealed the highest increase in antitumor activity in Ascites, and the studied drug combinations showed the highest dose-reduction index for OLL.

Discussion

To the best of our knowledge, this is the first attempt to prepare a liposome-based delivery system for topotecan and olaparib and to evaluate the effect of their combination in a 2D model of patient-derived ovarian cancer without BRCA1/2 mutation. Combination therapies

are being tested to prolong progression-free survival (PFS) and overall survival (OS) in most fields of oncology. The main rationales for these strategies are to utilize the synergistic or additive effect of drugs with different mechanisms of action and to overturn drug resistance mechanisms [17]. Unfortunately, overall survival of ovarian cancer patients has not improved significantly in recent decades. The high mortality rate is mainly connected with late disease detection due to a lack of practical screening tools. [40, 41]. Additionally, medical management has improved only for patients with BRCA mutations due to the PARPi revolution and the diagnostic detection of BRCA1/2 pathogenic variants. Unfortunately, PARPi monotherapy has limited efficacy in BRCA-wild-type cancers, especially HR-proficient or platinum-resistant [42]. Thus, PARPis in combination with other compounds, are currently being developed as a treatment strategy for advanced cancer patients with confirmed, partial, or without known DNA repair deficiency. The main goal is to enhance the antitumor potential of the chemotherapy partner and PARPi [13, 43]. PARP is involved in the processes of replication, recombination, chromatin remodeling, and DNA break repair. Due to the fact that this protein is essential in so many various cellular processes, its inhibition should potentiate the cytotoxic effect of DNA-damaging chemotherapy [43]. It is critical that the combination selections for clinical trials are based on a predefined understanding of drug mechanisms of action. Only on this basis, we can predict whether additive or synergistic effects are likely to occur clinically [44]. PARPis demonstrate several known mechanisms of action. The first is PARP trapping, in which inhibitors trap PARP on DNA during damage repair, and these complexes are lethal to HR-deficient cells. Next is the inhibition of DNA single-strand break (SSB) repair by inhibiting base excision repair (BER) caused by PARP inactivation, leading to double-strand breaks (DSBs). PARP trapping abilities vary among PARP inhibitors. Thus, veliparib is weaker than olaparib, rucaparib, and niraparib, although all four agents inhibit PARP catalytic activity. Furthermore, PARPis promote classic nonhomologous end joining (classic NHEJ), a more error-prone mechanism of DNA damage repair [45, 46]. Mechanisms leading to synergy between olaparib and topotecan are likely multifactorial. Topotecan-related DSBs induce multiple types of DNA damage, and olaparib diminishes the PARP enzyme's ability to repair it.

This study shows that the developed liposome-based delivery system improves drug performance, and the combination of topotecan and olaparib has higher cytotoxicity than each drug individually, demonstrating a synergistic antitumor effect at most of the selected concentrations. We successfully loaded neutral liposomes with olaparib and topotecan. However, the results indicate the need to optimize the composition of the TLL and OLL further to ensure better long-term stability. One solution to this problem may be pegylation, which significantly improves liposome stability during long-term storage without any negative impact on the delivery of the therapeutic [47]. The lower cytotoxic activity of TLL than the standard formulation on normal patient-derived ovarian cells suggests the possibility of better therapeutic efficacy and safety profile. Interestingly, different ovarian cancer subtypes exhibited a broad range of IC_{50} values for both tested compounds. Moreover, the concentration-dependent response of the tested ovarian cancer subtypes to combination therapy confirms the need for in vitro optimization to maximize drug cytotoxicity and synergistic drug interactions. The OLL and TLL combination is a promising subject for further animal studies, especially for eliminating BRCA-wild-type EOC. The development of PARPi/chemotherapy combinations requires preclinical solid data to support human clinical trial testing and extended in vitro studies to learn and understand the mechanisms of action.

To improve the outcomes for BRCA wild-type patients, several PARP inhibitor-based drug combinations have been tested in randomized trials. It is critical to determine whether synergistic effects can overcome the reduced activity of attenuated doses and demonstrate benefits beyond the standard of care. The first example is olaparib plus cediranib, an antiangiogenic agent. The combination showed an improved outcome in BRCA-wild-type patients with platinum-sensitive recurrent ovarian cancer compared to olaparib alone [48]. Based on the mechanism of action proposed by Kaplan et al., cediranib not only interrupts tumor blood supply but also sensitizes cancer cells to PARPi. The main mechanism of action is through decreasing the expression of the HDR factors BRCA1/2 and RAD51 recombinase. This occurs as a result of direct platelet-derived growth factor receptor (PDGFR) inhibition that suppresses this DNA repair gene expression and partially as a consequence of hypoxia. Moreover, the ability to inhibit DNA repair appears to be specific to tumor cells with no effect in several normal mouse tissues [49]. Interestingly, HRD-positive patients receiving frontline maintenance therapy with olaparib plus another antiangiogenic agent, bevacizumab showed a significant PFS benefit compared to bevacizumab alone [50]. In a phase II clinical trial from the GINECO group bevacizumab, olaparib, and durvalumab were tested in advanced ovarian cancer patients with platinum-resistant relapse. Durvalumab is a human immunoglobulin G1 kappa monoclonal antibody that blocks the interaction of PD-L1 with PD-1 and CD80. Durvalumab is

approved in multiple tumors as monotherapy or combination therapy. However, only modest clinical activity in recurrent ovarian cancer was noted. Clinically, the triplet combination was well tolerated in relapsed advanced OC patients without excess toxicity due to the combination [51, 52]. Additionally, results from the phase II MEDIOLA study confirmed the efficacy of this three-drug treatment also in platinum-sensitive relapsed ovarian cancer (PSROC) patients. This signal-seeking combination therapy study demonstrated encouraging clinical activity in women without BRCA1/2 mutations. Additionally, olaparib and durvalumab co-treatment show notable clinical activity in patients with germline BRCA1/2-mutated PSROC [53]. Many PARPi-based combination was tested as a therapy for different types of ovarian cancer with and without HRD. Unfortunately, not in every case the improvement in PFS is observed. Therefore, the need to test other conjunctions of drugs still exists.

A phase I study was performed to determine the safety, tolerability, and maximum tolerated dose (MTD) of orally administered olaparib in combination with topotecan in patients with advanced solid tumors. Further development of this combination was not explored due to dose-limiting hematological adverse events and the resulting subtherapeutic MTD [54]. In contrast, a phase I trial of veliparib in combination with weekly topotecan demonstrated a less myelosuppressive, manageable safety profile and early signs of activity. Therefore, a phase II clinical trial is now underway [55]. The GEICO-1601/ROLANDO study, a single-arm, open-label, nonrandomized, multicenter phase II clinical trial, regarded olaparib in combination with pegylated liposomal doxorubicin (PLD) in platinum-resistant ovarian cancer patients regardless of BRCA status. The results have shown promising efficacy and strong potential for further development [56].

Our study offers a potential therapeutic alternative with liposome-based delivery systems that can maximize efficacy by broadening the therapeutic index and minimizing the development of multidrug resistance while reducing systemic toxicity by delivering lower drug doses [23, 57]. Moreover, the proposed combination can expand the potential pool of olaparib beneficiaries.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12935-024-03469-0.

Additional file 1.

Additional file 2.

Acknowledgements
The authors thank Dr. Tan Ince, Weill Cornell Medicine, for providing the deidentified patient-derived OCI-E1p ovarian cancer cells.

Author contributions
Conceptualization, A.R.D., T.M., and A.H.; methodology, A.R.D., P.S.M., T.M., and O.G.; software, A.R.D.; validation, A.R.D., P.S.M., and O.G.; formal analysis, A.R.D., P.S.M.; writing—original draft preparation, A.R.D., P.S.M.; writing—review and editing, T.M., A.H. supervision, T.M.

Funding
The research was supported in part by grants from NIH/NCI R01 CA269513 (granted to T.M.) and R01CA251438 (granted to A.H.), the European Union's Horizon 2020 Research and Innovation program under the Marie Skłodowska-Curie grant agreement No 778051, and the Ministry of Science and Higher Education of Poland fund for supporting internationally co-financed projects in 2018 to 2022 (agreement No 3899/H2020/2018/2).

Availability of data and material
Source data and the sequencing data generated in this study can be found in Additional file 1.

Declarations

Ethics approval and consent to participate
It is not applicable as no humans were involved.

Consent for publication
All authors consent to publication.

Competing interests
The authors declare no competing interests.

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Received: 1 March 2024 Accepted: 31 July 2024
Published online: 12 August 2024

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