

Synthesis of EPD, an established cytotoxic agent for ovarian cancer cells, and its efficacy in cisplatin and paclitaxel resistant tumor cells

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Abstract

Ovarian cancer remains still the leading cause of death of gynaecological malignancy, for the greater part caused by tumour resistance to conventional treatment with cisplatin and paclitaxel. In the past a new compound, EPD, from the plant *Calomeria amaranthoides* has been isolated and proved to be a potential anti-cancer agent. The sesquiterpene lactone, EPD, or Eremophila-1(10)-11(13)-dien-12,8 β -olide, has an alpha-methylene gamma-lactone ring, essential for its apoptotic activity. In order to have sufficient EPD material for further anti-tumour studies, a synthetic route has been developed. Drug combinations with synthesized EPD, cisplatin and paclitaxel were studied in four ovarian cell lines, and normal fibroblasts. EPD showed in particular strength in its effects with the cisplatin resistant cell line COV362 cl 4 and with a paclitaxel resistant cell line A2780. Compared to cisplatin and paclitaxel, less effects of EPD were shown to effect the viability of normal fibroblasts. Synthesized EPD proved to be promising both as an anti-ovarian cancer agent as well as a chemo-sensitizing agent in combination with other anti-cancer drugs. Synthesized EPD will facilitate pre-clinical studies.

Introduction

Ovarian cancer is the leading cause of death from gynaecological cancer. Although initial response to surgery and chemotherapy is favourable, the long-term prognosis of this disease for women with advanced disease is still poor, in spite of the administration of platinum and paclitaxel [1, 2]. This phenomenon is thought to be caused by the development of tumour cell resistance to the standard cytotoxic drugs (3,4) In the past a new compound from the leaves of the biennial plant *Calomeria amaranthoides* (endemic to NSW, Australia) was isolated and named: **EPD**, Xanthanodiene, or Eremophila-1(10)-11(13)-dien-12,8 β -olide (Fig. 1). EPD, part of the subgroup Eremophilanolides, is a sesquiterpene lactone (SL). Belonging to the family of Asteraceae, a large number of natural products have been described, including SLs [5, 6]. These SLs are colourless, natural bitter compounds of the subfamily of 15-carbon terpenoids, with a lipophilic character. The alpha-methylene gamma-lactone ring is responsible for their bioactivity. The SLs are playing an important role in traditional medicine. To date, several SLs are of great interest for their potential as anti-cancer agents [7, 8]. In previous studies *in vitro* cytotoxicity (IC₅₀) tests were performed with different ovarian cell lines and other type of cancer cell lines, first with the crude extract of the leaves of *Calomeria a*, later with purified EPD [9]. Measurements were performed using a non-fluorescent substrate, Presto Blue (Bio Source, Invitrogen, UK) and based on dose response curves. Synergistic drug combinations with EPD and cisplatin or paclitaxel were also performed and caused apoptosis [10, 11].

To produce sufficient and reproducible EPD material for further anti-tumour studies, it was thought essential to develop a synthetic route to produce the compound. To see if it would be possible to synthesize purified EPD and establish a production line for synthesized EPD was one of the objectives of the present study. The second goal of the present study was to test synthesized EPD on ovarian cancer cell lines as well as on normal fibroblasts, with an emphasis on cell lines resistant to the standard current

chemotherapeutic agents: cisplatin and paclitaxel. Also a possible synergistic effect of synthesized EPD with cisplatin and paclitaxel was analysed.

Material and Methods

Agents

EPD, Eremophila-1-(10)-11(13)-dien-12,8B-olide, a sesquiterpene lactone (6) was used in the synthesis of EPD.

Paclitaxel and cisplatin were obtained from Sigma-Aldrich, USA.

Cell lines

Cell lines used for the cytotoxicity assays were, JC, JC-pl [10]; cisplatin resistant cell line COV362 cl 4 [3] and the multi-drug-resistant cell line A2780 [4], as well as normal human skin fibroblasts [10]. The cell lines were grown in RPMI 1640 (Invitrogen, The Netherlands), supplemented with 2 mM L-Glutamine (Gibco, Invitrogen, UK), 10% heat inactivated fetal calf serum (FCS) (Sigma), penicillin (50 units/mL) and streptomycin (50 µg/mL) (Invitrogen, UK). The cultures were maintained in a humidified atmosphere of 37 °C with 9% CO₂. The ovarian cancer cells (3x10⁴) were seeded in 24-well plates (Costar) in 1mL medium/well. After 24 hrs, exponential growing cultures were then treated in duplicate or triplicate with different concentrations of the compound EPD, dissolved in dimethyl sulfoxide (DMSO). Final concentration of DMSO was 0,02%, in 2 mL /well fresh medium. Control blanco (BL) cells were untreated. Experiments were performed twice or three times.

In vitro cytotoxicity tests with EPD

The cells were incubated with EPD, with different concentrations for 72 hrs to ensure doubling times of the cells. Presto Blue, a cell viability reagent (Bio Source, Invitrogen, UK), 10%, was then added to the cultures. After 2–3 hrs of incubation the percent of cell viability was measured by absorbance with a multi plate reader, Victor 3 V (Perkin Elmer) by transferring 100 µl of the medium with Presto Blue in a 96 well plate (Greiner Bio-one). Wave lengths used were 570 and 610 nm, with 570/8 and 610/10 filters. Viability was calculated relative to untreated cells.

Synergistic effects of EPD with cisplatin and paclitaxel

Synergistic drug interactions were evaluated using a simplified version of the Bliss independence model [10]. Combination of drugs was used: EPD + cisplatin; EPD + paclitaxel; cisplatin + paclitaxel and EPD + cisplatin + paclitaxel.

Results

EPD synthesis

EPD [9] was prepared in a number of steps, as shown in (Fig. 2). Starting from 2,3-dimethylcyclohexanone (1), which was obtained via oxidation of 2,3-dimethylcyclohexanol, the synthesis of racemic (2) was considerably improved over existing methods by performing the reaction of (1) with methylvinylketone in the presence of benzenesulfonic acid. The cis configuration was predominantly formed, which was crucial in obtaining the correct stereo chemistry in EPD.

Lithiation of (2) with LDA, followed by addition of tert-butyl bromoacetate, resulted in the known ester (3). Treatment of (3) with acetic anhydride and trimethylsilyl chloride to give (4), was followed by reduction with sodium borohydride to yield the deconjugated alcohol (5). Hydrolysis with base then gave racemic acid (6).

To get the right stereochemistry crude (6) was treated with the resolving agent 1-phenylethylamine which served to separate both enantiomers of (6), as well as to purify (6). After enantiomerically pure (6) had been liberated from the 1-phenylethylamine salt it was lactonized to (7) by dehydration with DCC in the presence of DMAP.

In order to access the desired EPD (11), the trans- configuration in lactone (7) had to be transformed into a cis configuration. To that end, lactone (7) was converted into bromolactone (8) via deprotonation with LDA and bromination with carbon tetrabromide. DBU-promoted elimination led to the unsaturated lactone (9). Reduction with sodium borohydride in the presence of nickel (II) chloride proceeded in the desired fashion, i.e. the proton approaches from the least hindered side, thus resulting into the formation of the desired cis-lactone (10). The final step, formation of a methylene lactone, was performed with potassium tert-butoxide, ethyl format and paraformaldehyde. Both enantiomers were prepared in this way and one of these enantiomers appeared to be fully identical with natural EPD.

Following these steps the synthesis of the natural product EPD, Eremophila-1-(10)-11(13)-dien-12,8β-olide, was established.

In vitro cytotoxicity tests with EPD

A range of drug combinations was tested for the four cell lines: JC-pl, COV362 cl4, JC, A2780 as well as for normal fibroblasts. Drug concentrations were then selected. Based on dose response curves (IC₅₀ tests) the various concentrations of EPD, cisplatin and paclitaxel are shown in Table 1.

Table 1
Drug concentrations in µg/ml, used for the ovarian cancer cell lines:
JC-pl, COV362 cl4, JC, A2780 and normal fibroblasts.

	JC-pl	COV362 cl 4	JC	A2780	Fibroblasts
EPD	1.5	1	1,25	1.5	2
Cisplatin	2.5	5			2.5
Paclitaxel	0.3	0.35	0,35	0.375	0.3

The relative cell viability of the four cell lines and normal fibroblasts, treated with these doses of synthesized EPD, cisplatin or paclitaxel showed reduced viability rates in the cell line JC-pl. EPD had little effect on the fibroblasts (88% viability), while paclitaxel showed strong reduction of cell viability (21% viability), with cisplatin in between (50% viability) (Fig. 3).

The cell line COV362 cl 4, resistant for cisplatin, showed no reduction with treatment of cisplatin and the cell line A2780 resistant for paclitaxel, showed no reduction with treatment of paclitaxel. Both cell lines were sensitive for EPD (Fig. 4A and 5).

Synergistic effects of EPD with cisplatin or with paclitaxel

The cell line COV362 cl 4, showed clear synergism when treated with the combination EPD + cisplatin, as well as the combination EPD + cisplatin + paclitaxel (Fig. 4A). The cell line JC showed clear synergism with the combination EPD + paclitaxel (Fig. 4B), as also was shown earlier with the natural compound EPD [10].

Discussion

In the last decades *in vitro* cytotoxicity of taxane and platinum agents has been widely reported and also has demonstrated clinically equal efficacy [12–14]. Due to relapses of ovarian cancer in patients with advanced epithelial ovarian cancer, most likely caused by the development of a drug resistance phenotype [3, 4], evaluation of alternative models of treatment is of interest.

The challenge in the synthesis of EPD has been the chirality, having four chiral centres present in the molecule. Especially the formation of the furan with the correct chiral centres was not straightforward. The desired form has been identified and confirmed by high pressure liquid chromatography (HPLC). It has taken several years to synthesize EPD. For the first mg quantities of synthesized EPD, Syncom (Symeres) developed the chemistry as depicted in the Results section. Separation of the two enantiomers was achieved by HPLC. In this way both enantiomers became available and one of the enantiomers appeared to be identical to natural EPD (Fig. 2). The anti-cancer agent EPD has exhibited potent cytotoxic effects towards ovarian cancer and other cancers, but much less towards normal cells [6, 10], while with synthesized EPD even less quantities of EPD are necessary to obtain reduction of tumour cell viability. In general, SLs induce apoptosis. Apoptosis, programmed cell death, is playing an important role in both carcinogenesis as well as cancer treatment [15–17] and it is believed that the α -methylene- β -lactone structure is essential for this process [7]. The newly synthesized EPD has been analysed by Oncolines™ and by Oncolines Profiler.™ Of the 102 analysed cell lines, EPD showed strong similarity with the IC₅₀ profile of Bay-11-7805 (Bayer Pharma A.G.), a well-known irreversible inhibitor of kappa B α [18]. EPD was then compared with Bay-11 in dose response curves. At least three times of the amount of EPD was necessary to induce cell death with Bay-11. Results of the Oncolines Profiler™ also showed that the working mechanism of EPD in the proliferation tests caused inhibition of nuclear factor kappa B (NF-kB), a protein complex that controls transcription of DNA, cytokine production and cell survival. The p65/p50

NF- κ B complex is localized in the cytoplasm of the cell when bound to inhibitor κ Ba (I κ Ba). Upon phosphorylation, I κ Ba undergoes degradation, releasing the p65/p50 complex, which then translocates to the nucleus and activates target genes. Of the many different target genes, regulation of cell survival as well as apoptosis play a role [15]. However, there is still a significant knowledge gap regarding the involved signalling pathways of sesquiterpene lactones [19]. EPD has also been compared with Isoalantolactone (IATL) [20], a sesquiterpene lactone of the eudesmanolide group (Specs, Zoetermeer, The Netherlands). In the dose response curves, much higher concentrations (almost twice as much) of IATL were necessary compared with EPD for cell death.

Over the past decades ex-vivo, three-dimensional (3D) tumour organoids have since become promising pre-clinical models to test novel treatment regimens for patients with cancer. These 3D organoids from solid tumour tissue as well as from ascites of patients, resemble the tumour epithelium they were derived from [21–23]. With the development of 3D organoids of high grade serous ovarian cancer (HGSOC) the time-consuming models of patient–derived xenografts might be overcome. However, the organoid models still have to overcome some challenges like limited success rates, limited assay runs and high labour intensity in the laboratory. Initial testing with EPD indicated that the results of 3D ex vivo tumour tests showed to be in line with the 2D *in vitro* results and it is expected that more extensive studies with EPD in 3D organoids will be performed.

Over the past years EPD has shown evidence to be of great interest as an anti-cancer agent. EPD showed synergistic interactions with paclitaxel and cisplatin and induced apoptosis, as is known for SLs. Also EPD caused cell reduction as well as synergism in the resistant cell lines A2780, CoV362 cl4 and JC. Several SLs are currently used in cancer clinical trials such as artemisinin, parthenolide and thapsigargin [24, 25] and synthetic derivatives have targeted tumour and cancer stem cells, while sparing normal cells as does EPD. Belonging to the subgroup of Eremophilanolides, EPD and other SLs from species of the genus *Senecio* [26] have all strong anti-cancer properties.

Conclusion

To overcome the clinical problems of resistance in ovarian- and other cancers, there is still a high need for alternative treatment regimens. Synthesized EPD has shown to be promising as an anti-ovarian cancer (and other cancers) agent. It was demonstrated that the compound is active in cisplatin- and paclitaxel resistant tumour cells as well as being a chemo-sensitizing agent in combination with other anti-cancer drugs.

Abbreviations

DBU: 1,8-diazabicyclo [5.4.0]undec-7-ene

DCC: N,N¹-dicyclohexylcarbodiimide

DMAP: 4-dimethylaminopyridine

LDA: lithium diisopropylamide

EPD: Eremophila-1-(10)-11(13)-dien-12,8B-olide

HPLC: high pressure liquid chromatography

HGSOC:high grade serous ovarian cancer

Declarations

Conflicting interests

The authors declare that they have no competing interest.

EPD is patented by C. van Haaften.

Funding

Leiden University Medical Center

Authors contribution

C. van Haaften drafted the manuscript and performed with J.D.H. van Eendenburg the experiments. The synthesis of EPD has been performed by W. ten Hoeve and J. Koek.

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Figures

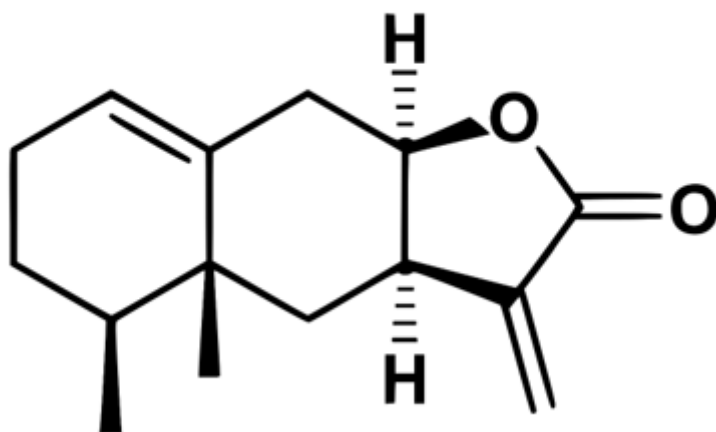


Figure 1

Eremophila-1-(10)-11(13)-dien-12,8 β -olide

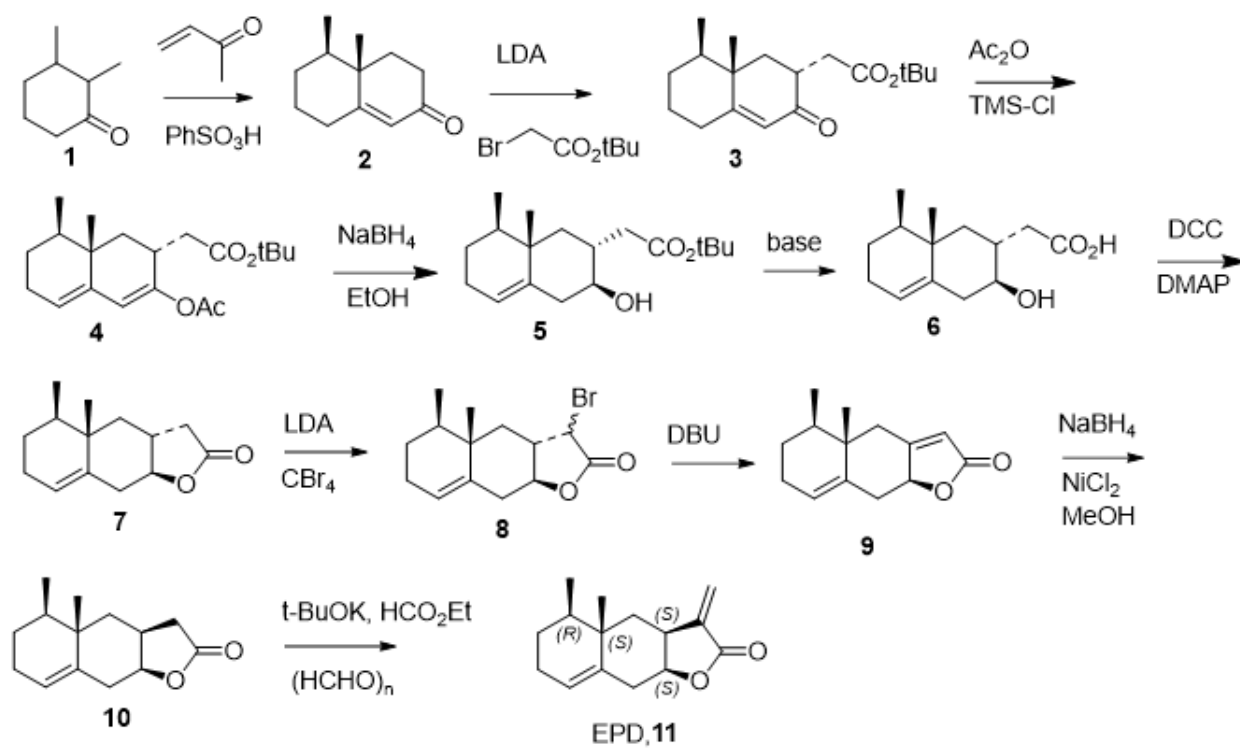


Figure 2

Synthesis of EPD

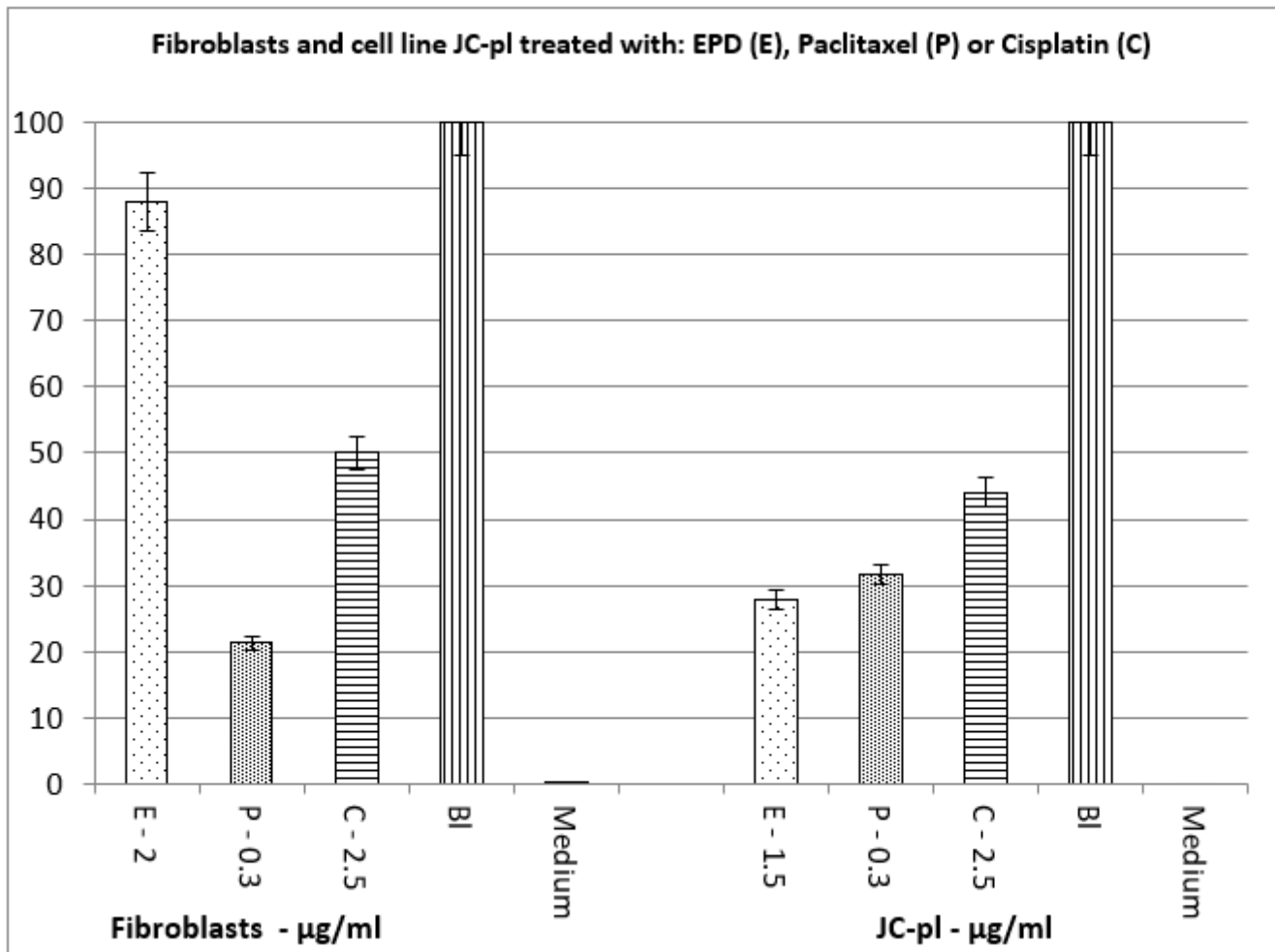
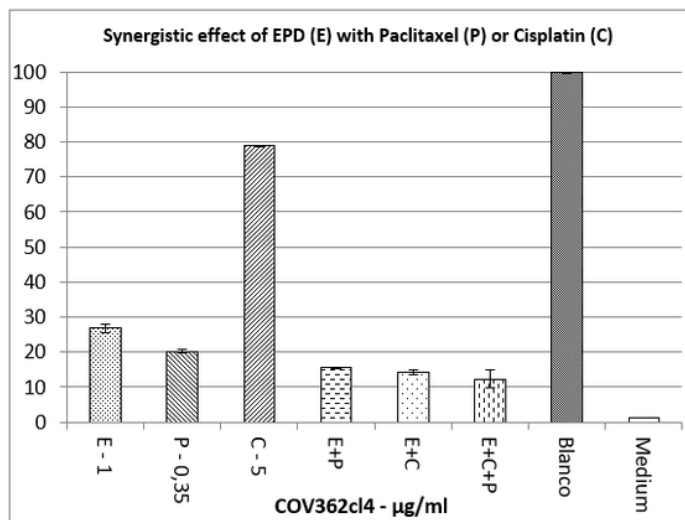
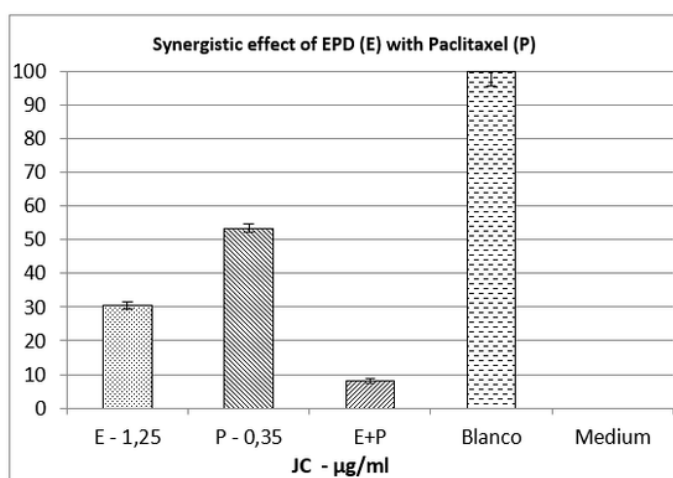


Figure 3

Viability percentages of normal fibroblasts and ovarian cancer cell line JC-pl after incubation with EPD (E), Paclitaxel (P) and Cisplatin (C). Bl indicates blanco control



a



b

Figure 4

A: The cell line COV362 cl4, resistant for cisplatin, showed synergism when treated with the combinations EPD + cisplatin as well as with the combination EPD + cisplatin + paclitaxel.

B: In the cell line JC, resistant for paclitaxel, synergism was shown when incubated with the combination EPD + paclitaxel.

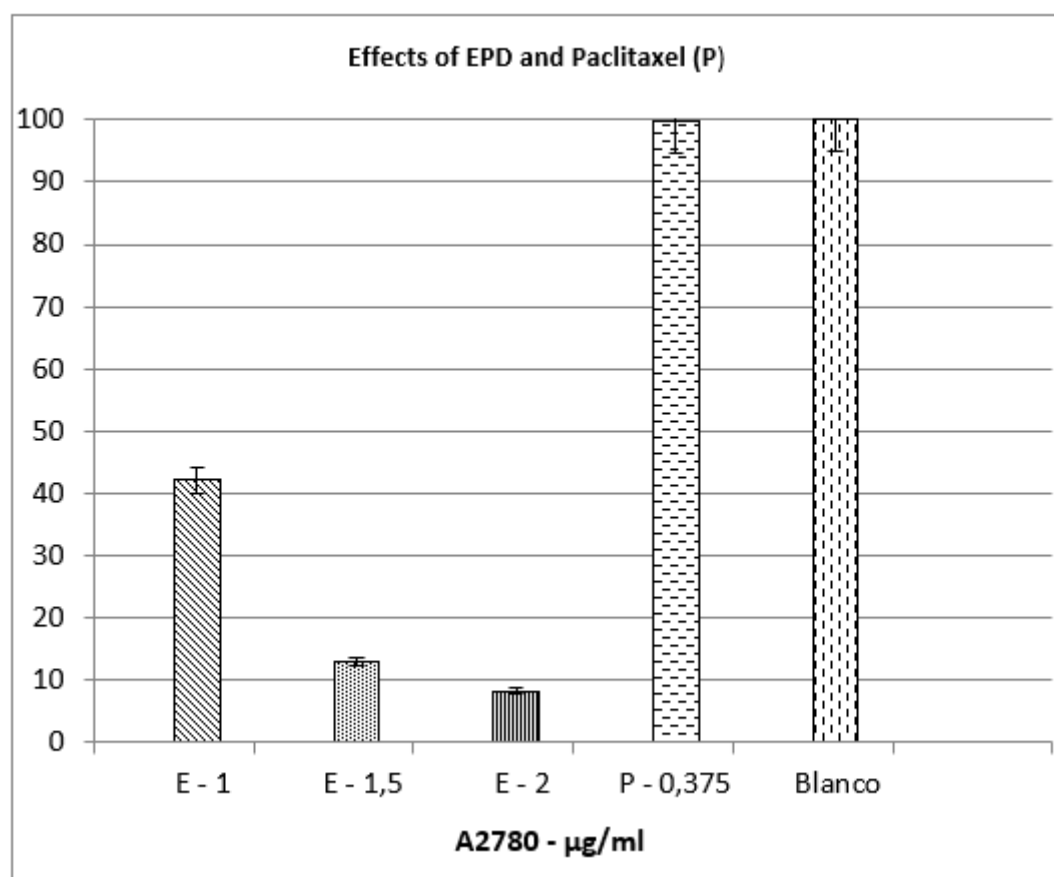


Figure 5

The paclitaxel resistant cell line A2780 with different concentrations of EPD. Paclitaxel alone had no effect at all.