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Research Article
Efficacy of Emodin/Paclitaxel Versus Paclitaxel for the Treatment of Ovarian Cancer *in vivo*

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Abstract
Background and Objective: Paclitaxel is widely used to treat ovarian cancer, but its toxic side effects and the development of drug resistance limit its usefulness as a long-term treatment. The aim of this study was to determine whether emodin can enhance the anticancer effect of paclitaxel but reduce its toxicity in ovarian cancer. **Materials and Methods:** Rats with DMBA-induced tumors were treated with emodin (12 mg kg^{-1}, p.o.) or paclitaxel (8 mg kg^{-1}, i.p.) twice a week for 8 weeks, then tumor sizes and body weight changes were measured and morphological observations were performed. The Kruskal-Wallis test and the chi-square test were used to compare tumor sizes and mortality rates, respectively in the non-treated and treated groups. **Results:** In a Sprague Dawley rats, emodin/paclitaxel combined treatment (2 mg i.p./3 mg p.o.) was associated with no change in tumor size after 8 weeks of treatment, but reduced mortality to 50%, whereas all rats treated with paclitaxel (3 mg i.p.) died. **Conclusion:** The present study shows emodin and paclitaxel combination therapy efficiently reduces the severe side effects of paclitaxel and thus, suggest emodin/paclitaxel co-administration be considered as a potential long-term treatment for ovarian cancer in the clinical setting.

Key words: Emodin, paclitaxel, ovarian cancer, *in vivo*, combination therapy, mortality, tumor size, histological observation

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ovarian cancer forms when genetic abnormalities errors occur in normal ovarian cells that drive uncontrolled cell growth. In 2012, ovarian cancer occurred in 239,000 women worldwide and resulted in 152,000 deaths, which makes it the 7th-most common cancer and the 8th-most common cause of cancer-related death among women[1]. Ovarian cancer is also the most fatal gynecological malignancy[2] and has one of the lowest 5 year survival rates[3].

Paclitaxel was discovered in Pacific yew (*Taxus brevifolia*) bark and is the agent usually used to treat ovarian cancer because of its potent cytotoxic effects (IC_{50}: 0.7-1.8 nM) on different ovarian cancer cell lines[4]. However, the toxicity of paclitaxel in man (LD_{50}: 31.8 mg kg^{-1})[5] and the development of drug resistance limit its usefulness as a long-term treatment.

Emodin is a naturally occurring anthraquinone that has been isolated from a number of plants, most recently from some herbs[6-9]. This versatile compound has been investigated for its broad spectrum anticancer effects on ovarian, gastric, pancreatic, lung, liver, gallbladder, colon, breast, prostate cancer and others *in vitro* and *in vivo*, furthermore, its anticancer effects have been reported to be due to different mechanisms[10]. In the previous report[11], emodin isolated from *Rumex acetosa* (a Chinese herbal medicine) exhibited potent cytotoxicity against human ovarian cancer cells, antimutagenicity against NPD and NaN_3 and antigenotoxic effects against the mutagens MNNG and NQO. In one study, emodin was found to inhibit drug resistant ovarian tumor growth by increasing the intracellular concentration of paclitaxel, thus re-sensitizing resistant cells to paclitaxel and in the same study, its possible use in combinatorial treatment strategies was suggested[12]. Further study demonstrated emodin improved the antitumor effect of gemcitabine, even at low doses and also decreased gemcitabine-induced toxicity in pancreatic cancer[13].

Given cytotoxicity, antimutagenicity and low toxicity (LD_{50}: 580 mg kg^{-1}) of emodin, emodin has been investigated whether it could reduce paclitaxel-induced toxicity and enhance paclitaxel efficacy in a rat model of ovarian cancer.

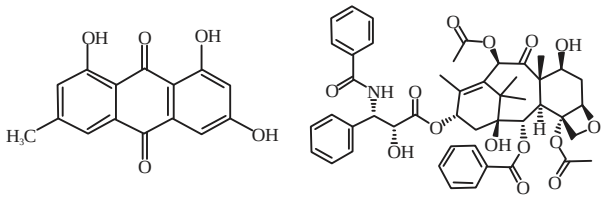
MATERIALS AND METHODS

General: Emodin was isolated from *Rumex acetosa* (Polygonaceae) collected from the suburbs of Ulsan city (Korea) in May, 2014. The plant was identified by Professor Byung-Soo Kang, College of Oriental Medicine, Dongguk University, Gyeongju, Korea. Paclitaxel was provided by Jeiljedang Pharmaceutical Co. (Seoul). Chemicals used for animal testing were obtained from Sigma-Aldrich (St., Louis, MO). All other chemicals and reagents were of the highest grade available. The NMR spectra were recorded using a varian unity INOVA 500 spectrophotometer (Varian, Palo Alto, CA, USA) and HPLC was performed using a Knauer Smartline system equipped with an Alltech 3300 ELSD detector and an Eclipse XDB-C18 column (4.6 mm×25 cm).

Isolation and purification of emodin: Powdered rhizomes of *Rumex acetosa* (600 g) were extracted with methanol (2 L) under reflux to give a brown oily extract (124.6 g), which was successively fractionated with n-hexane (0.5 L) and methylene chloride (0.5 L) to yield fractions of 9.6 and 22.5 g, respectively. The methylene chloride fraction (22 g) was subjected to column chromatography using a RP-C_{18} column (LiChroprep, 40-63 μm, Merck, Darmstadt, Germany) and 10% methanol-water as eluent to obtain emodin (Fig. 1), which was further purified by recrystallization from ethanol and identified by comparing its spectral data with a previously verified standard[11]. Some of the chemical assignments necessitated revision because of instrumental advances.

Emodin: Amorphous scarlet solid yield 1.25 g mp 262-263°C (264-265°C)[14], IR (nujol, cm^{-1}): 3435 (OH), 1680 (CO), 1628 (CO); UV λmax (logε) (CH_2Cl_2) 438 (3.07), 288 (3.24), 266 (3.24), 230 nm (3.25); ^{1}H NMR ($CDCl_3$+DMSO-d_6), δ (ppm): 2.40 (CH_3), 6.57 (br s, C7-H), 6.97 (br s, C2-H), 7.18 (br s, C-5H), 7.46 (br s, C-4H), 12.10 (br s, OH), 12.13 (br s, OH); ^{13}C NMR ($CDCl_3$+DMSO-d_6) δ: 22.4 (CH_3), 108.7 (C7), 109.5 (C5), 110.0 (C8a), 113.8 (C9a), 121.1 (C2), 124.4 (C4), 133.4 (C4a), 135.4 (C10a), 148.2 (C3), 162.4 (C1), 165.5 (C8), 166.1 (C6), 182.0 (C10), 190.4 (C9); EI-MS, m/z (relative intensity %): 270 (M^+, 100%), 242 (M-CO, 18%), 241 (M-CHO, 24).

The purity of the emodin isolated from *Rumex acetosa* was determined to be 98.2% by reverse-phase HPLC using a methanol-water gradient solvent system (from 10-50%)



Emodin Paclitaxel

Fig. 1: Chemical structures of emodin isolated from *Rumex acetosa* and paclitaxel a constituent of *Taxus brevifolia*

(Rt = 18.5 min). Emodin was isolated from the plant because commercially available emodin (Sigma-Aldrich) had a purity of only 90%.

Animals and treatment: Forty female Sprague Dawley rats (8 weeks old) supplied by the Korean Institute of Science and Technology (Daejeon, Korea) were used in this study. Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23; 1996 revision)[15]. The study protocol was approved beforehand by the Care of Animals Research Committee of Dongguk University, Republic of Korea. Animals were housed throughout in a controlled environment under a 12 h light/dark cycle at 22±2°C, provided a commercial diet with tap water *ad libitum* and weighed every 7 days. After a 1 week acclimatization period, a DMBA (7, 12-dimethylbenz[a]anthracene) coated suture was inserted into each left ovary as previously described[16]. Briefly, DMBA was heated to 130°C and the central portion of a 3-0 silk suture was then immersed in the molten DMBA such that it was coated with ~2.0 μg of the carcinogen as determined using a microchemical balance. Under isoflurane and N_2O general anesthesia, left ovaries were exposed and a DMBA-coated suture was then inserted into each ovary and knotted. Sixteen weeks later, left ovaries were re-exposed and tumor sizes were measured. Twenty rats with tumors were divided into four experimental groups: The non-treated group (n = 4), the emodin-treated group (n = 5), the paclitaxel-treated group (n = 5) and the emodin/paclitaxel group (n = 6). Emodin (3 mg; 12 mg kg^{-1}) was administered orally twice weekly for 8 weeks (from the beginning of the 3rd month post suture implantation to the end of the 4th month) to animals in the emodin-treated group and emodin/paclitaxel combined treated group and paclitaxel (2 mg; 8 mg kg^{-1}) was administrated intraperitoneally twice a week for 8 weeks in the paclitaxel treated group and emodin/paclitaxel combined treated group. Body weight changes were measured throughout the 8 weeks experimental period. At 9 weeks post administration, animals were sacrificed and tumor sizes were measured.

Morphological observations: Tissue sections (6 μm) were cut from the left ovarian muscles, then were fixed in 10% neutral buffered formalin. For morphological analysis of the tumor tissue[17] the cells in three sections from the non-treated and treated (emodin and emodin/paclitaxel) groups, respectively were stained using haematoxylin and eosin (H and E) and observed under an inverted microscope (Olympus, Tokyo). The tumors were subtyped according to the histologic characteristics of neoplastic cells.

Statistical analysis: Results are presented as Means±SDs. The Kruskal-Wallis test was used to compare tumor sizes in the non-treated and treated groups and the chi-square test was used to compare mortality rates. Statistical significance was accepted for $p<0.05$.

RESULTS

Emodin/paclitaxel suppressed ovarian tumor growth: At the beginning of the drug administration, mean tumor size in the 20 rats was 1.2±0.7 cm diameter in left ovaries. After 8 weeks of treatment, the non-treated group (n = 4) showed an increase in tumor size from 1.5±1.1 to 4.0±2.1 cm (166.7%) (Fig. 2a), however, tumor sizes were significantly smaller in the paclitaxel (n = 5), the emodin (n = 5) and the emodin/paclitaxel (n = 6) groups (Table 1). Figure 2b and c show observed tumor sizes in the emodin and emodin/paclitaxel groups, respectively. Mean tumor size in the paclitaxel group was 50% lower at treatment completion vs treatment commencement ($p<0.05$), whereas tumor size increased in the emodin group by 27.4% and remained almost unchanged in the emodin/paclitaxel group. The histological observations indicated that almost all carcinoma cells were well differentiated adenocarcinoma (Fig. 3a), with the exception one rat in the non-treated and emodin groups, which showed a mix of well differentiated adenocarcinoma (Fig. 3a) and squamous cell carcinoma (Fig. 3b).

Emodin/paclitaxel potently reduced paclitaxel induced mortality: Paclitaxel caused 100% mortality and this was diminished to 50% by emodin/paclitaxel treatment, although

Table 1: Effects of emodin/paclitaxel co-treatment on ovarian tumor growth and mortality

		Tumor size (cm, diameter)			
Groups	Drug dose (mg kg^{-1})	Before treatment	After treatment	Variation	Mortality (%)
Non-treated	0	1.5±1.1	4.0±2.1	2.5	0 (0/4)
Paclitaxel (P)	8	1.0±0.6	0.5±0.1	0.5[a]	100[a] (5/5)
Emodin (E)	12	1.1±0.7	1.4±2.0	0.3[a]	0[a] (0/5)
P+E	8P+12E	1.1±0.8	1.1±0.7	0.0[b]	50 (3/6)

[a]$p<0.05$ vs the non-treated group, [b]$p<0.05$ vs the non-treated group, P or E group

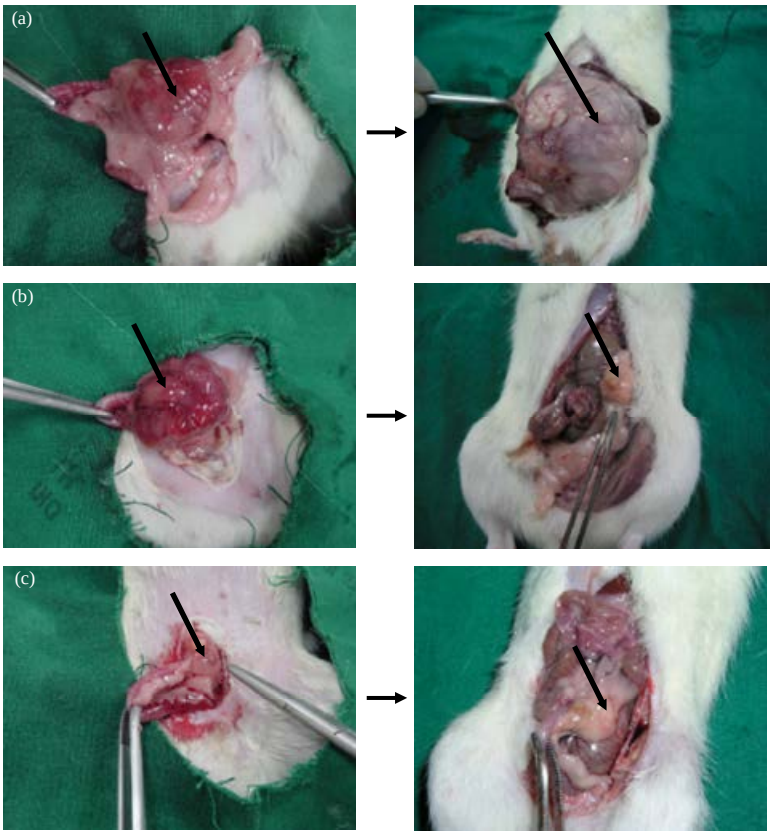


Fig. 2(a-c): Effects of treatments on tumor size. Tumor sizes increased in the (a) Non-treated group, (b) Decreased in the emodin group and (c) Emodin/paclitaxel group, significantly ($p<0.05$) compared to the non-treated group

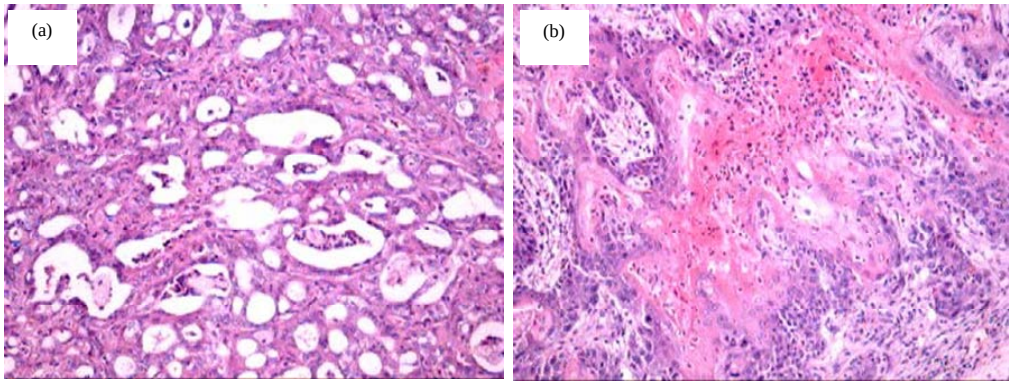


Fig. 3(a-b): Histological micrographs of (a) Well differentiated adenocarcinoma and (b) Squamous cell carcinoma. Cells were stained using haematoxylin and eosin (H and E)

the dose of emodin administered (12 mg kg^{-1}) was more than that of paclitaxel (8 mg kg^{-1}) (Table 1). This striking effect was attributed to the non-toxic nature of emodin. Up to date, no previous report on this effect of combination therapy on mortality in an animal model has been published.

Body weight changes: Animal body weights were measured for 8 consecutive weeks after commencing drug administration. Figure 4 shows the prior to drug administration mean body weight was 275 g in the non-treated group, but increased to 287.5 g (4.5% increase),

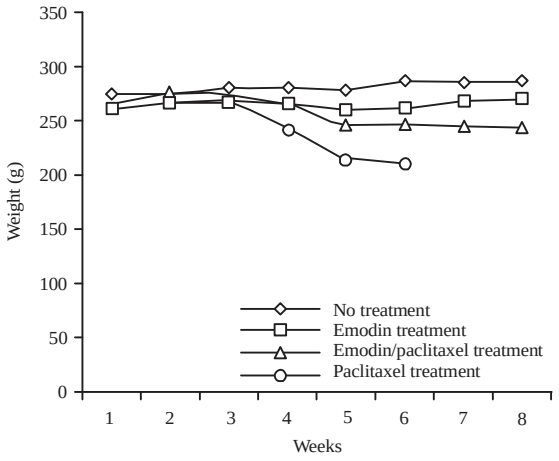


Fig. 4: Body weight changes in the experimental and non-treated groups during the 8 consecutive weeks of drug administration

respectively, after 8 weeks of treatment. The emodin group also showed an increase in body weight from 262-270 g after treatment (3.1% increase). However, rats in the emodin/paclitaxel group maintained showed a slight weight loss (7.6%) after treatment. These results are in-line with the effects of paclitaxel on body weight (ca. 20% decrease after 5 weeks).

DISCUSSION

Cancer starts when cells in the body begin to grow out of control. Several methods are used to treat ovarian cancer and of these chemotherapy most involves combinations of 2 or more drugs, such as of cisplatin or carboplatin and a taxane, such as, paclitaxel or docetaxel. Paclitaxel was first isolated from the bark of *Taxus brevifolia* (the Pacific yew) and is widely used to treat a number of malignancies including ovarian cancer[18]. However, its side effects and the occurrence of drug resistance following prolonged treatment are serious limitations[19]. Drug resistance to anti-cancer agents is a primary cause of treatment failure and mortality. Several possible reasons for paclitaxel resistance have been suggested, including increased drug cellular efflux and the deregulated expressions of anti-apoptotic or proapoptotic compounds[20].

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a constituent of many plants, in traditional medicine and has been used as a laxative, anti-bacterial and anti-inflammatory in Korea and China[21]. Emodin has been reported to increase paclitaxel-induced apoptosis at a low concentration, caused by Li *et al.*[12] to suggested the possibility of an innovative chemotherapeutic strategy based on a combination of emodin and paclitaxel[12]. In addition, Kurokawa *et al.*[22] found combined treatment with emodin and cisplatin inhibited platinum drug uptake by impacting the human copper transporter 1 and thereby, reduced the cytotoxicity of cisplatin. However, the anticancer and anti-mortality effects of emodin in combination with paclitaxel have not intensively been previously investigated in an animal model of ovarian cancer.

Pure emodin (>98% purity) was isolated from the plant (*Rumex acetosa*) due to the low purity (~90%) of commercially available material. In the present study, histologically determined well differentiated adenocarcinomas were significantly reduced in size by paclitaxel, whereas grew slightly in the emodin group. However, paclitaxel/emodin in combination almost completely inhibited tumor growth during the 8-week treatment period. Because paclitaxel[5] has a LD_{50} of 31.8 mg kg^{-1} in Wistar rats and thus, its toxicity restricts its long-term use. In the present study, the emodin/paclitaxel combination therapy greatly reduced mortality. Emodin and gemcitabine (an anti-pancreatic cancer drug) combined treatment has been reported to more effectively inhibit tumor growth than treatment with either agent[13]. However, the influence of emodin (LD_{50}: 580 mg kg^{-1})[23] on gemcitabine toxicity (LD_{50}: 500 mg kg^{-1})[13] has not been demonstrated. However, it has been reported that propofol induced apoptosis and increased the killing of paclitaxel-sensitive and resistant ovarian cancer cells[24]. It would be suggested that emodin/paclitaxel combination therapy might be used to reduce the side effects of paclitaxel and possibly enable long term paclitaxel treatment.

These observations of tumor sizes and mortalities after treatment are closely related to body weight loss, which may be one of the presenting manifestations of cancer or a symptom of advanced disease. The tissues and organs that exhibit low weights in cancer patients also diminish during starvation[25]. Thus, it appears body weight loss reflects extent of tumor growth. In the present study, emodin/paclitaxel treatment inhibited body weight loss induced by paclitaxel. These results suggest emodin/paclitaxel combination therapy prevented tumor size increases and substantially reduced mortality versus paclitaxel treated animals.

CONCLUSION

In summary, the present study shows that emodin/paclitaxel combined treatment can reduce mortality and inhibit body weight loss caused by paclitaxel and prevent

tumor growth significantly. These findings suggest the possibility that emodin/paclitaxel combinatorial therapy might offer a new means of treating ovarian cancer.

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