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SYNERGISTIC EFFECTS OF 9-METHOXYCANTHIN-6-ONE WITH CISPLATIN, PACLITAXEL, DOXORUBICIN, AND GEMCITABINE IN SKOV-3 OVARIAN CANCER CELLS

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Ovarian cancer is one of the most common gynaecological types of cancer among women with a high mortality rate when detected at a late stage. Most of the treatment for late-stage ovarian cancer is via chemotherapy, but it often causes poor prognosis due to toxic side effects. Combination therapy between two or more potent drugs, such as adjuvant therapy is one of the alternative modes of treatment currently being used to reduce the side effects when treating cancer. Natural products still remain an important source for discovering potent anti-cancer agents, whether they can be used singly or in combination with other treatment(s). Previously, an alkaloid 9-methoxycanthin-6-one (9M) was isolated from *Eurycoma longifolia* (Tongkat Ali) and had been identified to exert an anti-ovarian cancer effect *in vitro* and *in vivo*. However, no studies have been reported on the synergistic effects of 9M when combined with standard chemotherapy drugs in ovarian cancer cells. In this study, the SKOV-3 ovarian cancer cells were treated singly with 9-methoxycanthin-6-one (9M), cisplatin (Cis), paclitaxel (Tax), gemcitabine (Gem) or doxorubicin (Dox). The combined treatments were also performed in which 9M was paired and added with the drug simultaneously (0/0 h), 9M was added first following the drug 4 h later (0/4 h), and the drug was added first following 9M 4 h later (4/0 h) in the 96-well plate. The cells were incubated for 72 h and dead-end assay was conducted using Sulforhodamine B. The results were analysed via Compusyn software V.3.0.1 based on Chou-Talalay method that is based on the median-effect equation which allows quantitative determination of drug interactions in term of Combination Index (CI). CI less than one, equal to one or more than one indicates respectively synergism, additivity or antagonism in combined 9M-drug action. The results showed that 9M exerted synergistic effects when combined with each drug simultaneously (0/0 h) (CI_{50} ranged from 0.63 to 0.88), with the highest synergism activity when 9M was combined with Tax (CI_{50} was 0.63). 9M had also shown good synergistic effects when it was added 4 hours later after each drug (CI_{50} ranged from 0.69 to 0.75) except for Tax (CI_{50} was 1.24). This study suggests that 9M may have potential to be developed as an anti-ovarian cancer agent and may also be used as adjuvant therapy.

Keywords: 9-methoxycanthin-6-one, *Eurycoma longifolia*, cancer drugs, ovarian cancer cell line, Combination Index (CI)

INTRODUCTION

Cancer still remains one of the health burdens and it is responsible for one in six deaths worldwide (Bray et al. 2018). Ovarian cancer is one of the most common and leading causes of death among gynaecological cancers (Momenimovahed et al. 2019). Most cases are diagnosed at an advanced stage in which the cancer has metastasised. Chemotherapy drugs are still the main treatment option for treating advanced cancers, including ovarian cancer, but the five-year survival rate remains below 40% (Burke et al. 2023). Drug resistance and toxic side effects imposed by chemotherapy drugs remain a challenge to improve survival rates. Among the popular drugs used to treat ovarian cancer are cisplatin (Cis), paclitaxel (Tax), doxorubicin (Dox) and gemcitabine (Gem). However, these drugs cause several toxic side effects such as grade 3 to 4 hematologic toxicity, neutropenic sepsis, alopecia and uremia, among others (Gupta et al. 2005). Moreover, cancer is highly heterogeneous and different types of cancers are not necessarily treated by the same drug. The search for new anticancer agents is still ongoing worldwide to overcome these issues and one promising approach is to find drug candidate(s) that have different or multiple targets (Lu et al. 2012, Kenny & Marmion 2019) and often, are highly derived or inspired from natural products because they

have diverse molecular structures (Newman & Cragg 2020).

Eurycoma longifolia (Simaroubaceae), commonly known as tongkat ali is a popular herb that is native to most South East Asian countries, including Malaysia, Indonesia, Thailand, Philippines and Vietnam. Traditionally, its roots have been utilised for various medicinal purposes such as to treat intermittent fever, tonic after childbirth, ulcers, and headache (Burkill 1966). In addition, the plant has also been scientifically proven through various studies to have many therapeutic effects against several diseases such as cancer, malaria, diabetes and inflammation and promote overall well-being, including increased energy and strength, overcoming anxiety, appetite stimulants (Rehman et al. 2016). The roots of this plant are known for their bitter taste and have been extensively studied for their chemical contents. Apart from quassinoids, they are known to contain alkaloids including 9-methoxycanthin-6-one (9M) (Kardono et al. 1991, Nurhanan et al. 2005, Nurhanan et al. 2022). 9-methoxycanthin-6-one (9M) belongs to canthinone alkaloid and this group of compounds gained interest a decade ago and has been reported to have antitumour, antifungal, antimalarial, antibacterial and antiviral activities (Farouil et al. 2022). Earlier, the compound had been reported to have *in vitro* anticancer effects when treated alone against epidermoid, melanoma, lung, breast and colon cancer cell lines and also antimalarial property (Kardono et al. 1991, Kuo et al. 2003). Later, 9-methoxycanthin-6-one (9M) was also reported to have a cytotoxic effect on ovarian cancer (Nurhanan et al. 2005), and the mode of action was found to be via apoptosis (Nurhanan et al. 2022).

Chemotherapy drugs are commonly used in combination with other drug(s) or therapy to improve the survival rates while minimising toxicity side effects. For example, combinations between paclitaxel with either cisplatin or carboplatin (platinum drugs) have been used in first-line chemotherapy regimens to treat advanced stage III and IV ovarian cancer shown an improvement in the 5-year median survival rates by 39% as compared to the platinum drug when treated alone (Gupta et al. 2005). However, ovarian cancer often recurred after multiple chemotherapy regimens (Gupta et al. 2005). The recurrence was reported due to drug resistance since many drugs have underlying same modes of action and target such as platinum drugs (cisplatin, carboplatin, oxaliplatin) targeted mainly to DNA and cause DNA damage alterations in DNA damage repair (DRR) (Wang et al. 2024). Hence, there is a dire need to search for alternative drug candidates that are highly active in inhibiting cancer cells proliferation and has different modes of action than existing drugs which may improve the treatment outcome. Chemoresistance may also occur due to the malfunction of apoptosis in the cancer cells due to dysregulation of certain oncoproteins and apoptotic proteins. Earlier, 9M had shown to re-induced apoptosis in the ovarian cancer cells when treated alone (Nurhanan et al. 2022) but there was no report of the combined actions of 9M with any cancer drugs. Thus, the aim of this study is to evaluate on whether the 9M can cause synergism when combined in pair with commonly used ovarian cancer drugs, including cisplatin (Cis), paclitaxel (Tax), doxorubicin (Dox) and gemcitabine (Gem).

MATERIALS AND METHODS

Isolation of 9-methoxycanthin-6-one

Briefly, the dried cultivated root of *E. longifolia* (7.2 kg) was harvested from FRIM's Research Station in Maran, Pahang and extracted in chloroform by using soxhlet extraction at 35–45 °C for 18 hours. The chloroform solution was filtered and evaporated to yield chloroform extract (67.5 g, 0.94% (w/w)). This chloroform extract was then consecutively fractionated through column chromatography (15 mm × 300 mm) on silica gel 60 (70–230 mesh) using n-hexane-dichloromethane (DCM) (9:1, 7:3, 3:7 and 1:9) as mobile phase to obtain 7 fractions (FR1-FR7). A fraction 1 (FR1, 1.05 g) was then rechromatographed and purified by column chromatography on silica gel 60 and eluted with n-hexane-DCM (9:1, 7:3, 3:7 and 1:9) and DCM-methanol (1:1) to afford 9-methoxycanthin-6-one (89.6 mg, 0.001% (w/w)) (Figure 1). The 9-methoxycanthin-6-one was identified by comparison of its spectral data of 1H, 13C-NMR and MS with those reported in the literature (Mitsunaga et al. 1994, Nurhanan et al. 2022).

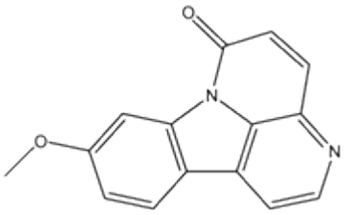


Figure 1 Structure of 9-methoxycanthin-6-one

Standard anticancer agents, cisplatin and paclitaxel were purchased from Sigma Aldrich, USA; doxorubicin and gemcitabine were purchased from Toronto Research Chemical Inc., Canada.

Cell culture preparations and treatments

The SKOV-3 ovarian cancer cell line was obtained from American Type Culture Collections (ATCC), USA. This cell line was grown in monolayer cultures and maintained in Dulbecco's modified Eagle's medium-high glucose (DMEM) supplemented with 10% foetal bovine serum (FBS), 0.25% amphotericin B, L-glutamine, 100 units penicillin, 100 µg/ml streptomycin and 0.01 mg/ml gentamicin, maintained in either T-25 or T-75 flasks and incubated in carbon dioxide incubator at 37 °C with 5% carbon dioxide.

The media was removed from the T-75 flask containing sub-confluent SKOV-3 cells, and the cells were washed three times with PBS. Next, 0.25 % trypsin was added and the flask was incubated for 15 min to allow the cells to detach from the surface of the flask. The cells suspension was then mixed with 10 ml of fresh DMEM supplemented with 5% FBS. Then, 100 µl of cells were seeded in each well of a 96-well plate. Cells were treated with 9-methoxycanthin-6-one (9M) and single drugs, and in combination studies, cells were treated with 9M and each drugs, at three different concentrations, generally at constant ratios of their median effect concentration; D_m (IC_{50}) values (Table 1). 9M was combined with the drug according to the following three sequences of administration: a) simultaneous addition (0/0 h), b) 9M was added first followed by the drug 4 h later (0/4 h), c) the drug was added first followed by 9M 4 hours later (4/0 h). Untreated cells served as control. The treated cells were then incubated for 72 hours, 37°C, pH 7.4 in the carbon dioxide incubator. Following this, Sulforhodamine B (SRB) assay was then performed (Skehan et al. 1990).

Sulforhodamine B Assay

After 72 hours incubation, the cells were fixed by adding 50 µl of ice-cold 50% (w/v) trichloroacetic acid (TCA) and then incubated at 21˚C for 30 minutes. Following this, the plates were washed three times with tap water and allowed to dry at room temperature. Then, 100 µl of 0.4% (w/v) Sulforhodamine (SRB) stain was added to each well and incubated for 30 min at room temperature. The plates were then washed with 50 ml of 1 % (v/v) acetic acid three times and then dried. Following this, 100 µl of

Table 1 Half-maximal inhibitory concentration (IC_{50}) and concentrations of 9-methoxycanthin-6-one, cisplatin, paclitaxel, doxorubicin, gemcitabine used to treat ovarian cancer cells (SKOV-3) when treated alone and in combination

Compound/ Drug	Half-maximal inhibitory concentration (IC_{50}, µM)	Concentrations used when treated alone (µM)	Concentrations used when treated in combination (µM)	Constant ratio used in when treated in combination
9-methoxycanthin-6-one (9M)	10.37	0.2, 2.0, 20.0	0.1, 1.0, 10.0	
Cisplatin (Cis)	1.43	04, 0.4, 4.0	0.02, 0.2, 2.0	9M: Cis (5:1)
Paclitaxel (Tax)	0.030	04, 0.04, 0.4	0.002, 0.02, 0.2	9M:Tax (50:1)
Doxorubicin (Dox)	0.42	6, 0.6, 6.0	0.03, 0.3, 3.0	9M:Dox (10:3)
Gemcitabine (Gem)	0.31	06, 0.6, 6.0	0.03, 0.3, 3.0	9M: Gem (10:3)

10 mM Trizma pH 10.5 was added per well and mixed with gentle agitation on a microtiter plate shaker (Fisher Scientific, USA). The optical density (OD) of the treated and untreated cells per well was then measured at 492 nm using a microplate reader (Tecan Infinite, Switzerland).

Combination index (CI) analysis

Combination indices (CIs), medium-effect concentration (D_m), coefficient signifying the shape of the dose-effect relationship (m) and linear correlation coefficient (r) from dose-response curves of 9M-drug combination results were automatically calculated using the CompuSyn software (V. 3.0.1). CI values <1, =1 and >1 indicate synergism, additivity and antagonism in combined drug action, respectively (Chou 2006, Chou 2018). Initially, OD values that were obtained from SRB assay were used to calculate f_u, that was the fraction of cells that survived. f_u was later used to calculate the f_a values.

f_u = OD_{492nm} of treated cells / OD_{492nm} of untreated cells

f_a is the fraction of cells that were killed so that f_a=1-f_u. In other words, f_a represents the proportion of cells that are inhibited or killed by a drug treatment. The combination index (CI) for binary combination of drugs was then calculated according to the following equation:

$$CI = \frac{D_1}{D_{1x}} + \frac{D_2}{D_{2x}}$$

D_1 and D_2 in the numerator represent respectively mean concentrations of compounds 1 and 2 in combination required to cause x% inhibition whereas D_{1x} and D_{2x} in the denominator represent the doses of compounds 1 and 2 required to cause x% inhibition when present alone.

D_x can be readily calculated from the following form of median effect equation, D_m (also reflect the values of IC_{50}).

$$D_x = D_m \times [f_a/f_u]^{1/m}$$

For statistical analysis, all pairwise comparisons (both simultaneous and sequential administrations) were analysed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey's multiple comparison test (Graphpad Prism v5, San Diego, CA, USA). A p-value of less than 0.05 indicates significant differences between the pairwise combinations that gave synergism across different time frames.

RESULTS

Fraction affected (F_a) and Median effect values (D_m)

The analysis of fraction affected (F_a) was shown in Table 2. F_a gives a direct measure of how effective a drug is in terms of reducing cell viability (i.e. increased cell death). As concentration of 9M and each drug either alone or in combination increased, the F_a values also increased (i.e. indicating dose-dependent effects). The median effect concentration needed to kill the respective cancer cells which reflects the IC_{50} value (D_m), the shape of dose-effect curve (m) and linear correlation coefficient (r) were shown in Table 3. Based on median effect concentration (D_m) values, all of the tested drugs and 9M were found to be active against ovarian cancer cells in which the most potent was Tax followed by Gem, Cis, Dox and 9M. m=1, >1 or <1 indicates hyperbolic, sigmoidal or flat sigmoidal curve, respectively. The linear correlation coefficient, r that was greater than 0.9 to indicate that the results were statistically good (Chou 2006, Chou 2018).

Combination index (CI)

CI values at different concentrations of F_a were calculated via CompuSyn software, as presented in Figure 2. The combination index (CI) analysis for each fraction affected (F_a) values is summarised in Table 4 and the dose-response curve between CI and F_a is shown in Figure 2. CI values <1, =1 and >1 indicate synergism, additivity and antagonism, respectively, in drug combinations. Based on the CI-F_a curves in Figure 2, the analysis on each 9M-drug combination had shown that the combination of 9M and Tax gave the best synergy at 0/0 h

Table 2 Concentration-effect parameters for the combinations of 9-methoxycanthin-6-one (9M) and each drug (cisplatin (Cis), paclitaxel (Tax), doxorubicin (Dox), gemcitabine (Gem)) in SKOV-3 ovarian cancer cells, n=3

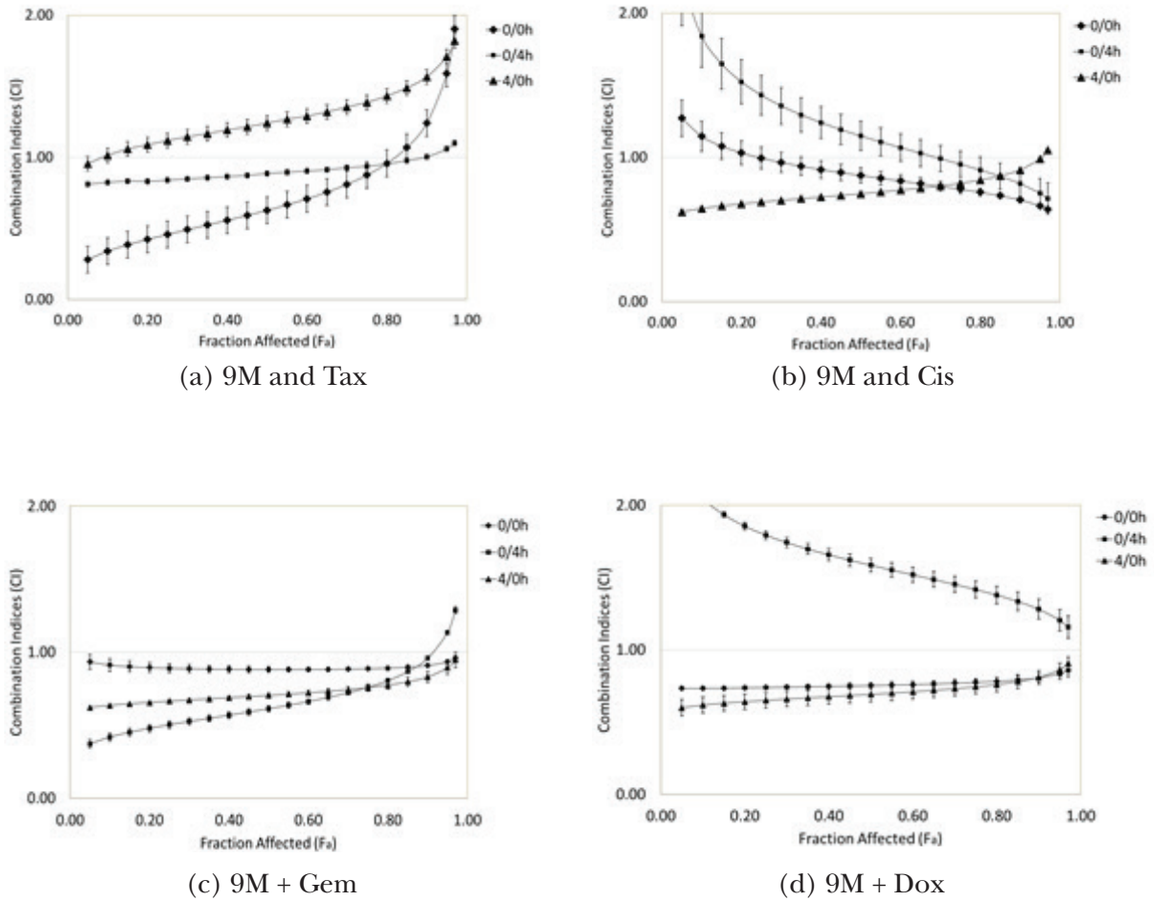
When used alone								
Drug Concentration (μM)	F_a	S.D.	Drug Concentration (μM)	F_a	S.D.	Drug Concentration (μM)	F_a	S.D.
9M			Cis			Dox		
0.20	0.01	0.001	0.04	0.11	0.005	0.06	0.04	0.001
2.00	0.30	0.010	0.40	0.32	0.028	0.60	0.62	0.027
0.20	0.01	0.001	0.04	0.11	0.005	0.06	0.04	0.001
20.00	0.94	0.006	4.00	0.89	0.010	6.00	0.92	0.014
			Tax			Gem		
			0.004	0.011	0.002	0.06	0.08	0.007
			0.040	0.70	0.034	0.60	0.76	0.004
			0.400	0.92	0.011	6.00	0.94	0.002

When used in combination

Drug Concentration (μM)		0/0 h		0/4 h		4/0 h	
		F_a	S.D.	F_a	S.D.	F_a	S.D.
9M	Cis						
0.10	0.02	0.08	0.011	0.02	0.001	0.10	0.008
1.00	0.20	0.31	0.004	0.32	0.007	0.36	0.037
10.00	2.00	0.92	0.008	0.91	0.011	0.90	0.010
9M	Tax						
0.10	0.002	0.05	0.03	0.03	0.02	0.03	0.001
1.00	0.02	0.62	0.043	0.59	0.057	0.22	0.086
10.00	0.20	0.86	0.013	0.86	0.025	0.86	0.027
9M	Dox						
0.10	0.03	0.04	0.003	0.01	0.009	0.04	0.021
1.00	0.30	0.57	0.068	0.19	0.013	0.63	0.023
10.00	3.00	0.94	0.007	0.92	0.023	0.93	0.008
9M	Gem						
0.10	0.03	0.04	0.014	0.13	0.022	0.07	0.010
1.00	0.30	0.76	0.020	0.74	0.008	0.78	0.027
10.00	3.00	0.93	0.007	0.93	0.003	0.93	0.013

at F_a <0.8 that gave CI below 1. Best synergism activities for 9M and Cis combination when they were treated at 4/0 h (Cis was added first followed by 9M 4 h later) at F_a <0.9 that gave CI value < 1. Although synergism between 9M and Gem had been shown at 0/0 h, 0/4 h and 4/0 h, the best synergism can be seen at 0/4 h (9M was added first followed by Gem) at F_a <0.9 that gave CI below 1. Synergism was also evident for 9M and Dox combination at 0/0 h and 4/0 h, but antagonism was observed when they were treated at 0/4 h.

For median effect analysis, CI values at F_a=0.5 were captured for further discussion as shown in Figure 3. The combination of 9M with Tax, Cis, Dox or Gem, gave synergistic effects when treated simultaneously (0/0 h). For sequential treatments based on CI_{50}, synergistic



(a) 9M and Tax (b) 9M and Cis

(c) 9M + Gem (d) 9M + Dox

Figure 2 Combination indices (CIs) versus fraction affected for 9M-drug combinations in pair treated at 0/0 h (simultaneous addition), 0/4 h (9M was added first followed by the respective drug) and 4/0 h (the drug was added first followed by 9M)

Table 3 Concentration-effect parameters (D_m), (m) and (r) for the combinations of 9-methoxycanthin-6-one (9M) and each drug (cisplatin (Cis), paclitaxel (Tax), doxorubicin (Dox), gemcitabine (Gem)) in SKOV-3 ovarian cancer cells

Single treatment	D_m	m	r			
9m	3.95	1.20	0.93			
Cis	0.53	0.91	0.98			
Tax	0.05	1.56	0.95			
Dox	0.64	1.25	0.99			
Gem	0.39	1,13	0.98			
Sequence of administration	D_m	m	r	D_m	m	r
Drug combination	9M and Cis			9M and Tax		
0/0 h	1.54	1.06	0.98	0.83	0.91	0.97
0/4 h	2.14	1.35	1.00	1.35	1.30	0.96
4/0 h	1.47	0.95	0.99	1.93	1.24	1.00
Drug combination	9M and Gem			9M and Dox		
0/0 h	1.12	1.25	0.96	1.33	1.24	1.00
0/4 h	0.72	0.97	0.98	3.57	1.82	1.00
4/0 h	0.89	1.12	0.96	1.45	1.32	0.99

effects were observed when 9M was treated first, followed by Tax or Gem 4 hours later and also when each drug was added first but Tax followed by 9M 4 hours later (Figure 3). Combination indices (CI_{50}) were analysed by one-way ANOVA followed by Tukey's multiple comparison test for the same 9M-drug combination that gave synergism at selected time frame. There were no significant differences for each pairwise that gave synergism, 9M and Tax combination at 0/0 h and 0/4 h, 9M and Cis combination at 0/0 h and 4/0 h, 9M and Dox combination at 0/0 h and 4/0 h, and 9M and Gem at 0/0 h, 0/4 h and 4/0 h.

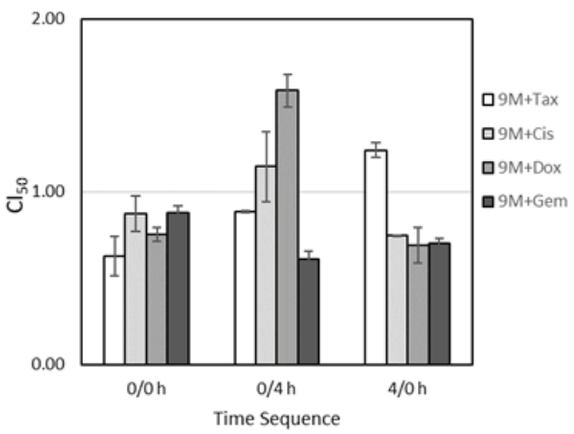


Figure 3 Combination indices with respect to F_a=0.5 (CI_{50}) of 9M when combined in pair with either Tax, Cis, Dox or Gem when treated simultaneously (0/0 h), 9M treated 4 hours before each drug (0/4 h), each drug added 4 hours before 9M (4/0 h). Drug combinations that give CI<1, CI>1 or CI=1 indicate synergism, antagonism or additive activities, respectively (Chou 2010 & 2018)

DISCUSSION

In ovarian cancer treatment, combinations of therapeutic agents showed an improvement in term of survival rates over single therapeutic agent (Zoń & Bednarek 2023). For example, one of the earliest first line treatment for

Table 4 Combination indices of 9-methoxycanthin-6-one (9M) when combined in binary with either Cisplatin (Cis), Paclitaxel (Tax), Gemcitabine (Gem) or Doxorubicin (Dox) at fraction affected (F_a) concentrations

Drug combination	Sequence of administration	Combination Index (CI) at F_a or IC:				
		0.25 or IC_{25}	0.50 or IC_{50}	0.75 or IC_{75}	0.90 or IC_{90}	0.95 or IC_{95}
9M and Tax	0/0 h	0.46	0.63	0.88	1.24	1.59
	0/4 h	0.84	0.89	0.94	1.00	1.06
	4/0 h	1.12	1.24	1.38	1.56	1.71
9M and Cis	0/0 h	0.99	0.87	0.78	0.70	0.66
	0/4 h	1.43	1.15	0.95	0.82	0.75
	4/0 h	0.69	0.75	0.82	0.91	0.99
9M and Dox	0/0 h	0.74	0.75	0.77	0.81	0.83
	0/4 h	1.79	1.59	1.42	1.28	1.20
	4/0 h	0.65	0.69	0.74	0.81	0.86
9M and Gem	0/0 h	0.89	0.88	0.89	0.91	0.93
	0/4 h	0.50	0.61	0.76	0.96	1.14
	4/0 h	0.66	0.70	0.76	0.83	0.89

ovarian cancer was with Cis or carboplatin (platinum drugs). Later, it was discovered that combination chemotherapy between Cis and Tax had been one of the main modality in advanced ovarian cancer treatment since it had shown significantly higher in efficacy, however, 20–30% of patients still failed to respond to this combination maybe due to drug resistance as one of the reasons (Judson et al. 1999). The combined chemotherapy such as Cis and Tax had also shown better median survival rates of 5-year as compared to when each drug was treated alone (American Cancer Society 2024, Burke et al. 2023). Development of more combinations of therapeutic agents are still ongoing worldwide in a race against resistance towards existing single therapeutic agent therapies. It may be noted that one of the strategies to combat Cis or other drug resistance is by combining the drug with natural products due to their highly diverse in molecular structures and may affect multiple and different targets (Dasari et al. 2022, Garrido et al. 2022, Pistollato et al. 2017). It may be noted that, one of the mechanisms of drug resistance is due to malfunction of apoptosis (Zoń & Bednarek 2023). Drug candidates can re-induce the apoptosis in the cancer cells in synergistic effect when combined with the existing drug. Approximately 65% of anticancer drugs were derived from natural products (Newman & Cragg 2020). Among the group of natural products of interest are alkaloids such as piperine, sanguinarine and dendrobine were found to increase the sensitivity to cisplatin therapy especially in ovarian cancers as being reviewed by Dasari et al. 2022.

For this discussion, we will compare the combined 9M and each drug action at CI_{50} (Combination Index at median effect concentration; F_a=0.5). Simultaneous treatments between 9M with either Cis, Tax, Gem and Dox gave synergistic effects at CI_{50}. This may due to 9M may have different modes of action than the tested drugs. 9M had also been reported to re-induce apoptosis in the ovarian cancer cells when treated alone by targeting a series of proteins which include pyruvate kinase isoenzyme M1/M2 (PKM), Annexin A2 (ANXA2), galectin-3 (LGALS3)) and also via glycolysis I pathway (glyceraldehyde-3-phosphate (GAPDH), phospolycerate mutase 1 (PGAM1), PKM and triosphosphate isomerase 1 (TPI1)) based on

proteomics analysis (Nurhanan et al. 2022). Whereas, Cis was found to target and crosslink with the purine bases on the DNA and cause DNA damage which lead to apoptotic cell death (Dasari & Tchounwou 2014). As mentioned earlier, combined chemotherapy such as Cis and Tax was reported that ANXA2 had been associated with Cis resistance in the ovarian cancer cells (Sato et al. 2012). Since ANXA2 had been one of the targeted proteins and being suppressed by 9M (Nurhanan et al. 2022), the combined action of 9M with Cis may increase the sensitivity of Cis, hence as shown in the synergism activities exerted by this combination from recent findings. Tax, Gem and Dox also had different modes of action than 9M in which Tax is an alkaloid that promotes the assembly of microtubules and caused mitotic arrest (Weaver 2014), Dox reacted by intercalating the DNA which then formed DNA adducts that lead to inhibition of topoisomerase II activity and stopped DNA replication (Varela-López et al. 2019) and Gem is a nucleoside analogue that functions to inhibit DNA synthesis (De Sousa Cavalcante & Monteiro 2014).

Whereas, sequential treatment within the 4 h gap between 9M and Cis, Tax, or Dox in pairs also showed synergistic activities in inhibiting the proliferation of ovarian cancer cells at selected time sequence. The 4 h gaps were chosen for sequential treatments based on our earlier combination studies utilising cisplatin and other natural products such as andrographolide, curcumin and epigallocatechin-3-gallate had shown synergistic effects when Cis was treated first followed by each of this compound 4 h later (Nurhanan et al. 2011, Nurhanan et al. 2013). Both 9M and each tested drug when combined within a 4 h gap may affect different phases of the cell cycle. Allowing a time gap can ensure that the cancer cells are in a more susceptible phase for each drug's action. From figure 3, 9M and Tax gave the synergism at 0/4 h (where 9M was treated 4 h before Tax). 9M is a cathinone alkaloid. There was no report on 9M studies on cell cycle phase. However, canthin-6-one (a canthinone) had been reported to affect the cell cycle in cancer cells primarily by inducing cell cycle arrest at the G1 phase. This arrest impedes the cells' progression from G1 phase to S phase (Torquota et al. 2017). Tax was reported to block the cell growth in the late G2 phase and M phase of the cell cycle, inhibiting the cell replication and eventually lead to cancer cell death (Panchagnula 1998). During G1 phase (earliest phase of cell cycle), the cell grows and prepares for DNA synthesis, whereas, in the G2/M phase, mitosis occurs. If 9M gave the same effect as canthin-6-one in affecting the cell cycle at G1 phase, it seems that inhibiting the cell cycle at G1 phase by 9M followed by Tax 4 h later had a more profound synergistic effect in inhibiting the cancer cells proliferation. Whereas, Cis, Dox and Gem when treated 4 h before 9M, showed synergistic activities in inhibiting the proliferation of ovarian cancer cells. Cis was reported to inhibit DNA replication by arresting at S-phase to early G2 phase in ovarian cancer cells and this finding may influence the synergism activities that are caused by the combined drug actions (Merlin et al. 1998, Judson et al. 1999). Dox was found to arrest ovarian cancer cells at the G2 phase of cell cycle (Malugin et al. 2007). Whereas, Gem arrested the cell cycle at S phase (DNA synthesis) rather than G1 phase (Merlin et al. 1998). All these three drugs (Cis, Dox and Gem) seem to exert mechanistic dominance affecting those cell cycle phases over 9M.

In vitro experiments on combination treatments were reported to have several limitations in terms of providing the data for pharmacokinetic effects including drug metabolism, drug clearance, drug distribution, half-life and plasma protein binding (Smith et al. 2005). To the best of our knowledge, there was limited information on the pharmacokinetic studies being performed on 9M. Nevertheless, this current studies may provide a proof-of-concept on the potential of 9M to be combined with the respective drug (either Cis, Tax, Gem or Dox) in exerting *in vitro* anticancer effects against the ovarian cancer cells.

The results of the present study indicate that combined drug action between 9M and Cis, Tax, Gem or Dox in pairs had shown synergistic effects in inhibiting the ovarian cancer cells proliferation, dependent on the sequence or simultaneous 9M-drug treatments and the concentrations used. Further studies on combination drug action between 9M and the respective drug against ovarian cancer resistance cells and cell cycle analysis shall be performed to validate on whether these combined treatments

may overcome drug resistance problem and whether the cell cycle phase-specific or non-specific affect the synergism or antagonism activities that they caused.

CONCLUSIONS

In conclusion, 9M has the potential to act as anti-ovarian cancer agent in synergy when combined in pairs with either Tax, Cis, Dox or Gem when added simultaneously at CI_{50}. Sequence of addition between the 9M and the drug within the 4 h gap may also give synergistic activities but each drug must be added first (except for Tax), followed by 9M. This study may suggest that 9M has the potential to be used as both primary and adjuvant therapies in treating ovarian cancer cells. Still, pre-clinical and clinical studies must first be conducted to validate these *in vitro* studies.

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