

Synergistic interactions between peloruside A and other microtubule-stabilizing and destabilizing agents in cultured human ovarian carcinoma cells and murine T cells

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Abstract

Purpose Microtubule-stabilizing agents are an important class of anticancer compounds. Peloruside A and laulimalide bind to a different site on the microtubule to taxoid site drugs such as paclitaxel (Taxol[®]), docetaxel (Taxotere[®]), ixabepilone (Ixempra[®]), the epothilones, and discodermolide. The purpose of this study was to examine the synergistic interactions of these drugs when given in combination in relation to the differences in their binding sites on the microtubule. **Methods** Human ovarian carcinoma cells (1A9 cells) and murine T cells were treated with different combinations of microtubule-stabilizing or destabilizing agents. The compounds were given individually and in combination, and the antiproliferative activity was assessed to calculate a combination index (CI) from the equation: $CI = D_1/Dx_1 + D_2/Dx_2$ in which D_1 and D_2 are the concentrations of drug 1 and drug 2 that when given together give the same response as drug 1 and 2 alone (Dx_1 and Dx_2). Thus, a CI value of less than 1.0 indicates a synergistic effect between the two drugs

in which the response to the two drugs given together is greater than the additive response of the two drugs if given on their own.

Results As anticipated from previous in vitro studies, peloruside A and laulimalide did not synergize with each other. They also failed to synergize with the microtubule-destabilizing agents vinblastine and 2-methoxyestradiol. Peloruside A and laulimalide did, however, synergize with the epothilones, as had been previously shown, but not with docetaxel or discodermolide.

Conclusions Combining two microtubule-targeting agents with different binding sites does not guarantee a synergistic interaction in cells, and additional factors are likely to be involved. This study highlights the importance of preclinical testing of actual combinations of drugs before proceeding into clinical trials.

Keywords Anticancer drug · Combination therapy · Laulimalide · Microtubule-targeting agent · Peloruside A · Synergy

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Abbreviations:

CFSE Carboxyfluorescein succinimidyl ester
MDA Microtubule-destabilizing agent
MDR Multiple drug resistance
MSA Microtubule-stabilizing agent
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Introduction

Microtubule-stabilizing agents (MSA), such as paclitaxel (Taxol[®]), docetaxel (Taxotere[®]), and ixabepilone

(Ixempra[®]), are members of an important class of compounds in anticancer therapy [24]. These drugs also show promise in delaying the onset of symptoms in a mouse model of multiple sclerosis [11] and in the treatment of neurodegenerative diseases [4]. Microtubule-targeting agents can be divided into MSA that bind to and stabilize the polymerized form of tubulin and microtubule-destabilizing agents (MDA) that inhibit microtubule polymerization and promote destabilization (see Fig. 1 for chemical structures). Even though MSA and MDA work in opposite ways, they both interfere with microtubule dynamics and lead to mitotic arrest [24]. Paclitaxel, the first identified MSA, is used clinically for the treatment of breast, ovarian, lung, and head and neck cancer [35], and MDA, such as the two *Vinca* alkaloids, vinblastine and vinorelbine, are in clinical use for the treatment of non-small-cell lung cancer (NSCLC) [13]. Toxicity, limited efficiency, and development of multiple drug resistance (MDR), however, limit their clinical use and highlight the need for continued development of new drugs. The use of combination therapy is an approach with great potential for solving some of these problems [3].

Peloruside A (peloruside), an MSA isolated from a marine sponge, binds to a similar or overlapping site on the microtubule as another marine sponge-derived MSA, laulimalide. These drugs bind to microtubules at a distinct site to that of the taxoid drugs [14, 23, 28, 33, 36]. Most other MSA such as paclitaxel, docetaxel, epothilone A and B, ixabepilone, and discodermolide bind to the well-defined taxoid site on β -tubulin and should therefore potentially be able to synergize with peloruside and laulimalide. In in vitro tubulin polymerization assays [15, 19], the theory that two MSA that bind to the same site (e.g., paclitaxel/epothilone A or peloruside/laulimalide) will not synergize together, but two that bind to different sites will (e.g., peloruside/paclitaxel or laulimalide/epothilone A), generally holds true. Indeed, the ability of peloruside and laulimalide to act synergistically with paclitaxel as well as with epothilone A has been verified in cultured cells [9, 37] and in vitro with isolated tubulin [15, 19].

Synergy between paclitaxel and discodermolide, two MSA that bind to the taxoid site, has also been demonstrated in cultured cells [21, 27] and in animals [22]. There is recent evidence, however, that discodermolide does not bind to the same peptide residues within the taxoid site as paclitaxel [25] and that the kinetics of polymerization of the taxoid site drugs differ from each other [16], which may explain the synergistic effects observed between paclitaxel and discodermolide. For example, the time required to achieve maximum tubulin polymerization under identical experimental conditions is not the same between discodermolide and other taxoid site agents. Discodermolide induced polymerization more rapidly than paclitaxel or epothilone B, and there were differences in

the physico-chemical properties of the tubulin polymers between discodermolide and paclitaxel- or epothilone B-induced polymerization [16]. MSA may also have secondary binding sites on microtubules or even on other proteins in the cell, and such complex actions and multiple targets may underpin some of the synergistic actions observed with particular drug combinations. It is also possible that two drugs act synergistically on one target and antagonistically or additively on another. Thus, the nature and causes of synergistic interactions in drug therapy are complex and merit further investigation.

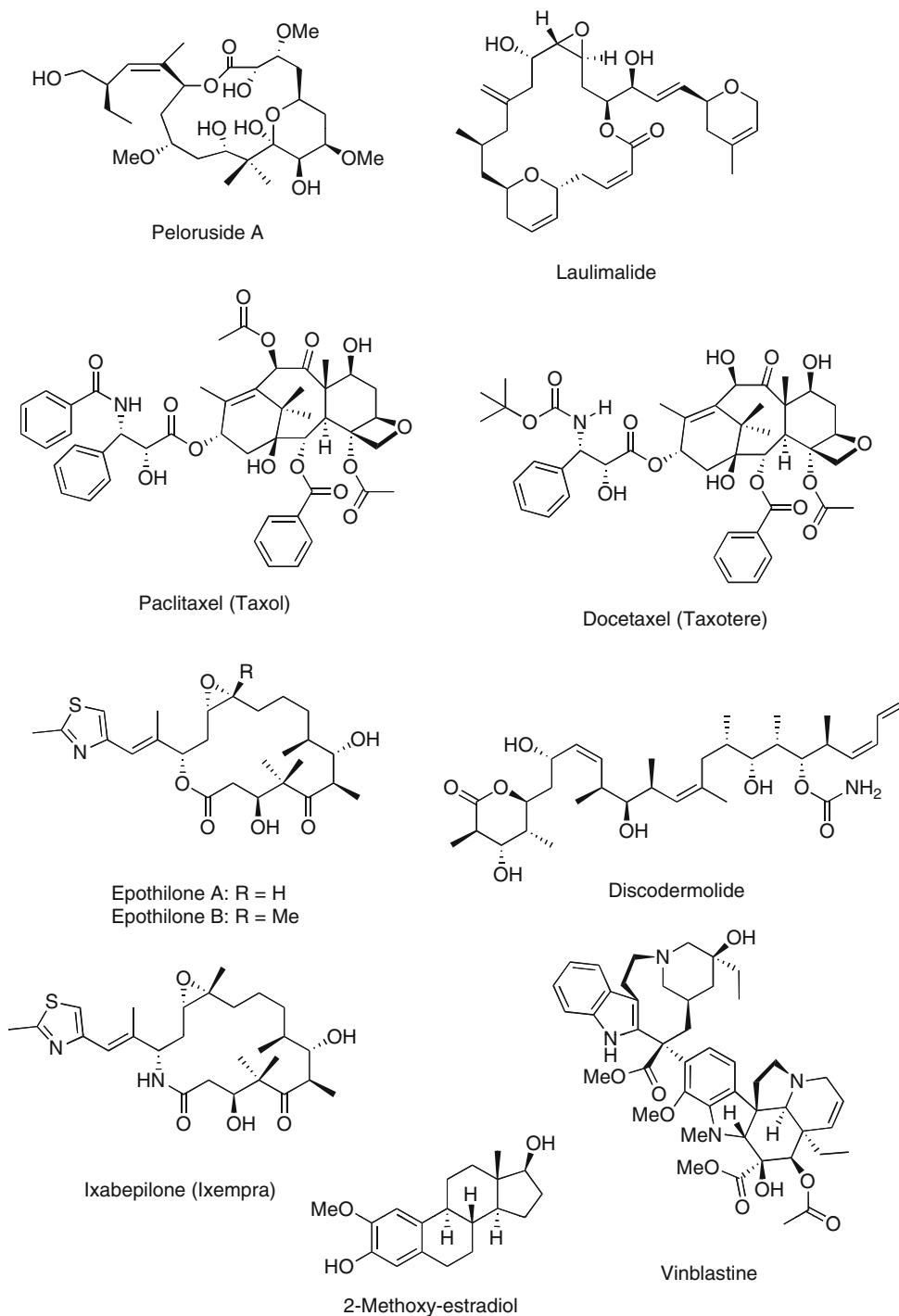
The aim of the present study was to extend these earlier studies to investigate synergy between peloruside, laulimalide and other MSA drugs such as docetaxel, discodermolide, and ixabepilone, an epothilone B derivative [26]. We also wanted to test for synergy between MSA and drugs that depolymerize tubulin, including 2-methoxyestradiol and vinblastine, since others have shown that laulimalide can synergize with the MDA 2-methoxyestradiol [9]. In addition, we wished to confirm the in vitro results of Hamel et al. [19] that peloruside and laulimalide, which bind to the same or overlapping site on tubulin, cannot interact synergistically. Because of recent studies showing potential benefits of MSA in the treatment of a mouse model of multiple sclerosis [10, 11, 34], we wished to extend our study from cancer cells to primary immune cells by investigating synergistic interactions in murine splenocytes.

Looking at additional combinations of MSA will provide more insight into the mechanism of synergy and highlight useful differences in the mode of action between microtubule-targeting agents and other drugs commonly used in the treatment of cancer, such as the alkylating agents (cisplatin, carboplatin, nedaplatin, cyclophosphamide), antimetabolites (mercaptopurine), anthracyclines (doxorubicin), and topoisomerase inhibitors. This study will help improve strategies for the use of these drugs in combination therapy, as well as provide evidence of the unique actions of individual MSA, given the premise that different activity profiles are needed for synergistic interactions to occur.

Experimental section

Materials

The structures of the microtubule-targeting agents used in this study are presented in Fig. 1. Peloruside A (peloruside) was isolated and purified from the marine sponge *Mycale hentscheli* [36]. Paclitaxel (Taxol[®]) and docetaxel (Taxotere[®]) were purchased from LC laboratories (Woburn, MA, USA). Discodermolide was synthesized by Paterson et al.

Fig. 1 Structures of microtubule-targeting agents

[31]; 2-methoxy-estradiol and epothilone A were purchased from Merck, vinblastine from Sigma, and ixabepilone (Ixempra®) from Bristol-Myers Squibb. Laulimalide was a gift from Dr Paraskevi Giannakakou of Cornell University Weill Medical College in New York, or was isolated and purified from the Tongan marine sponge *Cacospongia mycofijiensis*. All drugs were stored at -20 or -80°C as 1 mM solution in absolute ethanol. Working stocks were diluted in culture medium.

Cell culture

Human ovarian carcinoma 1A9 cells were cultured at 37°C in a 5% CO_2 in air atmosphere in RPMI-1640 medium supplemented with 10% FCS, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 units/ml insulin (Sigma).

Splenocytes were isolated from female C57BL/6 mice as previously described [11]. Briefly, spleens were

harvested, and single-cell suspensions were prepared using sterile 70- μ m-mesh cell strainers (BD Biosciences, Franklin Lakes, NJ, USA). Cells were stained with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Invitrogen) (see below) and cultured in 96-well round-bottom plates (BD Biosciences) at 5×10^5 cells/well in complete medium containing DMEM, 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM Hepes, 2 mM L-glutamine, and 5×10^{-5} M β -mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA). Following stimulation for 48 h in the presence or absence of concanavalin A (3 μ g/ml; Sigma Chemical Co.) and drug, the cells were harvested and stained with anti-CD4-cychrome *c* or an isotype control antibody (BD Biosciences) according to the manufacturer's recommendations. Combination treatments and individual drug concentration–response curves were conducted concurrently and in duplicate.

Cell proliferation assays

For 1A9 cells, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoyltetrazolium bromide) colorimetric, dye-reduction assay was used to estimate cell proliferation, as previously described [37]. Cells were treated with drugs for 48 h in 96-well plates. Combination treatments were performed at the same time as individual concentration–response curves for each drug. Concentrations for drug combinations were chosen according to the concentration–response curves of individual compounds (Table 5). Usually concentrations below the IC_{50} worked best for combination treatments [37]. At least 3 concentrations of each drug were tested for synergy (sometimes up to 7 concentrations), usually beginning at 50% or less of the IC_{50} concentration for growth inhibition but also including one concentration just over the IC_{50} . For example, 25, 50, and 75% of the IC_{50} represented good starting concentrations for testing for synergy. When a reasonable set of synergistic concentrations was found, multiple repeats of those concentrations were carried out to provide the robust statistical evidence of synergy. Concentrations greater than the IC_{50} usually gave additivity or antagonism rather than synergy because the effect of either drug alone was too strong and masked the subtle synergistic interactions. Low concentrations of drugs were also ineffective in generating a measureable synergistic response.

Isolated splenocytes were stained with CFSE and analyzed for proliferation following concanavalin A stimulation as described previously [11]. Briefly, isolated splenocytes were stained with 1 μ M CFSE in PBS for 8 min, then quenched with equal volumes of FCS and washed twice in medium. Proliferation, represented as a reduction in CFSE fluorescence in cell progeny, was analyzed using CellQuest Pro software (Becton–Dickinson,

Franklin Lakes, NJ, USA). A total of 10,000 events were collected for each sample, and results were calculated as percent of total splenocytes or $CD4^+$ cells that proliferated.

Statistical analysis

Drug interactions were assessed using the combination index equation of Chou and Talalay [8] and Berenbaum [2]:

$$CI = \frac{D_1}{D_{x1}} + \frac{D_2}{D_{x2}} \quad (1)$$

in which a CI value equal to one indicates additivity, values less than one indicate synergy, and values greater than one indicate antagonism. The doses D_1 and D_2 are those used in combination, and the doses D_{x1} and D_{x2} are the amounts of each drug given alone that would produce the same response as obtained with the two drugs together. In order to calculate the concentration of drug needed for a given response on its own, the following logit-log equation was used:

$$\text{Dose}(x) = IC_{50} \times (\max - \text{response} / \text{response} - \min)^{1/\text{hillslope}} \quad (2)$$

IC_{50} and values for minimum, maximum, and hillslope were obtained from Sigma plot software programs.

CI values were compared to a value of 1.0 using a one-sample Student's *t*-test (GraphPad Prism v4.0). Values for $P < 0.05$ were taken as significant. Only CI values of ≤ 0.8 were taken as representing effective synergy.

Results

Synergy between MSA in human ovarian carcinoma 1A9 cells

Peloruside and laulimalide share the same or overlapping binding sites on tubulin [14], and it is assumed for this reason that they are unable to synergize in an in vitro tubulin polymerization assay [19]. Our results in cultured 1A9 human ovarian cancer cells also showed that they were unable to synergize inside cells (Table 1). Martello et al. [27] reported no synergy between paclitaxel and epothilone B inside cells, yet we found significant synergy between paclitaxel and epothilone A (CI value of 0.45) in our earlier study in 1A9 cells [37]. In order to validate our methods in the present study, we repeated our earlier combination of 10 nM peloruside and 5 nM epothilone A and again obtained a significant CI value of 0.57 (Table 1).

Despite in vitro studies demonstrating synergy between peloruside and the taxoid binding site MSA discodermolide

Table 1 CI values for MSA combinations in human ovarian carcinoma 1A9 cells

Peloruside (nM)	Epothilone A (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
10	5	0.57 $\pm$ 0.13	4	0.044
15	5	0.57 $\pm$ 0.12	3	ns
Peloruside (nM)	Laulimalide (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
10	10	1.4 $\pm$ 0.3	5	ns
10	15	1.4 $\pm$ 0.2	5	ns
15	10	1.2 $\pm$ 0.1	4	0.018
15	15	1.5 $\pm$ 0.2	5	0.036
Peloruside (nM)	Discodermolide (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
10	10	1.4 $\pm$ 0.3	3	ns
10	15	1.6 $\pm$ 0.4	5	ns
10	25	1.5 $\pm$ 0.6	3	ns
Peloruside (nM)	Docetaxel (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
5	2	0.90 $\pm$ 0.07	6	ns
10	1.5	1.1 $\pm$ 0.1	6	ns
10	2	1.4 $\pm$ 0.1	6	0.003
Paclitaxel (nM)	Discodermolide (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
2	10	0.70 $\pm$ 0.001	2	0.010
2	15	0.69 $\pm$ 0.04	3	0.017
3	15	0.83 $\pm$ 0.02	2	ns
Paclitaxel (nM)	Docetaxel (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
2	2	1.7 $\pm$ 0.25	4	ns
2	3	1.9 $\pm$ 0.1	4	0.003
3	2	1.4 $\pm$ 0.24	4	ns
3	3	1.7 $\pm$ 0.25	4	0.045
Docetaxel (nM)	Epothilone A (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
1	5	1.3 $\pm$ 0.35	3	ns
2	5	1.4 $\pm$ 0.31	3	ns
3	5	1.8 $\pm$ 0.05	3	0.025

Calculated values for the combination index (CI) are presented for MSA paired with each other. Concentrations are given in nanomolar. *P* values are calculated from a one-sample Student's *t* test, and the number of biological replicates (*n*) is given. CI values showing significant synergistic interactions are presented in bold

in polymerizing isolated tubulin [19], no synergistic interactions between peloruside and discodermolide were seen in 1A9 cells (Table 1). As previously reported by others, [17, 21, 27], paclitaxel can synergize with discodermolide in cultured cells. We also found synergy between paclitaxel and discodermolide in 1A9 cells (CI values of 0.69 and 0.70) (Table 1); however, the values were less impressive than those originally reported by

Martello et al. [27] in four different cancer cell lines (CI range from 0.25 to 0.38) and Honore et al. [21] in A549 lung cancer cells (CI value of 0.23). Although the IC₅₀ for discodermolide (124 nM) was quite high in 1A9 cells relative to the other MSA (Table 5), synergy between paclitaxel and discodermolide was only seen at low concentrations of discodermolide (approximately 10–15 nM, Table 1). This is similar to that reported by Honore et al. [21], who found synergy between these two MSA on inhibition of microtubule dynamics at low 7 nM concentrations of discodermolide.

A surprising result in the present study was that peloruside was unable to synergize with the paclitaxel derivative docetaxel in 1A9 cells (Table 1). These results were unexpected, given the clear synergy seen between peloruside and paclitaxel in our previous study (CI value approximately 0.5 with 15 nM of each MSA) [37] and the results of Clark et al. [9] who reported synergy between laulimalide and paclitaxel in lung cancer cells. As might be expected, no synergy was found in 1A9 cells between combinations of the two taxoid site MSA paclitaxel and docetaxel or epothilone A and docetaxel (Table 1). This is consistent with the results of Budman and Calabro [5] who found combinations of paclitaxel and docetaxel were not just additive but were antagonistic in most cell lines, although synergy was seen in a Pgp-overexpressing cell line (CI value of 0.59). We also found significant antagonism in 1A9 cells between paclitaxel and docetaxel (Table 1; CI values of 1.7–1.9). One combination of epothilone A/docetaxel in our study was also significantly antagonistic (CI = 1.8).

Synergy between MSA in murine splenocytes and T cells

The synergy results in murine splenocytes (Table 2) and CD4⁺ T cells (Table 3) generally supported those seen in the 1A9 ovarian carcinoma cells (Table 2), although one combination of peloruside/laulimalide in splenocytes gave a significant CI value below 0.8 (CI = 0.74). As with 1A9 cells, no synergy was ever seen with combinations of peloruside/docetaxel, nor was this combination antagonistic as occasionally seen in 1A9 cells. Preliminary experiments in our laboratory have shown that the epothilone derivative, ixabepilone, can delay the onset of disease symptoms in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (unpublished results). A similar delay of disease progression had been previously shown for paclitaxel and peloruside [11]. We therefore tested ixabepilone combinations with peloruside and laulimalide in preparation for future in vivo combination dosing in mice. Peloruside, but not laulimalide, proved significantly synergistic with ixabepilone in

Table 2 CI values for MSA combinations in murine splenocytes

Peloruside (nM)	Laulimalide (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
50	6.25	0.97 $\pm$ 0.05	4	ns
50	3.125	0.81 $\pm$ 0.06	4	0.041
25	6.25	1.20 $\pm$ 0.08	4	ns
25	3.125	0.93 $\pm$ 0.06	4	ns
12.5	6.25	1.38 $\pm$ 0.20	4	ns
12.5	3.125	0.77 $\pm$ 0.09	4	ns
6.25	6.25	1.57 $\pm$ 0.27	4	ns
6.25	3.125	0.74 $\pm$ 0.03	4	0.016
Peloruside (nM)	Docetaxel (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
50	12.5	0.94 $\pm$ 0.09	4	ns
50	6.25	1.17 $\pm$ 0.20	4	ns
25	12.5	0.97 $\pm$ 0.15	4	ns
25	6.25	1.14 $\pm$ 0.14	4	ns
12.5	12.5	1.16 $\pm$ 0.13	4	ns
12.5	6.25	1.25 $\pm$ 0.18	4	ns
6.25	12.5	1.30 $\pm$ 0.10	4	ns
Peloruside (nM)	Ixabepilone (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
50	12.5	0.84 $\pm$ 0.04	4	0.028
50	6.25	0.81 $\pm$ 0.09	3	ns
25	12.5	0.81 $\pm$ 0.05	4	0.038
25	6.25	0.67 $\pm$ 0.01	3	0.001
12.5	12.5	0.72 $\pm$ 0.07	4	0.030
6.25	12.5	0.78 $\pm$ 0.10	4	ns
Laulimalide (nM)	Ixabepilone (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
12.5	12.5	1.21 $\pm$ 0.27	4	ns
12.5	6.25	1.08 $\pm$ 0.11	3	ns
6.25	12.5	0.94 $\pm$ 0.11	4	ns
6.25	6.25	1.12 $\pm$ 0.15	4	ns
3.125	12.5	0.81 $\pm$ 0.08	4	ns
1.263	12.5	1.03 $\pm$ 0.15	4	ns

CI values showing significant synergistic interactions are presented in bold

both splenocytes and CD4⁺ T cells. Only one of the laulimalide/ixabepilone combinations tested in CD4⁺ T cells was moderately but significantly synergistic (CI = 0.8). The synergy between peloruside and ixabepilone in both murine splenocytes and CD4⁺ T cells was expected, given the synergy seen between peloruside and epothilone A in human ovarian cancer cells in the present study (Table 1) and in previous studies in this same cell line and in HL-60 human promyelocytic leukemic cells [37]. The unexpected result in 1A9 cells (Table 1) that peloruside does not synergize with the taxoid site drug docetaxel was also seen in the murine leukocyte preparations (Tables 2, 3).

Table 3 CI values for MSA combinations in murine CD4⁺ T cells

Peloruside (nM)	Laulimalide (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
50	6.25	1.08 $\pm$ 0.14	4	ns
50	3.125	0.96 $\pm$ 0.12	4	ns
25	6.25	1.29 $\pm$ 0.21	4	ns
25	3.125	0.98 $\pm$ 0.08	4	ns
12.5	6.25	1.30 $\pm$ 0.27	4	ns
12.5	3.125	0.79 $\pm$ 0.09	4	ns
6.25	6.25	1.31 $\pm$ 0.27	4	ns
Peloruside (nM)	Docetaxel (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
50	12.5	0.88 $\pm$ 0.14	4	ns
50	6.25	1.03 $\pm$ 0.07	4	ns
25	12.5	1.01 $\pm$ 0.19	4	ns
25	6.25	1.21 $\pm$ 0.11	4	ns
12.5	12.5	1.19 $\pm$ 0.28	4	ns
12.5	6.25	1.26 $\pm$ 0.07	4	ns
6.25	12.5	1.25 $\pm$ 0.25	4	ns
Peloruside (nM)	Ixabepilone (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
50	12.5	0.67 $\pm$ 0.08	4	0.029
50	6.25	0.72 $\pm$ 0.05	3	0.036
25	12.5	0.76 $\pm$ 0.05	4	0.018
25	6.25	0.74 $\pm$ 0.06	3	0.045
12.5	12.5	0.74 $\pm$ 0.06	4	0.019
12.5	6.25	0.94 $\pm$ 0.15	3	ns
6.25	12.5	0.98 $\pm$ 0.12	4	ns
Laulimalide (nM)	Ixabepilone (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
12.5	12.5	1.14 $\pm$ 0.12	4	ns
6.25	12.5	0.97 $\pm$ 0.12	4	ns
3.125	12.5	0.82 $\pm$ 0.07	4	ns
1.263	12.5	0.80 $\pm$ 0.05	4	0.036
12.5	6.25	1.10 $\pm$ 0.13	3	ns
6.25	6.25	1.03 $\pm$ 0.15	4	ns

CI values showing significant synergistic interactions are presented in bold

Synergy between peloruside and MDA

Although other studies have demonstrated synergy between the destabilizing agents vinblastine and 2-methoxy-estradiol with both paclitaxel [18, 20] and laulimalide [9], we were unable to duplicate these results in 1A9 cells using peloruside or laulimalide in combination with these two MDA (Table 4). No synergy between peloruside or laulimalide and vinblastine or 2-methoxy-estradiol was seen at any of the combination doses tested except for 10 nM peloruside with 350 nM 2-methoxy-estradiol (CI = 0.75). Concentrations of 3–4 nM vinblastine were tested in triplicate. These concentrations were 2- to 3-fold higher than the IC₅₀ for

Table 4 CI values for peloruside or laulimalide in combination with MDA in 1A9 cells

Peloruside (nM)	2-Methoxy-estradiol (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
5	250	0.94 $\pm$ 0.18	5	ns
5	350	1.0 $\pm$ 0.11	7	ns
10	250	0.81 $\pm$ 0.10	8	ns
10	350	0.75 $\pm$ 0.06	8	0.003
Laulimalide (nM)	2-Methoxy-estradiol (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
3	250	1.02 $\pm$ 0.02	2	
3	350	1.00 $\pm$ 0.10	2	
4	250	0.93	1	
4	300	1.22	1	
4	350	1.10	1	
4	400	0.85	1	
5	250	0.97 $\pm$ 0.001	2	
5	350	1.09 $\pm$ 0.040	3	
7	250	1.11	1	
7	350	1.25	1	
8	300	0.96	1	
8	400	1.03	1	
Peloruside (nM)	Vinblastine (nM)	CI $\pm$ SEM	<i>n</i>	<i>P</i> value
10	3	1.4 $\pm$ 0.6	3	
10	4	1.4 $\pm$ 0.1	3	
15	3	1.2 $\pm$ 0.2	3	
10	4	1.3 $\pm$ 0.01	2	

CI values showing significant synergistic interactions are presented in bold

vinblastine; however, lower concentrations were also tested without any synergy being observed (data not presented).

IC₅₀ values for MSA and MDA

The effects of the microtubule-targeting agents, given on their own, on cell proliferation were tested simultaneously with the combination dose tests. IC₅₀ values for growth inhibition following 48-h exposure to drug are presented in Table 5. IC₅₀ values were determined from concentration–response curve analyses (Sigma Plot). The CI values for Tables 1, 2, 3, and 4 were calculated using the paired concentration–response curves to estimate Dx_1 and Dx_2 of Eq. 1.

Discussion

Synergy of MSA with each other

Combination therapy has become more and more strategically important in the clinic, although some caution is

Table 5 IC₅₀ values of individual compounds

Compound	IC ₅₀ $\pm$ SEM (<i>n</i>) [nM]		
	1A9 cells	Splenocytes	CD4 ⁺ cells
Peloruside	24 $\pm$ 2 (12)	86 $\pm$ 7 (12)	75 $\pm$ 6 (12)
Paclitaxel	7.7 $\pm$ 1.0 (5)		
Docetaxel	3.2 $\pm$ 0.6 (6)	12 $\pm$ 2 (4)	10 $\pm$ 1 (4)
Epothilone A	7.5 $\pm$ 2.5 (4)		
Ixabepilone	–	24 $\pm$ 3 (8)	30 $\pm$ 2 (8)
Discodermolide	124 $\pm$ 7 (7)		
Laulimalide	18 $\pm$ 2 (6)	9 $\pm$ 1 (8)	11 $\pm$ 1 (8)
Vinblastine	1.7 $\pm$ 0.3 (4)		
2-Methoxy-estradiol	592 $\pm$ 43 (9)		

The 48-h IC₅₀ values in nanomolar (mean $\pm$ SEM) are presented from MTT cell proliferation assays in 1A9 human ovarian carcinoma cells, and CFSE dilution assays in murine splenocytes (mixed T cells) and murine CD4⁺ T helper cells (*n* biological replicates)

advisable. Combining two drugs used clinically on their own may bring additional toxicities in combination, as was found in a preclinical study on docetaxel and vinorelbine [12]. In most studies, two drugs that target two independent pathways are used in combination, for example, paclitaxel in combination with platinum-containing drugs such as nedaplatin or carboplatin [1, 30]. Synergy between two drugs with the same mechanism of action, for example, peloruside and paclitaxel, could also prove clinically useful since less of each drug would be needed to reach a minimally effective concentration in the blood. In vivo studies in mice have shown that the combination of two MSA that bind to the same site on β -tubulin, paclitaxel and discodermolide, can also act synergistically in reducing tumor size in mice [22]. MSA that have different binding sites synergize with each other in polymerizing isolated tubulin, whereas two MSA that bind to the same site on tubulin are not synergistic [15, 19]. However, synergy studies in cells, as well as studies in mice [22], have shown that some MSA that bind to the same site, for example, paclitaxel and discodermolide, can interact synergistically [21, 27]. The synergistic interaction between paclitaxel/discodermolide in cells was confirmed in our study. This may be explained by the differences in binding configurations despite binding to the same site [25]; however, this does not account for the lack of synergy observed between these two taxoid site drugs in studies with isolated tubulin [19].

An unexpected finding in our study was that peloruside did not interact synergistically with two MSA that bind to the taxoid site, docetaxel and discodermolide. This is surprising since peloruside interacts synergistically in cells with three other MSA that bind to the taxoid site,

paclitaxel, epothilone A, and ixabepilone, in three different cell lines, 1A9 cells, HL-60 human promyelocytic leukemia cells (Table 1; [37], and murine T cells isolated from the mouse spleen (Tables 2, 3). In isolated tubulin studies, peloruside and docetaxel synergize similarly to peloruside and paclitaxel (personal communication, Ernest Hamel), and peloruside also synergizes with discodermolide *in vitro* [19]. This indicates that different binding sites are not sufficient for MSA to synergize, and perhaps other factors or secondary targets of individual drugs may also be important in synergy.

The reasons for a lack of synergy in the present study between peloruside and docetaxel or discodermolide could be due to different activity profiles of the drugs themselves on the primary or secondary targets or to the cell lines used. Although differences in binding sites seem to adequately explain the synergistic interactions reported with isolated tubulin polymerization, interactions in cultured cells appear to be more complex. There is support for different binding conformations within a single site, for example, the taxoid site (paclitaxel and discodermolide) [25], or novel stabilization mechanisms in the peloruside/laulimalide binding site [23], but these differences should also affect synergy in isolated tubulin studies. There is also evidence that the taxoid site drugs have distinctly different effects on tubulin polymerization and may interact with different β -tubulin isotypes [16]. This could explain differences between cell lines that express their own unique tubulin isotype compositions. Other factors presumably also play a role in synergy, including differences in drug effects on microtubule-associated proteins or potential unique secondary target profiles or downstream effects of the drugs [38]. Even drug efflux pump activity has been linked to synergy effects [5]. Thus, each MSA would have its own synergy profile with other drugs and in different cell lines that would determine whether synergistic interactions occurred or not.

A comparison of the results in human ovarian cancer cells with murine leukocytes shows both similarities and differences. In both types of cells, significant synergy was seen between peloruside and the epothilone derivative epothilone A (Table 1; [37]) or ixabepilone (Tables 2, 3), and no synergy was seen between peloruside/laulimalide or peloruside/docetaxel combinations (Tables 1, 2, 3). A novel and unexpected finding in the murine leukocytes was a lack of synergy between laulimalide and ixabepilone, despite the fact that peloruside/ixabepilone combinations were synergistic in these cells. The combination of laulimalide with ixabepilone would be expected to be comparable to peloruside/epothilone A (Table 1; [37]) or peloruside/ixabepilone combinations (Tables 2, 3) that did give synergy in these cell lines.

Synergy between MSA and MDA

An important property of microtubule-targeting agents is their ability to alter microtubule dynamics [21, 24]. This might explain why combinations of MSA and MDA, which have opposite effects on tubulin polymerization, give synergistic effects [9, 18, 20]. Both MSA and MDA disrupt microtubule dynamics (e.g., growth and shortening of microtubules) at concentrations 10- to 100-fold lower than those needed to alter the microtubule polymer mass [24]. Peloruside, like paclitaxel, epothilone A, and discodermolide, has recently been shown to inhibit microtubule dynamics [7]. In cultured cell studies, synergy has been reported between combinations of paclitaxel and the destabilizing agent vinorelbine [32], docetaxel and vinorelbine [5], paclitaxel and vinblastine [18], paclitaxel and 2-methoxy-estradiol [20], and laulimalide and 2-methoxy-estradiol [9]. The combination of docetaxel and vinorelbine has also been moderately successful in clinical trials for the treatment of metastatic NSCLC [29] and more recently in advanced or metastatic breast cancer [6]. Paclitaxel and 2-methoxy-estradiol also synergized in a human breast cancer cell xenograft study in immunocompromised mice [20]. Despite these examples of synergy between MSA and MDA, in the present study, peloruside and the two MDA tested failed to synergize together in 1A9 cells, suggesting that the synergy between stabilizing and destabilizing agents may be to some extent cell specific.

Conclusions

Current models of MSA-binding interactions do not fully explain synergy or lack of synergy between pairs of compounds. While different binding sites on tubulin might contribute in some instances to synergy between microtubule-targeting drugs, this is not always sufficient, and other factors, including unique microtubule structure/morphology, differences in kinetics of polymerization, unique interactions with microtubule-regulating proteins, altered tubulin isotype levels, or secondary non-tubulin targets, are likely to be involved. Because of the lack of predictability of synergistic combinations in cultured cells, potential drug combinations will need to be tested *in vivo* on a case-by-case basis.

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