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Research paper

Synergistic interaction of selected marine animal anticancer drugs against human diffuse large cell lymphoma

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We studied the antitumor effects of dolastatin 10, its structural modification, auristatin PE (TZT-1027), and vincristine alone and in combination with bryostatin 1 on a human diffuse large cell lymphoma line (WSU-DLCL$_2$) *in vitro* and *in vivo*. WSU-DLCL$_2$ cells were cultured in RPMI 1640 at a concentration of 2×10^5/ml using a 24-well plate. Agents were added to triplicate wells, and cell count, viability, mitosis and apoptosis were assessed. Dolastatin 10 showed no apparent inhibition of cell growth at concentrations less than 500 pg/ml. Auristatin PE showed significant growth inhibition at concentrations as low as 10 pg/ml, while vincristine had a minimal effect at 50 pg/ml. Dolastatin 10, auristatin PE and vincristine-treated cultures, at 50 pg/ml, exhibited 11, 1.7; 45, 11.8%; and 39, 25% mitosis and apoptosis, respectively. In the WSU-DLCL$_2$ SCID mouse xenograft model, the efficacy of these agents alone or in combination with bryostatin 1 was evaluated. Tumor growth inhibition (T/C), tumor growth delay (T–C) and $\log_{10}$ kill for dolastatin 10, auristatin PE, vincristine and bryostatin 1 were 30%, 14 days and 1.4; 0.0%, 55 days and 5.5; 29.6%, 16 days and 1.6; and 39%, 7 days and 0.7, respectively. When given in combination, two out of five mice treated with auristatin PE + bryostatin 1 were free of tumors for 150 days and were considered cured. Dolastatin 10 + bryostatin 1 and vincristine + bryostatin 1 combinations were highly active but no cure was observed. We conclude that: (i) auristatin PE is more effective in this model than dolastatin 10, vincristine or bryostatin 1, (ii) auristatin PE can be administered at a concentration 10 times greater than dolastatin 10, and (iii) there is a synergistic effect between these agents and bryostatin 1, which is more apparent in the bryostatin 1 + auristatin PE combination. The use of these agents should be further explored clinically in the treatment of lymphoma. [© 1998 Rapid Science Ltd.]

Key words: Auristatin PE, B cell, bryostatin 1, dolastatin 10, SCID mice, vincristine, Xenografts.

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Introduction

The diffuse large cell non-Hodgkin's lymphoma (DLCL) is classified as an intermediate grade lymphoma.[1] Statistics show that when using standard chemotherapy with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) only 30–40% of patients achieve long-term survival.[2] In the 1980s, several newer regimens were developed combining a variety of standard chemotherapy agents in an attempt to improve the cure rate. Unfortunately, randomized studies published in the 1990s showed that such newer regimens are more toxic, more expensive, but no more effective than the original CHOP.[3,4] One approach to maximize the antitumor effect of certain regimens and possibly improve cure rates is to integrate new agents that have unique modes of action.

The NCI natural products program has identified a number of novel marine animal products with significant anti-lymphoid activity. The bryostatins represent one such group. Bryostatin 1, isolated from the marine bryozoan *Bugula neritina*,[5] exhibited both *in vitro* and *in vivo* antitumor activity in a number of model systems.[5,6] In addition, bryostatin 1 has biological effects on both T and B lymphocytes as well as on the hematopoietic system.[6] This agent has undergone preclinical evaluation against a variety of human lymphoid tumors, and was found to have antitumor, immune modulating and differentiating effects on a number of B cell tumors, including acute lymphoblastic leukemia,[7] chronic lymphocytic leukemia,[8] non-Hodgkin's lymphoma[9] and Waldenstrom's macroglobulinemia.[10] Dolastatin 10 was isolated from the sea hare *Dolabella auricularia*.[11] It is a linear tetrapeptide linked to a complex primary amine[12] and interacts with tubulin to inhibit microtubule polymer-

ization.[13] Auristatin PE is a structural analog of dolastatin 10 with the dolaphenine unit substituted with phenethylamide.[14]

In this study, we investigated the antitumor effect of these agents, alone and in combination with bryostatin 1, in a SCID mouse xenograft model bearing the WSU-$DLCL_2$ cell line.

Materials and methods

Cell culture, chemicals and marine animal products

The human diffuse large cell lymphoma cell line (WSU-$DLCL_2$) was established recently in our laboratory at Wayne State University School of Medicine.[15] The cell line was maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. Cells were then incubated in a humidified 5% CO_2 atmosphere at 37 C. No growth factors, mitogens or Epstein–Barr virus were added to the cell culture medium.

Auristatin PE is a new structural modification of dolastatin 10 which was isolated from the sea hare *D. auricularia*.[12] Auristatin PE, dolastatin 10 and bryostatin 1 were dissolved in DMSO at 10^{-3} mg/ml for *in vitro* studies, and were further diluted with PBS and added to cell culture at final concentrations of 10, 50, 100, 500 and 1000 pg/ml. Vincristine was obtained from Sigma (St Louis, MO) and was diluted in RPMI 1640 media and added to the cultures. For *in vivo* studies, all agents were used at their maximum tolerated doses (MTD) as previously determined in our laboratory, i.e. auristatin PE at 2.0 mg/kg/injection, dolastatin 10 at 0.2 mg/kg/injection, bryostatin 1 at 75 μg/kg/injection and vincristine at 0.5 mg/kg/injection.

Cell growth

WSU-$DLCL_2$ cells were plated in 24-well culture cluster (Costar, Cambridge, MA) in triplicate. The plating density was 2×10^5 viable cells/ml per well. WSU-$DLCL_2$ cells were treated with auristatin PE, dolastatin 10 or vincristine at concentrations of 10, 50, 100, 500 or 1000 pg/ml. Plates were incubated at 37 C, 5% CO_2, in a humidified incubator. Cell count and viability were determined in control-, dolastatin 10-, auristatin PE-, and vincristine-treated cultures on days 1–4 using 0.4% Trypan blue stain (Gibco, New York, NY) and a hemacytometer.

Mitosis and apoptosis

For light microscopic examination, WSU-$DLCL_2$ cells were seeded in 24-well culture plates at a concentration of 2×10^5 viable cells/ml as previously described.[15,16] Briefly, untreated (control) and treated cells with either dolastatin 10, auristatin PE or vincristine were set in four replications. Aliquots from all cultures were cytocentrifuged using a Cytospin 2 centrifuge (Shandon Southern Instruments, Sewickley, PA). Cell smears were air-dried, stained with tetrachrome at full concentration for 5 min and then at 50% dilution in dH_2O for another 5 min. Slides were analyzed under light microscopy (Nikon, Garden City, NY). Five different high power fields were counted for viable, mitotic, apoptotic and dead cells. At least 50 cells per field were evaluated from each treatment. Features of apoptosis looked for included nuclear chromatin condensation, formation of membrane blebs and apoptotic bodies. Features of cell death (necrosis) included cell swelling, nuclear expansion and gross cytolysis.

WSU-$DLCL_2$ xenografts

Four-week-old female ICR-SCID mice were obtained from Taconic Laboratory (Germantown, NY). The mice were adapted and WSU-$DLCL_2$ xenografts were developed as described previously.[10] Each mouse received 10^7 WSU-$DLCL_2$ cells (in serum-free RPMI-1640) s.c. in each flank area. When s.c. tumors developed to approximately 1500 mg, mice were sacrificed, and tumors dissected and mechanically dissociated into single-cell suspension. Mononuclear cells were separated by Ficoll-Hypaque density centrifugation and washed twice with RPMI-1640 medium. These cells were subjected to phenotypic analysis for comparison with the established tumor line to insure the human origin and its stability. After formation of s.c. tumors, serial propagation was accomplished by excising the tumors, trimming extraneous material and cutting the tumors into fragments of 20–30 mg that were transplanted s.c. via a 12 gauge trocar into the flanks of a new group of mice.

Efficacy trial design

For the subsequent drug-efficacy trials, small fragments of the WSU-$DLCL_2$ xenograft were implanted s.c. bilaterally into naive, similarly conditioned mice, using a 12 gauge trocar. Mice were checked three times a week for tumor development. Once transplanted

WSU-DLCL$_2$ fragments developed into palpable tumors (100–200 mg), groups of five animals were removed randomly and assigned to different treatment groups. Each experimental group received i.v. injections of dolastatin 10, auristatin PE or vincristine via a tail vein every second day for a total of three injections. For bryostatin 1, the injections were given i.p. daily for a total of 5 days. The combination groups were treated concurrently with dolastatin 10 i.v. and bryostatin 1 i.p., auristatin PE i.v. and bryostatin 1 i.p., or vincristine i.v. and bryostatin i.p., every second day for a total of three injections. Mice were observed for measurement of s.c. tumors, changes in weight and side effects of the drugs. The s.c. tumors were measured three times weekly. Animals were euthanized when their total tumor burden reached 2000 mg to avoid discomfort. All studies involving mice were performed under Institutional Review Board-approved protocol. Tumor weights in SCID mice were plotted against time on a semilog sheet. The growth pattern was close to an S-shape. Tumor doubling (T_d) is the time (in days) required in order for the tumor to double its weight during the exponential growth phase.

Assessment of tumor response

The end points for assessing anti-tumor activity were according to standard procedures used in our laboratories[10,17] and as follows. Tumor weight (mg) = $(A \times B^2)/2$, where A and B are the tumor length and width (in mm), respectively; Tumor growth inhibition (T/C) is calculated by using the median tumor weight in the treated group (T) when the median tumor weight in the control group (C) reached approximately 900 mg. Tumor growth delay (T–C) is the difference between the median time (in days) required for the treatment group tumors (T) to reach 900 mg and the median time (in days) for the control group tumors (C) to reach the same weight. Tumor cell kill net ($\log_{10}$) = (T–C) − (duration of treatment in days)/(3.32)(T_d). Tumor cell kill total (gross) ($\log_{10}$) = (T–C)/(3.32)(T_d). In this study the antitumor activity is considered highly active (++++) when the $\log_{10}$ kill (net) is more than 2.0 and (gross) is more than 2.8. Activity rating scores of (++++) or (+++) are needed for translation to clinical activity and equate with complete and partial tumor regression, respectively. A score of either (+) or (++) is not considered active by usual clinical criteria.[17]

Challenging the cured mice

After 150 days, cured mice were challenged by re-implanting tumor fragments (about 30 mg) of WSU-DLCL$_2$ bi-laterally into their flanks using a 12 gauge trocar. Mice were checked three times a week for tumor development.

Immunophenotyping of WSU-DLCL$_2$

The cell line and the s.c. xenograft cells underwent immunophenotyping by flow cytometric analysis (FCM) on a FACScan (Becton-Dickinson Immunodiagnostics, San Jose, CA) as previously described.[9] Monoclonal antibodies used in this study were as follows: anti-CD10, CD19, CD20, CD22, HLA-DR, Leu10 for B lineage and CD2, CD5, CD8 for T lineage. All monoclonal antibodies were obtained from Becton-Dickinson.

Results

Effects of dolastatin 10, auristatin PE and vincristine on cell growth *in vitro*

WSU-DLCL$_2$ cells were exposed to varying concentrations of dolastatin 10, auristatin PE and vincristine. The drug effect was observed over 4 days. Dolastatin 10, auristatin PE and vincristine showed complete inhibition of cell growth at different concentrations. Dolastatin 10 showed its maximum effect at 500 pg/ml (Figure 1A), auristatin PE at 50 pg/ml (Figure 1B) and vincristine at 100 pg/ml (Figure 1C). The results highlight the superiority of auristatin PE over dolastatin 10 and to some degree over vincristine.

Mitosis and apoptosis

Auristatin PE at 50 pg/ml exhibited 45 and 11.8% mitosis and apoptosis, respectively, at 24 h of treatment (Figure 2A). Dolastatin 10 at the same concentration arrested only 11% in mitosis and 1.7% in apoptosis (Figure 2B), while vincristine exhibited 39% in mitosis and 25% in apoptosis (Figure 2C). At higher doses (500 and 1000 pg/ml), all three agents arrested cells in mitosis and induced apoptosis at comparable levels.

In vivo efficacy of dolastatin 10, auristatin PE and vincristine

When five SCID mice were injected s.c. with equal numbers of WSU-DLCL$_2$ cells (10^7) in each flank, three

developed tumors (60% take rate). The tumors in each flank were palpable by 21 days. Tumor volume doubling time (T_d) in SCID mice was 3.0 days. When WSU-DLCL$_2$ was passaged in SCID mice, the take rate was 100%. Drug efficacy trials were conducted on animals with palpable tumors (100–200 mg).

Table 1 shows the antitumor activity of dolastatin 10, auristatin PE, bryostatin 1 or vincristine given at

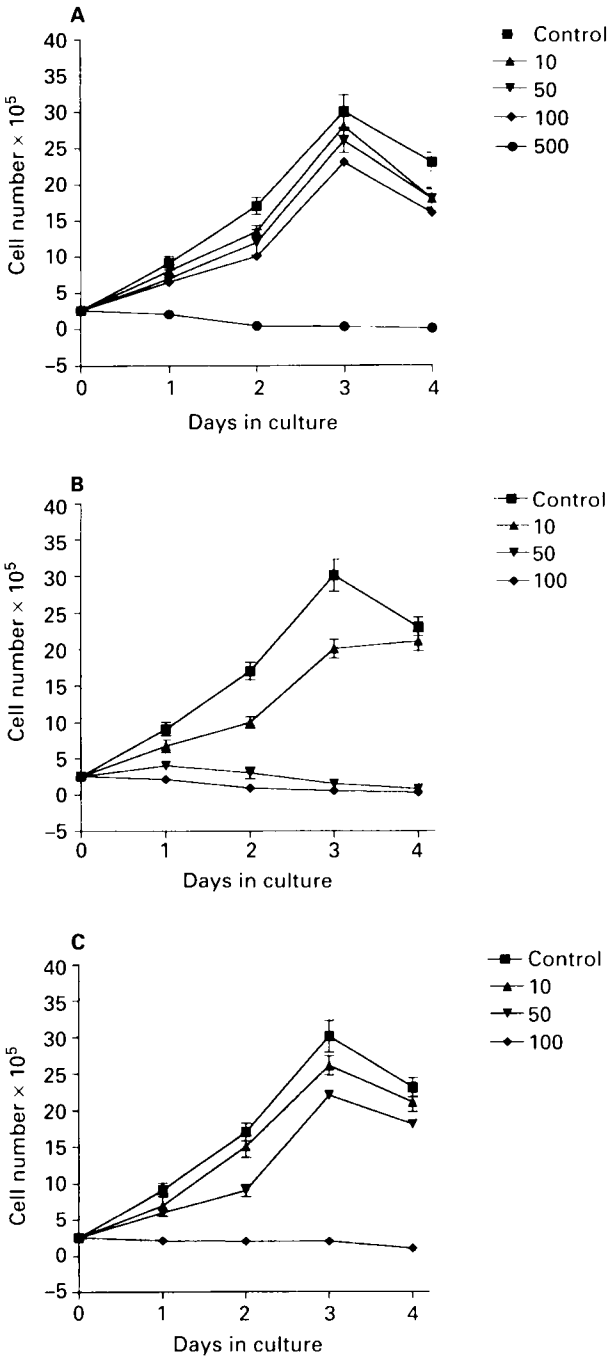

Figure 1. Dose–response curves of (A) dolastatin 10 at concentrations of 10, 50, 100 and 500 pg/ml, (B) auristatin PE at 10, 50 and 100 pg/ml, and (C) vincristine at 10, 50 and 100 pg/ml. WSU-DLCL$_2$ cells were seeded at 2×10^5/ml at time 0. The culture medium was not changed throughout the duration of the experiment. The numbers are expressed as mean ± SD.

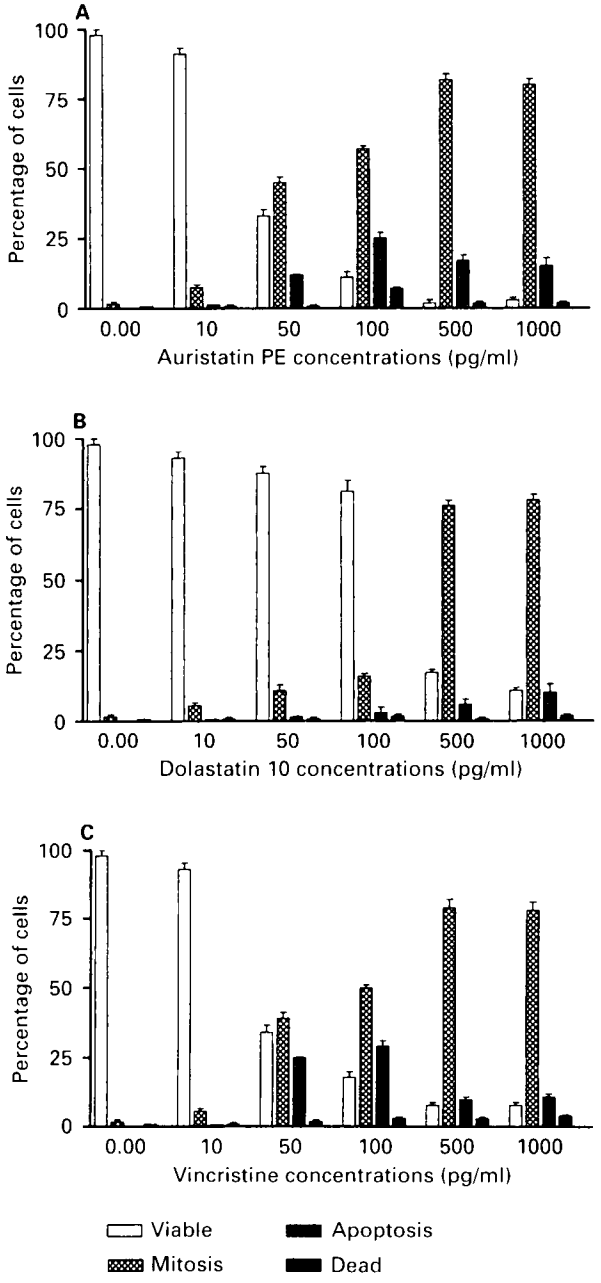

Figure 2. Bar-chart showing the percentage of viable, mitotic, apoptotic and dead cells in control and auristatin PE treated WSU-DLCL$_2$ cells. WSU-DLCL$_2$ cells were assessed morphologically. Features of apoptosis looked for include cell shrinkage, nuclear condensation and apoptotic bodies. WSU-DLCL$_2$ cells were treated with auristatin PE (A), dolastatin 10 (B) and vincristine (C) at concentrations of 10, 50, 100, 500 and 1000 pg/ml.

MTDs, against WSU-DLCL$_2$-bearing SCID mice. When tumor responses are determined by the T/C value, all agents are considered active against this type of human tumor (a T/C value of 42% or higher is indicative of anti-tumor activity). However, if $\log_{10}$ kill values (net and gross) are added as criterion, only auristatin PE had a clinically meaningful activity, while the others were not considered active by usual clinical criteria. It should be noted that an activity rating score of (+++, active) or (++++, highly active) is needed to effect partial or complete tumor regressions. Thus a score of (+) or (++) is not considered active by usual clinical criteria.[17] Noteworthy, dolastatin 10, auristatin PE and bryostatin 1 at 0.4 mg/kg, 4.0 mg/kg and 100 μg/kg, respectively, are toxic (data not shown). The MTD of dolastatin 10 in this model was equal to 0.2 mg/kg/injection, while that of auristatin PE was 2.0 mg/kg/injection.

Antitumor activity of combinations of dolastatin 10, vincristine or auristatin PE with bryostatin 1

In the combination treatment experiments, auristatin PE at a concentration of 2.0 mg/kg + bryostatin 1 at 75 μg/kg killed all treated mice and was considered toxic. Reducing auristatin's dose to 1.5 mg/kg and combining it with bryostatin 1 (75 μg/kg) was well tolerated, and resulted in better antitumor activity compared with combinations of either dolastatin 10 + bryostatin 1 or vincristine + bryostatin 1. The auristatin PE + bryostatin 1 combination achieved an excellent activity score (++++) and two out of five treated mice were free of tumors for 150 days, and were considered cured (Table 2). On the other hand, the dolastatin 10 + bryostatin 1 combination also received an activity score of (++++) but no cures were observed.

Challenged mice

Both challenged mice developed bi-lateral tumors after 3 weeks from their implantations. Mice were euthanized when their total tumor burden reached 2000 mg.

In vitro and *in vivo* immunophenotyping of WSU-DLCL$_2$

Comparison of the immunophenotypes of the WSU-DLCL$_2$ line *in vitro* and in the SCID mice as a xenograft showed the same characteristics as the original lymphoma. Cells expressed B cell markers with a monoclonal IgG λ. There was no expression of T lymphoid markers. *In vitro* and *in vivo* cytogenetic analysis revealed the same chromosomal abnormalities (data not shown).

Discussion

In this study we showed that auristatin PE given to SCID mice bearing human diffuse large cell lymphoma tumors leads to superior antitumor activity compared with those of dolastatin 10, bryostatin 1 or vincristine. Moreover, administering auristatin PE concurrently with bryostatin 1 resulted in significantly higher antitumor activity compared with all other combination treatments and resulted in a cure of 40% of the WSU-DLCL$_2$-bearing SCID mice.

Two decades ago, using a combination of chemotherapy agents, 30–40% of advanced stage DLCL was found to be curable.[18] Although numerous attempts were made to improve the cure rate, no further progress has thus far been made.[3,19] The WSU-DLCL$_2$ line was established from a relapsed DLCL patient that was clinically resistant to therapy.[15] The

Table 1. Antitumor activity of dolastatin 10, auristatin PE, byrostatin 1 and vincristine in WSU-DLCL$_2$-bearing SCID mice

Agent	Dose[a]	Route	No. of animals	T/C (%)	T–C (days)	Log_{10} kill		Activity score[b]	Cure
						Net	Gross		
Diluent (control)	0.0	i.v.	5	100	0.0	0.0	0.0	–	0/5
Dolastatin 10	0.2 mg/kg	i.v.	5	30	14	1.1	1.4	++	0/5
Auristatin PE	2.0 mg/kg	i.v.	5	0	55	5.2	5.5	++++	0/5
Bryostatin 1	75 μg/kg	i.p.	5	39	7	0.4	0.7	+	0/5
Vincristine	0.5 mg/kg	i.v.	5	29.6	16	1.3	1.6	++	0/5

[a]Doses are determined based on previous experiments with these drugs.
[b]Rating score of (+++, active) or (++++, highly active) is needed to effect partial or complete tumor regressions; (+) or (++) is not considered active by usual clinical criteria.[17]

Table 2. Activity of combinations of dolastatin 10, vincristine or auristatin PE with byrostatin 1 in WSU-DLCL$_2$-bearing SCID mice

Agent	Dose[a]	Route	No. of animals	T/C (%)	T–C (days)	Log_{10} kill		Activity score[b]	Cure
						Net	Gross		
Diluent (Control)	0.0	i.v.	5	100	0.0	0.0	0.0	–	0/5
Dolastatin 10 + Bryostatin 1	0.2 mg/kg 75 μg/kg	i.v. i.p.	5	5	39	3.6	3.9	++++	0/5
Vincristine + Bryostatin 1	0.5 mg/kg 75 μg/kg	i.v. i.p.	5	6	38	3.5	3.8	++++	0/5
Auristatin PE + Bryostatin 1	1.5 mg/kg 75 μg/kg	i.v. i.p.	5	0	100	9.7	10.1	++++	2/5

[a]Rating score of (+++, active) or (++++, highly active) is needed to effect partial or complete tumor regressions; (+) or (++) is not considered active by usual clinical criteria.[17]

disease relapsed after high-dose chemotherapy and radiation, followed by bone marrow transplantation. The ability of these cells to grow as xenografts in SCID mice makes it a useful preclinical model to search for effective drugs against resistant lymphoma.

Among the large number of marine invertebrate-derived anticancer agents, dolastatin 10 was selected for clinical development. Pettit *et al.*[14] have explored structural modifications of dolastatin 10, and have successfully developed a useful method to improve the synthesis of dolastatin 10's right terminal unit dolaphenine and to completely eliminate the need for the thiazole derivative. Auristatin PE is closely related structurally to dolastatin 10 where the dolaphenine unit is replaced by a phenethylamide group.[14] In this *in vitro* study the WSU-DLCL$_2$ cell line was exposed to varying concentrations of dolastatin 10, auristatin PE or vincristine (Figure 1A–C). The results highlighted the obvious superiority of auristatin PE over dolastatin 10 and to some degree over vincristine. Auristatin PE at 50 pg/ml arrested more cells in mitosis compared with dolastatin 10 and vincristine at the same concentration (Figure 2A–C). Apparently, for dolastatin 10 to exert its effect, it must reach a threshold concentration of 100 pg/ml. However, at higher doses (500 and 1000 pg/ml), dolastatin 10 arrested more cells in mitosis compared with auristatin PE (Figure 2A and B). Previously, our group[15] reported that in order for dolastatin 10 to cause a significant arrest in mitosis and to initiate apoptosis in WSU-DLCL$_2$, a concentration of 1000 pg/ml is needed. Thus the replacement of the dolaphenine unit of the dolastatin 10 with a phenethylamide had an impact on the interaction with tubulin and induction of apoptosis in WSU-DLCL$_2$ cells.

Recently a Japanese group evaluated the antitumor activity of TZT-1027 (auristatin PE) against a variety of tumors in mice.[20] They found intermittent injections of auristatin PE were more effective than single or repeated injections in mice bearing p388 leukemia and B16 melanoma. Interestingly, they used the same dose as we did in their experiments (2.0 mg/kg/injection) and they found auristatin PE to be more effective when administered as an i.v. injection rather than i.p. Moreover, the antitumor activity against colon 26 adenocarcinoma, B16 melanoma and M5076 sarcoma was superior or comparable to that of dolastatin 10, vincristine, cisplatin, 5-fluorouracil and E7010.[20] Also its moderate activity in vincristine-resistant P-388 leukemia suggests that it might not be cross-resistant with vincristine.

The concept of arresting cells in mitosis and inducing apoptosis is clinically plausible. Dolastatin 10 is more effective in inhibiting tubulin polymerization at lower concentrations as compared with vincristine[21] and has additional mechanisms of action on tubulin which are quite different from those caused by vinca alkaloids.[11,22] We sought to study the *in vivo* antitumor activity of dolastatin 10 and compare it with its new structurally modified derivative, i.e. auristatin PE. Dolastatin 10, auristatin PE and bryostatin 1 were tested against the WSU-DLCL$_2$ xenograft model. At 75 μg/kg, bryostatin 1 showed marginal activity against WSU-DLCL$_2$ and was not considered active (activity score = +) by usual clinical criteria. Although dolastatin 10 and vincristine showed higher growth inhibition, growth delay and log_{10} kill compared with bryostatin 1, they were not considered active against WSU-DLCL$_2$ (activity score = ++). Auristatin PE, however, showed the highest activity and was considered highly active aginst WSU-DLCL$_2$ (activity score= ++++). The replacement of the dolaphenine unit of dolastatin 10 with a phenethylamide enabled us to administer auristatin PE at a concentration 10 times higher than that of dolastatin 10. Similar high levels of dolastatin 10 proved to be toxic in our hands.

Previously, we reported that replacement of the dolaphenine unit of dolastatin 10 with a phenethylamide was found to afford exceptionally high activity against several human cancer cell lines and murine P-388 lymphocytic leukemia.[13]

Because of promising preclinical data on the combined therapy of bryostatin 1 and vincristine against the Waldenstrom's macroglobulinemia xenograft model,[9] and since vincristine and dolastatin 10 interact with tubulin, we evaluated the antitumor activity of the dolastatin 10 + bryostatin 1 combination and compared it with the auristatin PE + bryostatin 1 combination in SCID mice bearing WSU-DLCL$_2$ tumors. While the response criteria we adopted in our animal studies are standard, they do not necessarily directly translate to partial (PR) or complete (CR) response criteria in humans. Our drug discovery group, therefore, has adopted a 'scoring' system of drug activity which correlates better with response criteria in humans.[17] According to this system, the response seen in animals to these agents used alone will not translate to PR or CR. Only the combination use of auristatin PE + bryostatin 1 led to what is considered clinically meaningful activity. In the combination treatment experiments, auristatin PE + bryostatin 1, when given at the MTD in SCID mice (1.5 mg/kg + 75 μg/kg), were well tolerated at both drug levels, and provided significant antitumor activity as measured by tumor growth inhibition, tumor growth delay and tumor $\log_{10}$ kill (Table 2). The auristatin PE + bryostatin 1 combination achieved an activity score of (++++) and two out of five treated mice were free of tumors for 150 days and were considered cured. These cured mice were challenged (re-implanted) with WSU-DLCL$_2$ tumors. All challenged mice developed bi-lateral tumors after 3 weeks from their implantations and were euthanized when their total tumor burden reached 2000 mg. This indicates that mice were free from tumors due solely to drug efficacy and not to mice rejection. On the other hand, the dolastatin 10 + bryostatin 1 and vincristine + bryostatin 1 combinations reached activity scores of (++++), but no cures were observed in either of these combinations.

Collectively, the results obtained from this work highlighted the obvious superiority of auristatin PE over dolastatin 10 and vincristine. Auristatin PE can be administered to the mice at a concentration 10 times greater than that of dolastatin 10, and there is a synergistic effect between these agents and bryostatin 1, which is more apparent in the auristatin PE + bryostatin 1 combination. The results from this study should prove useful in guiding the clinical application of these novel agents in the treatment of lymphomas.

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