Successful Treatment of Human Chronic Lymphocytic Leukemia Xenografts with Combination Biological Agents Auristatin PE and Bryostatin 1

Ramzi M. Mohammad,2 Mary L. Varterasian, Victor P. Almatchy, Ghadeer N. Hannoudi, George R. Pettit, and Ayad Al-Katib

Division of Hematology and Oncology, Department of Internal Medicine, Wayne State University School of Medicine, Karmanos Cancer Institute, Detroit, Michigan 48201 [R. M. M., M. L. V., V. P. A., G. N. H., A. A-K.], and Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287 [G. R. P.]

ABSTRACT

We tested the activity of dolastatin 10 (a natural product derived from the shell-less marine mollusk, Dolabella auricularia, a sea hare) and its structural modification, auristatin PE, alone and in combination with bryostatin 1 (a protein kinase C activator derived from the marine bryozoan Bugula neritina) on a human B-cell chronic lymphocytic leukemia cell line (WSU-CLL) and in a severe combined immune deficient (SCID) mouse xenograft model bearing this cell line. WSU-CLL cells were cultured in RPMI 1640 at a concentration of 2 x 10⁵/ml using a 24-well plate. Agents were added to triplicate wells, and cell count, viability, mitosis, and apoptosis were assessed after 24 h of incubation at 37°C. Results showed that dolastatin 10 had no apparent inhibition of cell growth at concentrations less than 500 pg/ml. Auristatin PE, on the other hand, showed significant growth inhibition at concentrations as low as 50 pg/ml. Auristatin PE-treated cultures, at this concentration, exhibited 27 and 4.5% mitosis and apoptosis, respectively. Dolastatin 10, at the same concentration, did not exert any effect and was comparable with that of control cultures. In the WSU-CLL-SCID mouse xenograft model, the efficacy of these agents alone and in combination with bryostatin 1 was evaluated. Tumor growth inhibition (T/C), tumor growth delay (T−C), and log₁₀ kill for dolastatin 10, auristatin PE, and bryostatin 1 were 14%, 25 days, and 1.98; 2%, 25 days, and 1.98; 19%, 13 days, and 1.03, respectively. Auristatin-PE produced cure in three of five mice, whereas dolastatin 10 showed activity but no cures. When given in combination, auristatin PE + bryostatin 1-treated animals were all free of tumors (five of five) for 150 days and were considered cured. Dolastatin 10 + bryostatin 1-treated animals produced cure in only two of five mice. We conclude that: (a) auristatin-PE is more effective in this model than dolastatin 10; (b) auristatin PE can be administered at a concentration 10 times greater than dolastatin 10; (c) 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 explored clinically in the treatment of CLL.

INTRODUCTION

B-cell CLL1 is the most common adult leukemia accounting for approximately 30% of all cases in Western countries (1). When the diagnosis is made in an asymptomatic patient, the course can be indolent for months and even years (2). When therapy is required, alkylating agents, steroids, and more recently the newer purine analogue, fludarabine, are used. Over time, patients become resistant to these agents, and treatment options at this point are limited. There is no curative therapy for CLL, and there has been little success in extending the overall duration of survival. The discovery of antilymphoid agents with novel mechanism of action and their subsequent incorporation into combination regimens should improve outcome in this disease.

As part of the National Cancer Institute natural products program, a number of novel agents with antilymphoid activity derived from marine products have been identified and are now entering clinical trials. The bryostatins represent one such group of novel agents. Bryostatin 1 was isolated from the marine bryozoan Bugula neritina in 1982 by Pettit et al. (3) and is one of a family of more than 20 compounds with a multiringed macrocyclic lactone structure. The wide range of activities of bryostatin, thought to be secondary to its ability to modulate the family of protein kinase C enzymes, include hematopoietic and immune stimulation (4) and induction of differentiation of both myeloid and lymphoid cell lines. The antitumor properties of bryostatin 1 have been demonstrated against a number of marine tumors (5), human myeloid and lymphoid leukemias (6–8), and human lymphoid xenografts (9). Dolastatin 10 was isolated from the shell-less marine mollusk Dolabella auricularia (sea hare) in 1984 and reported in 1987 (10). It is a linear tetrapeptide

1 The abbreviations used are: CLL, chronic lymphocytic leukemia; SCID, severe combined immune deficiency.
Auristatin PE, Dolastatin 10, and Bryostatin 1 in CLL

1338

**Table 1** Activity of dolastatin 10, auristatin PE, and bryostatin 1 in WSU-CLL SCID xenografts

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose* (mg/kg)</th>
<th>Route</th>
<th>No. of animals</th>
<th>T/C (%)</th>
<th>T–C (days)</th>
<th>Log_{10} kill (days)</th>
<th>Activity score</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent (control)</td>
<td>0.0</td>
<td>i.v.</td>
<td>5</td>
<td>100</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0/5</td>
</tr>
<tr>
<td>Dolastatin 10</td>
<td>0.1</td>
<td>i.v.</td>
<td>5</td>
<td>65</td>
<td>5</td>
<td>0.24</td>
<td>0.40</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>i.v.</td>
<td>5</td>
<td>14</td>
<td>25</td>
<td>1.80</td>
<td>1.98</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>i.v.</td>
<td>5</td>
<td>Died of toxicity</td>
<td></td>
<td></td>
<td></td>
<td>0/5</td>
</tr>
<tr>
<td>Auristatin PE</td>
<td>2.0</td>
<td>i.v.</td>
<td>5</td>
<td>2</td>
<td>25</td>
<td>1.80</td>
<td>1.98</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>i.v.</td>
<td>5</td>
<td>Died of toxicity</td>
<td></td>
<td></td>
<td></td>
<td>0/5</td>
</tr>
<tr>
<td>Bryostatin 1</td>
<td>75</td>
<td>i.p.</td>
<td>5</td>
<td>Died of toxicity</td>
<td></td>
<td></td>
<td></td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>i.p.</td>
<td>5</td>
<td>Died of toxicity</td>
<td></td>
<td></td>
<td></td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Doses are determined based on previous experiments with these drugs.

**Materials and Methods**

**WSU-CLL Cell Line.** The human chronic lymphocytic leukemia cell line (WSU-CLL) was recently established in our laboratory at Wayne State University School of Medicine (14) and maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. No growth factors, mitogens, or EBV were added to the cell culture medium.

**Tested Agents.** Auristatin PE is a new structural modification of dolastatin 10 that was isolated from the sea hare *Dolabella auricularia* (10). Auristatin PE and dolastatin 10 were dissolved in DMSO at 10 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. For the in vivo studies, auristatin PE was used at 2.0 and 4.0 mg/kg injection, whereas dolastatin 10 was used at 0.1, 0.2, and 0.4 mg/kg injection. Bryostatin 1, a macrocyclic lactone, was used at its maximum tolerated dose of 75 μg/kg injection.

**Cell Growth.** WSU-CLL cells were plated in 24-well culture cluster (Costar, Cambridge, MA) in triplicate. The plating density was 2 × 10^5 viable cells/ml-pel well. WSU-CLL cells were treated with auristatin PE or dolastatin 10 at concentrations 10, 50, 100, 500, and 1000 pg/ml. Plates were incubated at 37°C in CO2 in a humidified incubator. Cell counts and viability were determined in control-, dolastatin 10-, and auristatin PE-treated cultures on days 1–4 using the trypan blue dye exclusion method. activity score was noted for included nuclear chromatin condensation, formation of membrane blebs, and apoptotic bodies. Features of cell death were counted in viable, mitotic, apoptotic, and dead cells. At least 50 cells/fiber were evaluated from each treatment. Features of apoptosis included nuclear condensation, formation of membrane blebs, and apoptotic bodies. Features of cell death included cell swelling, nuclear expansion, and gross cytolysis.

**WSU-CLL Xenografts.** Four-week-old female ICR-SCID mice were obtained from Taconic Laboratory (Germantown, NY). The mice became adapted, and WSU-CLL xenografts were developed as described previously (14). Each mouse received 10^7 WSU-CLL cells (in serum-free RPMI 1640) s.c. in each flank area. When s.c. tumors developed to ~1500 mg, mice were sacrificed, and tumors were dissected and mechanically dissociated into single-cell suspension. Mononuclear cells were separated by Ficoll-Hypaque density centrifugation and washed twice with RPMI 1640. These cells were subjected to a second drug-efficacy trial designed to compare the established tumor line to the established 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, which were transplanted s.c. via a 12-gauge trocar into the flanks of the next group of mice.

**Efficacy Trial Design.** For the subsequent drug-efficacy trials, small fragments of the WSU-CLL xenograft were implanted s.c. bilaterally into naive similarly conditioned mice, using a 12-gauge trocar. Mice were checked three times every week for tumor development. Once transplanted, WSU-CLL fragments developed into palpable tumors (100–200 mg), groups of five animals were removed randomly and assigned to different treatments. Each experimental group received an i.v. injection of dolastatin 10 or auristatin PE via a tail vein every second day for a period 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 i.v. and bryostatin 1 i.p. or auristatin PE i.v. and bryostatin 1 i.p., every second day for a total of three injections, as shown in Table 1. Mice were observed for measurement of s.c. tumors, changes in weight, and side effects of the drugs three times weekly. All studies involving mice were performed under Institutional Review Board-approved protocol. Tumor weights in SCID mice were plotted against time on semilog sheets. The growth pattern was close to an S-shape. Tumor doubling (Td) is the time (in days) required for the tumor to double its weight during the exponential growth phase.

Assessment of Tumor Response. The end points for assessing antitumor activity were according to standard procedures used in our laboratories (14, 17) and as follows: tumor weight (mg) = (A x 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 ~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)(Td); and tumor cell kill total (gross) (log_{10} = (T - C)/(3.32)(Td)). In this study, the antitumor activity is considered highly active (++++) when the log_{10} kill (net) is >2.0 and (gross) is >2.8. Activity rating score of ++++ or +++ is 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). To avoid discomfort and stress, animals were euthanized when their total tumor burden reached 2000 mg (~10% of body weight). Percentage of survival curves were plotted when tumors in mice reached 2000 mg. Student’s unpaired, two-tailed t test with a 95% confidence interval was used to estimate statistical significance between control (placebo), dolastatin 10 + bryostatin 1, and auristatin PE + bryostatin 1 groups.

Challenging the Cured Mice. After 150 days, all cured mice [auristatin PE (three mice), dolastatin 10 + bryostatin 1 (two mice), and auristatin PE + bryostatin 1 (five mice) combinations] were challenged by re-implanting tumor fragments (~30 mg) of WSU-CLL bilaterally into their flanks using a 12-gauge trocar. Mice were checked three times a week for tumor development.

RESULTS

Effects of Dolastatin 10 and Auristatin PE on WSU-CLL Growth. The WSU-CLL cell line was exposed to varying concentrations of dolastatin 10 and auristatin PE. The growth-inhibitory effect was observed over 4 days. Dolastatin 10 showed no apparent inhibition of cell growth at concentrations <500 pg/ml (Fig. 1A). Auristatin PE, on the other hand, showed significant growth inhibition effect, even at concentrations as low as 50 pg/ml (Fig. 1B). Dolastatin 10 showed complete growth inhibition of WSU-CLL cells at 1000 pg/ml, whereas auristatin PE showed the same degree of growth inhibition at 100 pg/ml. The results highlighted the obvious superiority of auristatin PE over dolastatin 10.

Mitosis and Apoptosis. Auristatin PE at 50 pg/ml exhibited 27 and 4.5%, mitosis and apoptosis, respectively, at 24 h of treatment (Fig. 2A). Dolastatin 10, at the same concentration, did not exert any effect and was comparable with that of control cultures (Fig. 2B). At a higher dose (100 pg/ml), both agents arrested cells in mitosis and induced apoptosis, which reached 35 and 12% for auristatin PE and 21 and 4% for dolastatin 10.
respectively. Dolastatin 10 at 500 and 1000 pg/ml arrested more cells in mitosis compared with auristatin PE at the same concentrations. The effects of auristatin PE + bryostatin 1 and dolastatin 10 + bryostatin 1 combinations on apoptosis and cell death were compared with untreated WSU-CLL cells, treated with auristatin PE alone or dolastatin 10 alone. Results show significant statistical differences in apoptosis and cell death between the auristatin PE + bryostatin 1 combination and auristatin PE alone (8 and 9% versus 4.4 and 5.0%, respectively), whereas only cell death was significant in the dolastatin 10 + bryostatin 1 combination compared with dolastatin 10 alone (9.0% versus 5.0%; Fig. 2B).

In Vivo Drug Efficacy. When five SCID mice were injected s.c. with equal numbers of WSU-CLL cells (10^7) in each flank, four developed tumors (80% take rate). The tumors were palpable by the end of the third week. Tumor volume doubling time (Td) in SCID mice was 3.8 days. When WSU-CLL 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 activity of dolastatin 10, auristatin PE, or bryostatin 1 given at different doses to WSU-CLL-bearing SCID mice. When tumor responses are determined by T/C, dolastatin 10 at 0.2 mg/kg/injection, auristatin PE at 2.0 mg/kg/
injection, or bryostatin 1 at 75 μg/kg/injection are considered active against this type of human tumor. (T/C is an indicator of antitumor effectiveness, where a value of 42% or less is considered significant antitumor activity.) However, if log_{10} kill values (net and gross) are added as a criterion, dolastatin 10 and auristatin PE had a clinically meaningful activity, whereas bryostatin 1 is not considered active by the usual clinical criteria (Table 1). Here it should be noted that an activity rating score of + or + + is not considered active by the usual clinical criteria (17). However, if log_{10} kill values (net and gross) are added as a criterion, dolastatin 10 and auristatin PE had a clinically meaningful activity, whereas bryostatin 1 is not considered active by the usual clinical criteria (Table 1). Here it should be noted that an activity rating score of + or + + is not considered active by the usual clinical criteria (17). However, if log_{10} kill values (net and gross) are added as a criterion, dolastatin 10 and auristatin PE had a clinically meaningful activity, whereas bryostatin 1 is not considered active by the usual clinical criteria (17).

**Challenged Mice.** All challenged mice (total, 10) developed bilateral tumors after 3 weeks from their implantations. Mice were euthanized when their total tumor burden reached 2000 mg.

**DISCUSSION**

In this report, we show that auristatin PE given to SCID mice bearing human CLL tumors leads to improved antitumor activity over that of dolastatin 10. Moreover, administering auristatin PE concurrently with bryostatin 1 resulted in significantly higher antitumor activity compared with all other treatments and resulted in cures of all mice.

Among the rapidly increasing number of marine invertebrate-derived antineoplastic agents, dolastatin 10 has been selected for further clinical development. Auristatin PE is an improved analogue that is closely related structurally and in which the dolaphenine unit is replaced by a phenethylamide group (13). In this in vitro study, using a human CLL cell line, WSU-CLL (14), our results showed that dolastatin 10 had no apparent inhibition of cell growth at concentrations <500 pg/ml (Fig. 1A). Auristatin PE, on the other hand, showed significant growth inhibition effect, even at concentrations as low as 50 pg/ml (Fig. 1B). These results highlighted the obvious superiority of auristatin PE over dolastatin 10. Although the sequential addition of bryostatin 1 followed by auristatin PE inhibited the growth more than the concurrent or the reverse sequential addition, statistically it was not significant. However, the concurrent addition of bryostatin 1 + dolastatin 10 inhibited cell growth more than the sequential addition of the two drugs (data not shown). Our results also revealed that auristatin PE exhibited more pronounced mitotic arrest and apoptosis compared with dolastatin 10. Auristatin PE at a very low concentration (50 pg/ml) exhibited 27 and 4.5%, mitosis and apoptosis, respectively, at 24 h of treatment of WSU-CLL cells (Fig. 2A), whereas dolastatin 10 at the same concentration did not exert any effect and was comparable with that of control cultures (Fig. 2B). 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 (Fig. 2). Previously, our group (15) reported that for dolastatin 10 to cause a significant arrest in mitosis and to initiate the apoptosis process in diffuse large cell lymphoma, a concentration of 1000 pg/ml is
needed. Clearly, 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 of WSU-CLL cells. The effect of auristatin PE + bryostatin 1 and dolastatin 10 + bryostatin 1 combinations on apoptosis and cell death were studied. Our results show significant statistical differences in apoptosis and cell death between the auristatin PE + bryostatin 1 combination and auristatin PE alone (8 and 9.0% versus 4.4 and 5.0%, respectively), whereas only cell death was significant in the dolastatin 10 + bryostatin 1 combination, compared with dolastatin 10 alone (9.0% versus 5.0%; Fig. 2). Dysregulation of apoptosis appears to be involved in the pathogenesis of CLL (18, 19). Thus, it has been proposed that prolonged life span, due to dysregulation of the apoptosis process, is a critical factor contributing to the delayed senescence of CLL (20). Cladribine, pentostatin, and fludarabine, agents that induce apoptosis, are presently being used in the treatment of CLL patients (21). Despite their therapy, the relapse rate underscores the need to search for new agents with novel therapeutic effects.

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 (22) and has additional mechanisms of action on tubulin that are quite different from those caused by Vinca alkaloids (12, 23). We sought to study the in vivo antitumor activity of dolastatin 10 and compare it with its new structural modification, auristatin PE. WSU-CLL is the first B-CLL line established without EBV infection or growth factor stimulation. The line grows in liquid culture and forms s.c. tumors in SCID mice bearing WSU-CLL (14). Dolastatin 10, auristatin PE, or bryostatin 1 were tested against this xenograft model, and the maximum tolerated doses were 0.2 mg/kg, 2.0 mg/kg, and 75 μg/kg, respectively (Table 1). At 75 μg/kg, bryostatin 1 showed marginal activity on WSU-CLL and was not considered active (activity score, +) by usual clinical criteria. On the other hand, dolastatin 10 and auristatin PE at 0.2 and 2.0 mg/kg/ injection showed significant growth inhibition, growth delay, and log10 kill and were considered active against WSU-CLL (activity score, ++ +). The replacement of the dolaphenine amino acid of the dolastatin 10 with a phenethylamide enabled us to administer auristatin PE at a concentration 10 times higher than that of dolastatin 10. This resulted in producing cure in three of five mice when treated with auristatin PE. 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 the promising preclinical data of the combined therapy of bryostatin 1 and vincristine against the Waldenstrom macroglobulinemia xenograft model (9), and because vincristine and dolastatin 10 interact with tubulin, we attempted to study the antitumor activity of a dolastatin 10 + bryostatin 1 combination and compare that with auristatin PE + bryostatin 1 combination in SCID mice bearing WSU-CLL tumors. Although the response criteria we adopted in our animal studies are standard, they do not necessarily directly translate to partial or complete response criteria in humans. Our drug discovery group, therefore, has adopted a “scoring” system of drug activity that correlates better with response criteria in humans (17). According to this system, the response seen in animals by these agents used alone will not translate to partial response or complete response. Only the combined use of dolastatin 10 + bryostatin 1 or auristatin PE + bryostatin 1 led to what is considered a significant activity in the mouse that is clinically meaningful. In the combination treatment experiments, auristatin PE, when given at the maximum tolerated dose by the SCID mice (2.0 mg/kg) + bryostatin 1 at 75 μg/kg, was too toxic (Table 2). Reducing the auristatin PE dose to 1.5 mg/kg resulted in better antitumor activity compared with the dolastatin 10 + bryostatin 1 combination. The combination treatments were well tolerated at both drug levels and provided significant antitumor activity as measured by tumor growth inhibition, tumor growth delay, and tumor log10 kill (Table 2). The auristatin PE + bryostatin 1 combination achieved an activity score of ++ +++, and all treated mice were free of tumors for 150 days and were considered cured. On
the other hand, the dolastatin 10 + bryostatin 1 combination reached an activity score of only + + +, and responses were seen in only two of five mice. Survival curves of WSU-CLL xenografts revealed that the dolastatin 10 + bryostatin 1 combination was significantly (P = 0.024) better compared with the dolastatin 10 + bryostatin 1 combination (Fig. 3) Noteworthy was the observation that all challenged mice developed bilateral tumors 3 weeks after 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 murine rejection.

Dolastatin 10 and auristatin PE interact with tubulin to inhibit microtubule polymerization (10, 11). Bryostatin 1 activates protein kinase C and induces differentiation of CLL cells (7), arrests cells in G0-G1, down-regulates bcl-2 and up-regulates wild-type p53 in lymphoma cell lines (15, 16), and potentiates apoptosis (24). The combination of agents with different mechanisms of action has proven to be the most beneficial approach to the treatment of cancer.

Collectively, the results obtained from this work highlighted the obvious superiority of auristatin PE over dolastatin 10. Auristatin PE can be administered to 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 CLL.

REFERENCES


Successful treatment of human chronic lymphocytic leukemia xenografts with combination biological agents auristatin PE and bryostatin 1.

R M Mohammad, M L Varterasian, V P Almatchy, et al.