# The cytotoxicity of anti-CD22 immunotoxin is enhanced by bryostatin 1 in B-cell lymphomas through CD22 upregulation and PKC-βII depletion

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## ABSTRACT

### Background
In spite of potent first-line therapies for chronic lymphocytic leukemia, treatment remains palliative and all patients frequently relapse. Treatment options for these patients are more limited. BL22 is a recombinant protein composed of the variable region of a monoclonal antibody that binds to CD22 and of PE38, a truncated Pseudomonas exotoxin. BL22 is a very potent drug already used in patients with hairy cell leukemia, whereas in chronic lymphocytic leukemia its cytotoxicity is limited by a lower expression of CD22. Here we demonstrate that this limitation can be overcome by pre-activation of chronic lymphocytic leukemia cells with bryostatin 1.

### Design and Methods
Primary malignant B cells from chronic lymphocytic leukemia and mantle cell lymphoma patients were used *in vitro* to assess the therapeutic impact of drug combinations using BL22 and bryostatin 1.

### Results
We demonstrate that bryostatin 1 sensitizes chronic lymphocytic leukemia cells for the cytotoxic effects of BL22 through activation of protein kinase C and subsequently increased CD22 surface expression. Dose and time response analysis reveals that activation of protein kinase C further activates an autocrine feedback loop degrading protein kinase C-βII protein. Depletion of protein kinase C-βII and upregulation of CD22 persist for several days following pre-stimulation with bryostatin 1. Therefore, our data provide a rationale for the sequential administration of BL22 following bryostatin 1 treatment. In addition to primary chronic lymphocytic leukemia cells, bryostatin 1 also sensitizes diffuse large B-cell lymphoma and mantle cell lymphoma cells to BL22 induced apoptosis.

### Conclusions
Our data suggest that the combination of bryostatin 1 with antibodies directed against CD22 is a potent drug combination for the treatment of low- and high-grade B-cell lymphoma.

Key words: CLL, bryostatin 1, CD22, immunotoxin, B-cell lymphoma.

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## Introduction

New treatment options for patients with CLL have demonstrated high efficacy and complete remission rates of over 50%.[1-3] The combination of the monoclonal antibody rituximab and fludarabine-based chemotherapies has now become the standard first-line therapy for young and fit CLL patients. However, in spite of excellent overall response rates, the disease still remains incurable with increasing shorter progression free survival (PFS) following salvage therapies. Therefore, new treatment options are needed for patients who relapse after immunochemotherapy and those who are ineligible for such treatments.

BL22 is a recombinant protein composed of the variable region of a monoclonal antibody that binds to CD22 on the surface of normal and malignant B cells and of PE38, a truncated Pseudomonas exotoxin A.[4] BL22 has demonstrated significant *in vivo* cytotoxicity in patients diagnosed with relapsed hairy cell leukemia following treatment with cladribine.[5] We previously demonstrated that BL22 induces cell death in CLL, involving the intrinsic apoptotic pathway. However, apoptosis induction correlates with the expression of CD22 on the surface of CLL cells and is only moderate in 'CD22 low'-expressing cells.[6] The aim of this study was to increase BL22 cytotoxicity by modulating the surface expression of CD22 on leukemic cells.

Bryostatin 1 is a macrocyclic lactone which was isolated from the marine *bryozoan Bugula neritina* more than 30 years ago. It modulates the family of protein kinase C (PKC) enzymes due to the structural similarities to the PKC-activating second messenger diacylglycerol.[7] Evidence from several groups indicates that PKC activity plays an important role in the pathogenesis of CLL and is crucial for cell survival by regulating anti-apoptotic proteins such as Mcl-1 and Bcl-2.[8,9] The effects of bryostatin 1 are complex and include induction of differentiation of CLL cells,[8] modulation of Fas/CD95 signaling[10] and downregulation of PKCs.[11] However, after phase I/II evaluation, it is now evident that bryostatin 1 has minimal single agent activity and, therefore, combined treatments of bryostatin 1 and chemotherapeutics were investigated in clinical trials.[12,13]

The ability of bryostatin 1 to induce a 'hairy cell phenotype' in CLL cells, including the marked upregulation of CD22, prompted us to investigate whether it could enhance the cytotoxicity of BL22. By using dose-response evaluation of bryostatin 1 we demonstrate that the combination of BL22 and bryostatin 1 increases the cytotoxicity of the immunotoxin not only through upregulation of CD22, but also through modulation of PKC-βII. The upregulation of Mcl-1 appears to be an undesirable effect of bryostatin 1 and may account for an impaired activity in CLL cells when used as monotherapy. Notably this upregulation of Mcl-1 was not sufficient to block the cytotoxicity of BL22. Furthermore, we demonstrate *in vitro* that the combination of bryostatin 1 and BL22 can be separated temporally, allowing enhanced cytotoxicity and potentially decreasing side effects *in vivo*.

## Design and Methods

### Cell samples

Approval for the study was obtained from the local ethics committee and all patients taking part gave their informed consent. Peripheral blood was obtained from patients with a diagnosis of B-CLL who had not been treated for at least three months. At the time of analysis, all patients were clinically stable, free from infectious complications and undergoing routine clinical outpatient review.

All cell lines used for the experiments were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen; GmbH, Braunschweig, Germany). Cells were characterized by morphology, immunphenotyping, cytogenetics and molecular genetics. A detailed description of each cell line can be found on *http://www.dsmz.de/*

### Statistical analysis

Data from individual experiments are presented as mean +SEM. Statistical significances were determined using unpaired and paired t-tests as appropriate. $P<0.05$ was considered statistically significant.

Further details of study Design and Methods are available in the *Online Supplementary Appendix*.

## Results

### Bryostatin 1 enhances the cytotoxic effects of BL22 in primary CLL cells

BL22 is a genetically engineered antibody directed against CD22 and is composed of the variable region of a monoclonal antibody coupled to a truncated pseudomonas exotoxin A.[4] We have previously reported that BL22 is active in a subset of CLL patients, but this activity is reduced in patients expressing only low levels of CD22.[6] In contrast to CLL, BL22 has demonstrated strong *in vivo* activity in hairy cell leukemia, characterized by high expression levels of CD22.[5]

Bryostatin 1 is a PKC-modulator with minimal single agent activity in CLL. Interestingly, bryostatin 1 induces a 'hairy cell'-phenotype in CLL. These morphological changes include cell enlargement and formation of cytoplasmatic extensions and are associated with an upregulation of CD22[14] (Figure 1A). We, therefore, hypothesized that bryostatin 1 may enhance the cytotoxic effects of BL22. To test this, CLL cells were incubated in the absence or presence of bryostatin 1 (1 and 50 ng/mL) and BL22 (1 μg/mL). In order to reduce spontaneous apoptosis of CLL cells *ex vivo* and to mimic microenvironment survival signals, CLL cells were cultured on a murine fibroblast cell line Ltk$^-$[15] (kindly provided by P. Pérez-Aciego). Leukemic cells were sufficiently protected from spontaneous apoptosis when cultured on Ltk$^-$ cells (Figure 1B, bars 1 and 2). Bryostatin 1 showed no cytotoxic effects on CLL cells cultured on feeder cells. However, the cytotoxic effect of BL22 was not abolished in the presence of survival signals derived from bystander cells (Figure 1B). Importantly, bryostatin 1 significantly sensitized primary CLL cells to the cytotoxic effects of BL22. (Relative apoptosis induction compared to CLL cells cultured on Ltk$^-$ cells: BL22 alone, mean 9%, range 0-33%; BL22 + bryostatin 1 (1 ng/mL), mean 28%, range 10-65%; BL22+ bryostatin 1 (50 ng/mL), mean 45%, range 24-78%). The expression of ZAP70 and CD38 in CLL cells is a surrogate marker for poor prognosis. Patients' characteristics according to the samples used for our experiment (Figure 1B) are shown in Table 1. These show that the combination of BL22 and bryostatin 1 is highly effective in low- and high-risk patients (*Online Supplementary Table S1*).

We hypothesized that an increased binding and uptake of the immunotoxin occurred due to an increased expression of CD22 by bryostatin 1 on the surface of CLL cells. To test this hypothesis, surface protein expression of CD22 was determined by flow cytometry 24 h after stimulation of leukemic cells with bryostatin 1. An isotype matching, non-specific antibody was used as negative control. We observed a significant upregulation of CD22 at a bryostatin1 dose of 1 ng/mL (Figure 1C). Importantly, the surface expression of CD22 varies between CLL patients, ranging from low to moderate expression.[6] By analyzing the expression of CD22 in response to bryostatin 1 (1 ng/mL) we observed that 'CD22-low' and 'high' expressers up-regulate CD22, although the effect was more pronounced in CLL cells with a higher baseline expression of CD22 (Figure 1C).

Surprisingly, higher doses of bryostatin 1 (50 ng/mL) further enhanced the cytotoxic effect of BL22 compared to 1 ng/mL (Figure 1B), but CD22 upregulation peaked at a dose of 5 ng/mL and was significantly less pronounced with 50ng/mL bryostatin 1 (Figure 1D). These data indicate that additional mechanisms than enhanced CD22 expression must account for the sensitizing effects of bryostatin 1 on BL22.

### Bryostatin 1 up-regulates the expression of CD22 through PKC-β, but not Erk

As reported above, bryostatin 1-induced differentiation of CLL cells requires both PKC and extracellular signal-related kinase (Erk)-activity.[14] Of all classical PKC-isoforms PKC-βII is over-expressed in CLL and contributes significantly to the pathogenesis.[16-18] In order to characterize which of these pathways was required for CD22 upregulation, CLL cells were exposed to bryostatin 1 (1 ng/mL) in the presence of the small molecule inhibitors enzastaurin and PD98059. Enzastaurin is an acyclic bisindolylmaleimide that was developed as a selective and specific PKC-β inhibitor.[19] PD98059 is a widely used inhibitor of the Erk1/2 pathway. Leukemic cells were pre-incubated with the inhibitors 2 h before stimulation with bryostatin 1. Blockage of PKC-β abrogated the upregulation of CD22 by bryostatin 1, whereas inhibition of the Erk-pathway had no effect on the expression of CD22 (Figure 2A). Conclusively, bryostatin 1 up-regulates CD22 using PKC-β as main conduit.

Since our data suggested that PKC-β is a mediator of bryostatin 1-induced upregulation of CD22, we analyzed the expression of PKC-βII 24 h after stimulation with bryostatin 1. We noticed that 'low dose' bryostatin 1 (1 ng/mL) did not affect the expression levels of PKC-βII (Figure 2B, lanes 2 and 3). In contrast, higher doses of bryostatin 1 (50 ng/mL) depleted the expression of PKC-βII in CLL to levels undetectable by immunoblotting (lane 4). Inhibitory effects of PKC activators have been observed on the expression of PKC isoforms and may be related to a negative feedback loop involving proteasomal protein-degradation, protein-proteolysis and vesicle-dependent degradation.[20,21] Importantly, even though low-dose bryostatin 1 (1 ng/mL) is sufficient to up-regulate CD22 on CLL cells (Figure 1), this activation seems to be incapable of activating this feedback loop. The dose-dependency of PKC-βII degradation appeared to be isoform-specific, since the expression levels of PKC-ε were unaltered upon 'low'- and 'high-dose' bryostatin 1 treatment (Figure 2B).

In the light of the pro-apoptotic effects of PKC-inhibitors in CLL,[17,18] we hypothesized that depletion of PKC-βII augmented the cytotoxic effects of BL22. In order to prove this hypothesis and to rule out the possibility that the down-regulation of PKC-βII by bryostatin 1 was merely an epiphenomenon, PKC-βII activity was blocked with the small molecule inhibitor enzastaurin. BL22-induced cell death was significantly enhanced in the presence of enzastaurin. The pro-apoptotic effect of the combined treatment of BL22 and enzastaurin exceeded the additive effects of single agent treatment, suggesting a synergism between blockage of PKC-β and BL22 induced cell death (mean apoptosis induction: enzstaurin+BL22 41%; enzastaurin 13.8%; BL22 18.7%; *Online Supplementary Figure S1*). Notably, expression levels of CD22 remained unchanged in enzastaurin-treated CLL cells (*data not shown*).

### Bryostatin 1 primes primary CLL cells for the cytotoxic effects of BL22

To further characterize the kinetics of CD22 upregulation and PKC-βII depletion following bryostatin 1 exposure, bryostatin 1 was removed after 24 h and CD22/ PKC-βII expression was monitored by flow cytometry and immunoblotting. Successful removal of bryostatin 1 from the cell culture was monitored by expression of Mcl-1, a putative downstream target of activated PKC[8] (*data not shown*). The upregulation of CD22 following a 24-h exposure to bryostatin 1 was maintained for at least 72 h after removal of bryostatin (Figure 3A). Remarkably, we observed that PKC-βII expression did not recover within 72 h after removal of bryostatin 1 (Figure 3B, lanes 6, 8 and 10).

This result prompted us to investigate whether sensitizing effects with BL22 could last for several days *after* bryostatin 1 treatment. From a clinical point of view, sequential therapies offer a strategy to reduce drug-interactions and side effects while increasing the cytotoxic effect. To address whether this was a feasible approach for BL22-based therapies, CLL cells were primed with bryostatin 1 (1 ng/mL and 50 ng/mL) for 24 h. Forty-eight and 72 h after removal of bryostatin 1, leukemic cells were exposed to BL22 for an additional 24 h. Priming of CLL cells with bryostatin 1 significantly enhanced the cytotoxic effects of sequentially administered BL22 (Figure 3C). Due to a significant amount of apoptosis in the medium control after several days of *in vitro* culture, the pro-apoptotic effect of BL22 was less pronounced than in CLL cells protected by pro-survival factors (Figure 1B).

### BL22 overcomes bryostatin 1-induced upregulation of Mcl-1 and p-Ser70-Bcl-2

Based on results from phase I/II clinical trials, it is evident that bryostatin 1 has only minimal single agent activity in CLL.[12,13,22] Mechanistically this may be related to the fact that bryostatin 1 acts as a mixed antagonist/agonist of PKC. Bryostatin 1 has been shown to induce Mcl-1 expression in CLL cells, thus enhancing apoptosis resistance.[23] Accordingly, we also observed that bryostatin 1 attenuated the cytotoxic effect of the chemotherapeutic drugs fludarabine, doxorubicine and vincristine (*data not shown*). In order to further evaluate the relative contribution of Mcl-1 induction by bryostatin 1 to BL22 toxicity, we analyzed the expression of apoptosis-regulating proteins after treatment with BL22, bryostatin 1 or a combination hereof. As expected, a strong upregulation of Mcl-1 in CLL cells upon bryostatin 1 treatment was observed. Low-dose (1 ng/mL)

bryostatin 1 was significantly more potent in inducing Mcl-1 than high-dose (50 ng/mL) bryostatin 1 (*Online Supplementary Figure S2A*, compare lane 2 to lanes 7 and 8). Accordingly, single agent treatment with bryostatin 1 significantly reduced spontaneous apoptosis of CLL cells (*Online Supplementary Figure S2B*). In addition to the upregulation of Mcl-1, bryostatin 1 induced phosphorylation of Bcl-2 at serine-70. This phosphorylation increases the binding of the pro-apoptotic Bim to Bcl-2 and enhances the anti-apoptotic properties of Bcl-2.[18] In addition, Bim-function was further impaired due to degradation of BimEL in bryostatin 1-treated cells. We observed a reduced electrophoretic mobility of BimL in broystatin 1-treated cells, indicative of posttranslational modifications mediated by PKC. These modifications of Bim were not affected by BL22, excluding the possibility that BL22-mediated apoptotic effects were mediated by Bim. In contrast, single agent BL22 diminished the expression of Mcl-1 and XIAP (*Online Supplementary Figure S2A*, lanes 2 and 3). The combination of BL22 and bryostatin 1 attenuated the upregulation of Mcl-1 and phosphorylation of Bcl-2 (*Online Supplementary Figure S2A*, compare lanes 4 and 5 to 7 and 8). Importantly, expression levels of Mcl-1 and phospho-ser70-Bcl-2 were still above baseline-levels (*Online Supplementary Figure S2A*, compare lanes 4 and 5 to lane 1), indicating that bryostatin 1 has unfavorable effects on the expression of anti-apoptotic proteins. However, BL22 can overcome the anti-apoptotic effects of bryostatin 1 mediated by upregulation of Mcl-1 and phosphorylation of Bcl-2 (*Online Supplementary Figure S2B*). The pro-apoptotic effect of BL22 in combination with bryostatin 1 was not related to induction of Noxa or Puma (*Online Supplementary Figure S2A*).

Under co-culture conditions of leukemic B cells and stromal cells (LTK$^-$), bryostatin 1 lacked the anti-apoptotic effect we had observed in the previous experiment (compare *Online Supplementary Figure S2B* and Figure 1B). This raised the question as to whether bryostatin also mediated the upregulation of Mcl-1 and downregulation of PKC-βII in the presence of stromal cell-derived survival factors. In order to address this question, primary CLL cells were cultured on LTK$^-$ cells for up to 72 h. Continuous administration for 72 h had been used in clinical trials with bryostatin 1 in order to shift the balance from PKC-agonistic to antagonistic properties.[22] Notably, even in the presence of stromal cells, bryostatin 1 significantly up-regulated the expression of Mcl-1 (*Online Supplementary Figure S2C*). Continuous exposure to bryostatin 1 for 72 h depleted

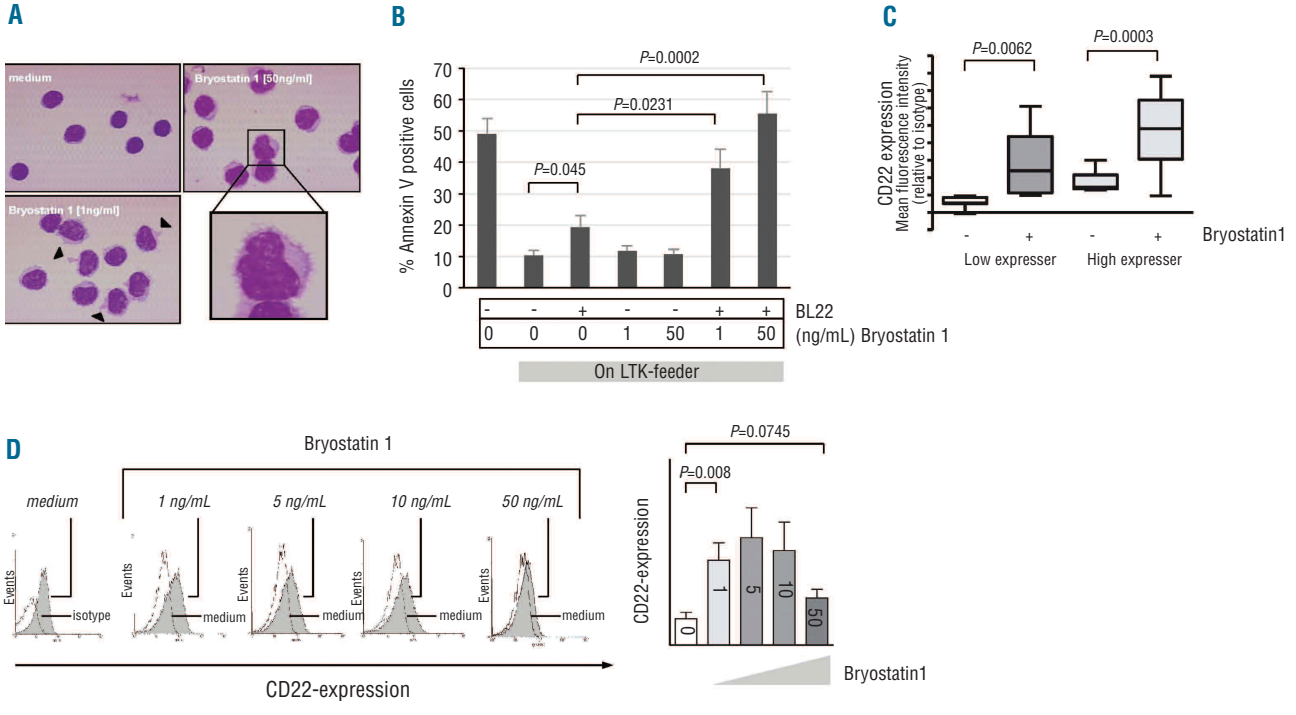


**Figure 1.** Bryostatin 1 sensitizes CLL cells for the cytotoxic effect of BL22. (A) Primary CLL cells were incubated with bryostatin 1 (1 ng/mL and 50 ng/mL). After 48 h, cells were spun onto a slide, stained according to "Pappenheim staining" and analyzed for morphological changes using light microscopy. Treatment results in an increase in cell size and formation of cytoplasmatic extensions (black arrows). One representative experiment out of 3 is shown. (B) Co-culture of CLL cells on a murine fibroblast cell line Ltk$^-$ significantly reduced spontaneous apoptosis (bars 1 and 2). Co-cultured CLL cells were exposed to BL22 (1 μg/mL), bryostatin 1 (1 ng/mL and 50 ng/mL) or a combination hereof. Apoptotic cells were analyzed by Annexin-V/ PI staining after 48 h (n=12). Whilst bryostatin 1 alone had no effect on survival of co-cultured CLL cells, it sensitized CLL cells for apoptosis induction by BL 22 immunotoxin. (C) Baseline expression of CD22 was analyzed by flow cytometry. Samples were categorized as "low expresser" when less than 10% of cells were above the threshold determined by the isotype control. CD22 expression was analyzed 24 h after treatment with bryostatin 1 (1 ng/mL); (n=8 for low expresser and n=10 for high expresser). (D) CLL cells were exposed to increasing doses of bryostatin 1. The shift in graph profile according to dose is shown. After 24 h, surface expression of CD22 was assessed by flow cytometry using fluoresced primary antibodies. Left panel: one representative patient is shown: histograms show fluorescence intensities (shaded), superimposed with that of an isotype control (solid line; left panel) or medium control (solid line; panel 2-5). Right panel: CD22 expression following bryostatin 1 treatment of 10 patients was quantified and revealed a strong upregulation of CD22 at a dose range from 1 ng/mL to 5 ng/mL; however, levels declined with higher doses of bryostatin 1.

Mcl-1 expression only in cells treated with higher doses of bryostatin 1 (50 ng/mL), indicating that PKC-antagonistic effects of bryostatin 1 are dose- and time-dependent. PKC-βII expression was depleted upon high-dose bryostatin 1 expression according to our results obtained from CLL cells cultured in the absence of feeder cells (compare *Online Supplementary Figure S2C* and Figure 3B).

### Combined treatment with BL22 and bryostatin 1 is active in diffuse large B-cell and mantle cell lymphomas

The expression of surface CD22 is not limited to CLL cells, but it can also be detected at even higher levels in B-pro-lymphocytic leukemia, mantle cell lymphomas, marginal zone lymphomas, diffuse large B-cell lymphomas, and in 70% of follicular B-cell lymphomas.[24-26] Clinical trials with either 'naked' CD22 antibodies or labeled-antibodies are currently underway in nearly all these B-cell malignancies (see *http://www.clinicalTrials.gov*). We were, therefore, interested in seeing whether enhanced cytotoxic effects with bryostatin 1 and BL22 could also be achieved in other B-cell malignancies. Karpas422,[27] DB[28] and Sudhl-4[29] are cell lines derived from patients with diffuse large B-cell lymphomas. Cells were treated for 24 h with bryostatin 1 at a dose of 1 ng/mL and then expression of CD22 was analyzed. All cell lines up-regulated surface CD22 in response to bryostatin1 (Figure 4A). Notably, the upregulation was much stronger in DB and Sudhl-4 cells compared to Karpas422 cells (Figure 4A). BL22-induced cell death when used as single agent in all cell lines, but its pro-apoptotic effect was dramatic only in Sudhl-4 cells (Figure 4C). The combined treatment of bryostatin 1 (1 ng/mL) and BL22 displayed synergistic effects in DB cells according to an increase in CD22 expression. Due to the high sensitivity of Sudhl-4 cells to BL22, no further increase in apoptosis-induction could be detected in spite of a significant upregulation of CD22. In contrast, Karpas422 cells were relatively resistant to single agent or combined treatment of BL22 and bryostatin 1 (Figure 4C).

Similar to DLBCL cells, mantle cell lymphoma (MCL) cells were equally sensitive to single agent treatment with BL22 or bryostatin 1 (Figure 4D). However, bryostatin 1 failed to induce an upregulation of CD22 on the surface of Granta[30] cells (Figure 4B). Accordingly, no increase in BL22 induced toxicity was observed in these cells upon treatment with bryostatin 1. Additionally, high levels of Bcl-2 in Granta cells may also contribute to the lack of synergism between bryostatin 1 and BL22.[31] In contrast, the upregulation of CD22 in Jeko-cells[32] was associated with significantly increased cell death induced by BL22 (Figure 4B and D).

These cell lines were suitable models to exclude the remote possibility that free-dissociated pseudomonas-toxin from BL22 was responsible for the lymphoma cell death. Therefore, we performed knockdown experiments for CD22 on Sudhl-4 cells. The surface expression of CD22 could be reduced by repeated transfection with an siRNA directed against CD22 (Figure 4E). Such modified cells were exposed to BL22 for 24 h. Reduced expression of CD22 attenuated the cytotoxic effects of BL22, indicating that surface expression of CD22 determines the efficacy of BL22 (Figure 4F).

In addition, we tested whether primary mantle cell lymphoma cells derived from patients with leukemic variants of MCL were susceptible to the drug combination of bryostatin 1 and BL22. Single agent bryostatin 1 had no pro-apoptotic effect, but rather displayed an anti-apoptotic effect. However, bryostatin 1 enhanced the cytotoxic effects of BL22 on primary MCL cells (Figure 4G).

Surprisingly, we noticed that single agent bryostatin 1 exerts cytotoxic effects in 5 out of 5 cell lines (Figure 4C and D) in contrast to primary CLL and MCL cells (Figure 1B and Figure 4G).

These data suggest that the combination of bryostatin 1 and BL22 may be useful not only for the treatment of CLL, but also in other B-cell malignancies.

## Discussion

Targeting CD20 with monoclonal antibodies directed against various cell surface proteins has become a common

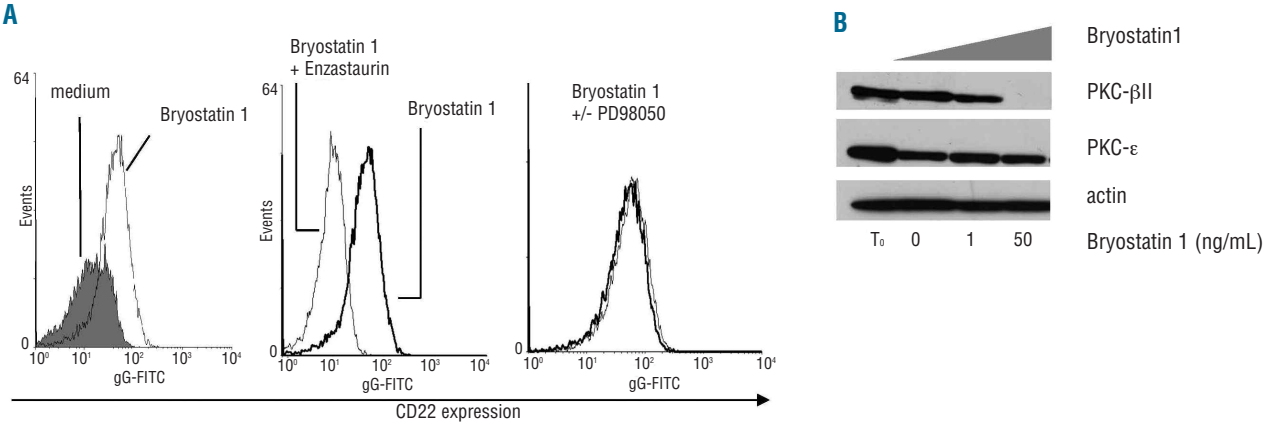


**Figure 2.** Bryostatin 1 induced upregulation of CD22 depends on PKC-β activity. (A) CD22 upregulation by bryostatin 1 is blocked by the PKC inhibitor enzastaurin (10 μM) but not by the ERK inhibitor PD98059 (50 μM). Cells were pre-treated with inhibitors for 120 min and then cultured with bryostatin 1 (1 ng/mL) for 24 h. The same results were obtained in 3 out of 4 individual experiments. (B) Primary CLL cells were exposed to either 1 ng/mL or 50 ng/mL bryostatin 1 for 24 h. Cells were then harvested and analyzed for the expression of PKC-βII or PKC-ε. To indicates cell lysates from CLL cells freshly isolated from peripheral blood. Results from 3 experiments revealed the same results.

approach to treat patients with B-cell lymphomas. However, in CLL, CD20 is a weak target and requires excessively high doses of CD20 directed antibodies such as rituximab[33] or ofatumumab[34] in order to achieve significant clinical responses with monotherapies. Alternative target proteins on CLL cells are CD22, CD23, CD40, CD70 and HLA-DR. Monoclonal antibodies directed against each of these proteins have been developed and demonstrated pre-clinical activity, and some of them have already been tested in clinical trials.

Epratuzumab is an anti-CD22 humanized antibody and has shown single agent activity in patients with relapsed and refractory B-cell lymphomas.[35] However, its activity in CLL patients was only modest, possibly related to variations in CD22 expression on the surface of leukemia cells. The conjugation of protein toxins to monoclonal antibody is a neat and efficient way of increasing their toxicity. BL22 is a monoclonal antibody directed against CD22 and fused to a truncated Pseudomonas exotoxin.[4] Of 11 CLL patients treated in an early phase I clinical trial with BL22, only one patient had a partial response and 3 patients showed minor responses.[12] In sharp contrast, this drug has demonstrated noteworthy clinical responses in patients with refractory hairy cell leukemia (HCL) with a median progression free survival of 36 months in patients achieving a complete remission.[5] This discrepancy between CLL and HCL in terms of response to BL22 is most likely related to a much lower surface expression of CD22 on CLL cells compared to HCL cells. In addition, all CLL patients included in this trial had been more heavily pre-treated than HCL patients before receiving BL22. Interestingly, in spite of excessive prior treatments, BL22 was surprisingly well tolerated with reversible toxicities not exceeding grade 1-2 at the MTD level. Most common side effects of BL22 included transfusion-related fever, fatigue, elevation of liver enzymes and hypoalbuminemia.[36] Based on these results, BL22 appears to be a potent drug for the treatment of B-cell malignancies, but its efficacy in CLL is hampered by low expression of

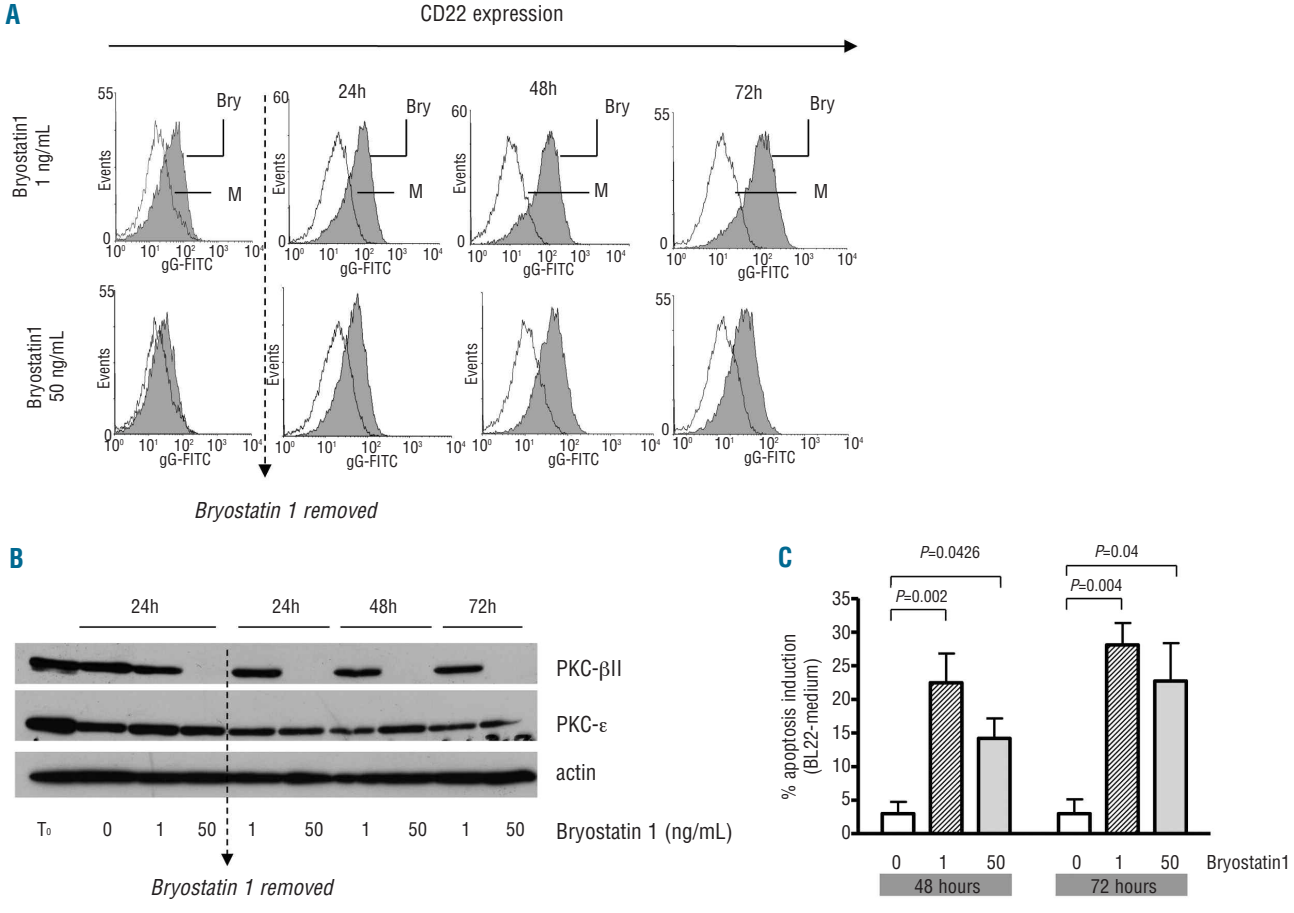


Figure 3. Sequential treatment of BL22 following bryostatin 1. (A) Primary CLL cells were exposed to either 1 ng/mL or 50 ng/mL bryostatin 1 for 24 h. Cells were then washed three times in PBS/10% FCS in order to remove bryostatin 1 and re-cultured in medium/ FCS. After the time points as indicated, cells were harvested and surface CD22 expression was analyzed by flow cytometry. Histograms show fluorescence intensities following bryostatin 1 treatment (shaded; (Bry), superimposed with that of medium control (solid line; (M)). Experiments were carried out in triplicates: one representative experiment is demonstrated. (B) Cells were treated as described in (A). After the time points as indicated, cells were harvested and analyzed for the expression of PKC-βII or PKC-ε. $T_0$ indicates cell lysates from CLL cells freshly isolated from peripheral blood. Results from 3 experiments revealed the same results. (C) CLL cells were either pre-treated with 1 ng/mL or 50 ng/mL bryostatin 1 or kept in medium alone. After 24 h of incubation, bryostatin 1 was removed by washing cells three times in PBS/ FCS. Cells were re-cultured in medium/ FCS. After 48 or 72 h BL22 was added to pre-treated cells at a dose of 1 μg/mL. Apoptotic cells were determined following Annexin-V/PI staining after an additional 24 h (n=3). Apoptosis-induction by BL22 was calculated by subtracting spontaneous apoptosis of CLL cells cultured in medium.

surface CD22 in some cases of CLL. In line with this observation, we have previously reported that the expression of CD22 is low in 50% of CLLs, irrespectively of disease stage, age, sex or leukocyte counts.[6] The pro-apoptotic effect of BL22 *in vitro* is strongly correlated to the density of CD22 on the surface of CLL cells, indicating that the expression of CD22 is crucial for BL22 toxicity to malignant cells.[6]

The purpose of this study was to overcome this limitation by increasing the cytotoxicity of BL22 through an upregulation of CD22. Phorbol esters have been reported to induce differentiation of CLL cells.[37] Bryostatin 1 is a structurally related compound lacking the tumor-promoting effects of phorbol esters. Bryostatin 1 induces morphological changes in CLL, including an increase in cell size, irregularity in cell shape, and formation of cytoplasmatic extensions (Figure 1A). These changes into a 'hairy cell phenotype' are accompanied by an upregulation of CD22.[14] Bryostatin 1 exerts a wide range of biological activities and differentiation of CLL cells is related to activation of PKCs. The molecular mechanisms of apoptosis induction with bryostatin 1 treatment are complex and little understood. Bryostatin 1 and related compounds were described to act as mixed PKC agonist/antagonists. Short-term exposure to bryostatin 1 causes activation of PKC and subsequent upregulation of anti-apoptotic proteins.[23] In contrast, pro-apoptotic effects of bryostatin 1 are thought to be related to diminished enzyme activity due to protea-

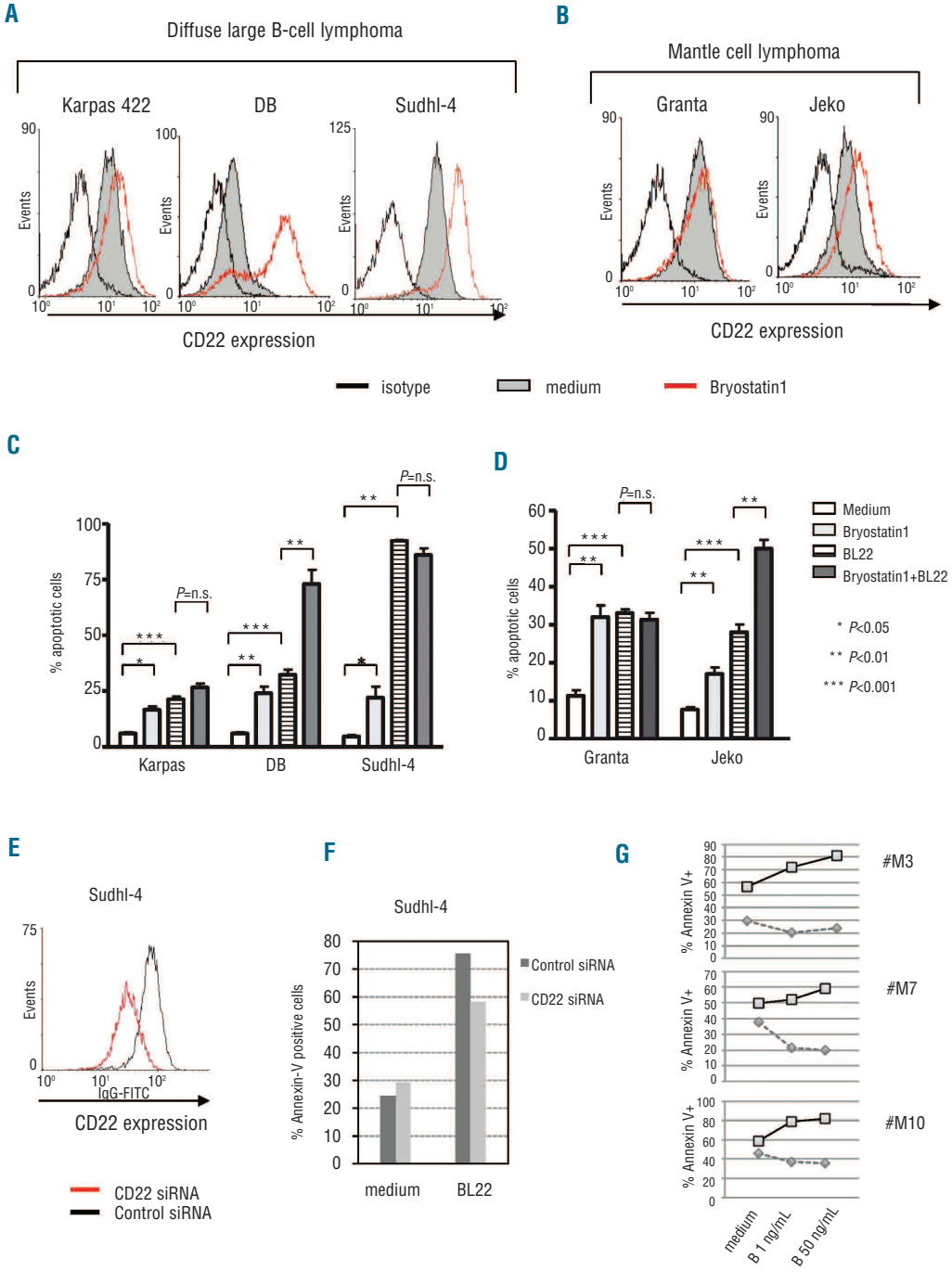


**Figure 4.** Bryostatin 1 and BL22 exert anti-lymphoma effects in DLBCL and mantle cell lymphoma cells. (A) Diffuse large B-cell lymphoma cell lines or mantle cell lymphoma cell lines (B) were exposed to bryostatin 1 (1 ng/mL) (red line). After 48 h the expression of CD22 was determined by flow cytometry and compared to untreated cells (shaded histograms). (C) DLBCL cells or (D) mantle cell lymphoma cell lines were treated with bryostatin 1 (1 ng/mL) or BL22 (100 ng/mL) or a combination of both drugs. After 48 h the amount of apoptotic cells was analyzed by Annexin-V/PI staining. Experiments were carried out in triplicates. (E) Sudhl-4 cells were either transfected two times with an siRNA against CD22 or a control siRNA. CD22 surface expression was reduced as determined by flow cytometry analysis. (F) CD22-modified cells were exposed for an additional 24 h to BL22 (100 ng/mL) before cell death was analyzed by Annexin-V staining. One representative experiment out of 4 is shown. (G) Primary mantle cell leukemic cells from 3 different patients (#M3,7,10) were treated with bryostatin 1(B) at a concentration of 1 and 50 ng/mL for 48 h. Single agent treatment reduced spontaneous apoptosis of primary MCL cells (◊). In contrast, the cytotoxicity of BL22 (1000 ng/mL) was enhanced in the presence of bryostatin 1 ( □ ).

somal protein degradation following long-term exposure to bryostatin 1.[38]

To date, few data are available on the clinical activity of bryostatin 1 in CLL. Over ten years ago, bryostatin 1 was given to refractory or relapsed CLL patients. Of the 3 CLL patients treated with bryostatin 1, 2 showed a significant, but only transient decrease in peripheral-blood lymphocyte counts, whereas the third patient did not respond to therapy.[12] A few years later, the same group conducted a phase II clinical trial. Of the 8 patients treated with bryostatin 1, 2 showed a partial remission and disease stabilized in an additional 2 patients.[22] In an independent trial, NHL patients were treated sequentially with bryostatin 1 and fludarabine (or vice versa). The overall response rate was reported to be 40% with only one patient achieving a complete remission.[13] Based on all available data, bryostatin 1 appears to be a safe drug in CLL patients. The most frequently reported side effects were dose-dependent myalgia that resolved spontaneously after several weeks. There were no reports of severe hematotoxicity or myelosuppression in patients receiving single agent bryostatin 1;[13,22,39,40] however, this might be of greater concern in combination with other chemotherapeutic drugs. Conclusively, these clinical data indicate that bryostatin 1 has only modest activity in CLL and its effect is rather cytostatic than cytotoxic. On a molecular base, this may be explained by the properties of bryostatin 1 not only acting as a PKC inhibitor, but also partially activating PKCs.

Our findings confirm that the combination of bryostatin 1 and BL22 has positive effects on apoptosis induction in CLL cells. Using two arbitrarily chosen concentrations of bryostatin 1, we discovered that two distinct mechanisms contribute to this: at low-dose bryostatin 1 (1 ng/mL), predominate activation of PKC causes a strong upregulation of CD22 on CLL cells (Figures 1 and 2, *Online Supplementary Figure S1*). Subsequently, increased binding of BL22 and uptake of the immunotoxin kills leukemia cells. Interestingly, the upregulation of CD22 following bryostatin 1 treatment occurred in CD22 low- and high-expressing CLL cells (Figure 1C). In addition, high-dose bryostatin 1 (50 ng/mL) depletes PKC-βII from CLL cells in association with a more moderate upregulation of CD22 and Mcl-1. The precise mechanisms of apoptosis induction by BL22 in the presence of bryostatin 1 remain elusive. In spite of a predominant role for Mcl-1 in CLL, high levels of Mcl-1 did not protect CLL cells from BL22 induced cell death. Du *et al.* demonstrated that release of the pro-apoptotic Bak from Mcl-1 was crucially dependent for Pseudomonas exotoxin induced apoptosis in MEFs.[41] However, elevated levels of Mcl-1 in BL22 and bryostatin 1-treated CLL cells (*Online Supplementary Figure S2A*) suggest that also other mechanisms need to be taken into account. Based on the observation that inhibition of PKC-β by enzastaurin strongly induces apoptosis in CLL cells[17,18] (*Online Supplementary Figure S1*), we conclude that loss of PKC-βII and increased binding of BL22 following bryostatin 1 treatment is inducing cell death. We demonstrated that the combination of enzastaurin and BL22 strongly induced cell death of CLL cells by an amount exceeding the pro-apoptotic effect of either compound alone (*Online Supplementary Figure S1*). Since CD22 is up-regulated by bryostatin 1 in spite of a loss of PKC-βII (Figure 1C and D, Figure 2B) it remains unclear whether this is a persistent effect due to a transient activation of PKC-βII (before protein degradation) or dependent on the activation of other PKC-isoforms.

One intriguing question arising from our work is what plasma levels of bryostatin 1 are achievable *in vivo*. Applying our findings to *in vivo* settings, plasma concentrations of at least 1 ng/mL of bryostatin 1 are needed to sensitize cells to the cytotoxic effects of BL22. One problem of the clinical application of bryostatin 1 has been the lack of reliable pharmacological studies. This is related to both a shortage of sensitive analytical methods and to the rapid clearance of bryostatin 1 after intravenous administration.[39] To tackle the problem, PKC activity has been assessed instead in patients receiving bryostatin 1.[40] Based on several reports, either continuous or bolus administration of bryostatin 1 was able to down-regulate PKC in PBMC. Since the concentration of bryostatin 1 required to increase CD22 expression on CLL cells is below the dose necessary to deplete PKC (Figure 1D and 2B), one can envisage that achievable *in vivo* concentrations will be sufficient to increase BL22 toxicity. Another pharmacokinetic aspect of bryostatin 1 is its ability to accumulate in lipophilic tissues. Therefore, bone marrow concentrations might even exceed plasma levels.

Sequential treatment of monoclonal antibodies and cytotoxic drugs is a common way of increasing cytotoxicity and avoiding potentially harmful drug interactions and side effects. For instance, rituximab is given one day before chemotherapy in nearly all patients diagnosed with B-cell lymphomas. Sequential treatment of bryostatin 1 and fludarabine has already been proven to be effective in CLL patients.[13] Here we provide *in vitro* evidence that priming CLL cells for 24 h with bryostatin 1 significantly increases the cytotoxicity of BL22 administered even several days after (Figure 3C). The initial treatment with bryostatin 1 increases the expression of CD22 and depletes PKC-βII from CLL cells. Both effects last for several days (Figure 3A and B) allowing administration of BL22 several days after bryostatin 1. Based on our *in vitro* experiments and the strong clinical activity of BL22 in hairy cell leukemia, we believe that the combination of BL22 and bryostatin 1 could constitute a very effective treatment for relapsed or refractory CLL patients. Both drugs have already been given to CLL patients and displayed moderate side effects when given as monotherapy.

In addition to CLL, we show that BL22 and bryostatin 1, either given as single agent or in combination, exert strong pro-apoptotic effects in diffuse large B-cell lymphomas and mantle cell lymphoma (Figure 4). Therefore, this drug combination may be a promising new treatment option for a variety of B-cell malignancies. However, our study is limited to *in vitro* experiments and we can only speculate that the synergism between the two drugs can be translated *in vivo*. Therefore, clinical trials are needed to prove whether or not this is a feasible therapeutic approach in the treatment of CLL and B-cell lymphoma patients.

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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## References


1. Keating MJ, O'Brien S, Albitar M, Lerner S, Plunkett W, Giles F, et al. Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. J Clin Oncol. 2005;23(18):4079-88.
2. Hallek M. Chronic lymphocytic leukemia (CLL): first-line treatment. Hematology Am Soc Hematol Educ Program. 2005:285-91.
3. Bosch F, Abrisqueta P, Villamor N, Terol MJ, Gonzalez-Barca E, Ferra C, et al. Rituximab, fludarabine, cyclophosphamide, and mitoxantrone: a new, highly active chemoimmunotherapy regimen for chronic lymphocytic leukemia. J Clin Oncol. 2009;27(27): 4578-84.
4. Kreitman RJ, Margulies I, Stetler-Stevenson M, Wang QC, FitzGerald DJ, Pastan I. Cytotoxic activity of disulfide-stabilized recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) toward fresh malignant cells from patients with B-cell leukemias. Clin Cancer Res. 2000;6(4):1476-87.
5. Kreitman RJ, Wilson WH, Bergeron K, Raggio M, Stetler-Stevenson M, FitzGerald DJ, et al. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. N Engl J Med. 2001;345(4):241-7.
6. Decker T, Oelsner M, Kreitman RJ, Salvatore G, Wang QC, Pastan I, et al. Induction of caspase-dependent programmed cell death in B-cell chronic lymphocytic leukemia by anti-CD22 immunotoxins. Blood. 2004;103 (7):2718-26.
7. Mutter R, Wills M. Chemistry and clinical biology of the bryostatins. Bioorg Med Chem. 2000;8(8):1841-60.
8. Thomas A, Pepper C, Hoy T, Bentley P. Bryostatin induces protein kinase C modulation, Mcl-1 up-regulation and phosphorylation of Bcl-2 resulting in cellular differentiation and resistance to drug-induced apoptosis in B-cell chronic lymphocytic leukemia cells. Leuk Lymphoma. 2004;45(5):997-1008.
9. Bellosillo B, Dalmau M, Colomer D, Gil J. Involvement of CED-3/ICE proteases in the apoptosis of B-chronic lymphocytic leukemia cells. Blood. 1997;89(9):3378-84.
10. Gomez-Angelats M, Cidlowski JA. Protein kinase C regulates FADD recruitment and death-inducing signaling complex formation in Fas/CD95-induced apoptosis. J Biol Chem. 2001;276(48):44944-52.
11. Kortmansky J, Schwartz GK. Bryostatin-1: a novel PKC inhibitor in clinical development. Cancer Invest. 2003;21(6):924-36.
12. Varterasian ML, Mohammad RM, Eilender DS, Hulburd K, Rodriguez DH, Pemberton PA, et al. Phase I study of bryostatin 1 in patients with relapsed non-Hodgkin's lymphoma and chronic lymphocytic leukemia. J Clin Oncol. 1998;16(1):56-62.
13. Roberts JD, Smith MR, Feldman EJ, Cragg L, Millenson MM, Roboz GJ, et al. Phase I study of bryostatin 1 and fludarabine in patients with chronic lymphocytic leukemia and indolent (non-Hodgkin's) lymphoma. Clin Cancer Res. 2006;12(19): 5809-16.
14. al-Katib A, Mohammad RM, Dan M, Hussein ME, Akhtar A, Pettit GR, et al. Bryostatin 1-induced hairy cell features on chronic lymphocytic leukemia cells in vitro. Exp Hematol. 1993;21(1):61-5.
15. Cuni S, Perez-Aciego P, Perez-Chacon G, Vargas JA, Sanchez A, Martin-Saavedra FM, et al. A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. Leukemia. 2004;18(8):1391-400.
16. Abrams ST, Lakum T, Lin K, Jones GM, Treweeke AT, Farahani M, et al. B-cell receptor signaling in chronic lymphocytic leukemia cells is regulated by overexpressed active protein kinase CbetaII. Blood. 2007;109(3):1193-201.
17. Holler C, Pinon JD, Denk U, Heyder C, Hofbauer S, Greil R, et al. PKCbeta is essential for the development of chronic lymphocytic leukemia in the TCL1 transgenic mouse model: validation of PKCbeta as a therapeutic target in chronic lymphocytic leukemia. Blood. 2009;113(12):2791-4.
18. zum Buschenfelde CM, Wagner M, Lutzny G, Oelsner M, Feuerstacke Y, Decker T, et al. Recruitment of PKC-betaII to lipid rafts mediates apoptosis-resistance in chronic lymphocytic leukemia expressing ZAP-70. Leukemia.24(1):141-52.
19. Faul MM, Gillig JR, Jirousek MR, Ballas LM, Schotten T, Kahl A, et al. Acyclic N-(azacycloalkyl)bisindolylmaleimides: isozyme selective inhibitors of PKCbeta. Bioorg Med Chem Lett. 2003;13(11):1857-9.
20. Parker PJ, Bosca L, Dekker L, Goode NT, Hajibagheri N, Hansra G. Protein kinase C (PKC)-induced PKC degradation: a model for down-regulation. Biochem Soc Trans. 1995;23(1):153-5.
21. Kang BS, French OG, Sando JJ, Hahn CS. Activation-dependent degradation of protein kinase C eta. Oncogene. 2000;19(37): 4263-72.
22. Varterasian ML, Mohammad RM, Shurafa MS, Hulburd K, Pemberton PA, Rodriguez DH, et al. Phase II trial of bryostatin 1 in patients with relapsed low-grade non-Hodgkin's lymphoma and chronic lymphocytic leukemia. Clin Cancer Res. 2000;6(3): 825-8.
23. Kitada S, Zapata JM, Andreeff M, Reed JC. Bryostatin and CD40-ligand enhance apoptosis resistance and induce expression of cell survival genes in B-cell chronic lymphocytic leukaemia. Br J Haematol. 1999;106(4):995-1004.
24. Tedder TF, Tuscano J, Sato S, Kehrl JH. CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. Annu Rev Immunol. 1997;15:481-504.
25. Strauss SJ, Morschhauser F, Rech J, Repp R, Solal-Celigny P, Zinzani PL, et al. Multicenter phase II trial of immunotherapy with the humanized anti-CD22 antibody, epratuzumab, in combination with rituximab, in refractory or recurrent non-Hodgkin's lymphoma. J Clin Oncol. 2006; 24(24):3880-6.
26. Cesano A, Gayko U. CD22 as a target of passive immunotherapy. Semin Oncol. 2003;30(2):253-7.
27. Dyer MJ, Fischer P, Nacheva E, Labastide W, Karpas A. A new human B-cell non-Hodgkin's lymphoma cell line (Karpas 422) exhibiting both t (14;18) and t(4;11) chromosomal translocations. Blood. 1990;75(3): 709-14.
28. Beckwith M, Longo DL, O'Connell CD, Moratz CM, Urba WJ. Phorbol ester-induced, cell-cycle-specific, growth inhibition of human B-lymphoma cell lines. J Natl Cancer Inst. 1990;82(6):501-9.
29. Winter JN, Variakojis D, Epstein AL. Phenotypic analysis of established diffuse histiocytic lymphoma cell lines utilizing monoclonal antibodies and cytochemical techniques. Blood. 1984;63(1):140-6.
30. Jadayel DM, Lukas J, Nacheva E, Bartkova J, Stranks G, De Schouwer PJ, et al. Potential role for concurrent abnormalities of the cyclin D1, p16CDKN2 and p15CDKN2B genes in certain B cell non-Hodgkin's lymphomas. Functional studies in a cell line (Granta 519). Leukemia. 1997; 11(1):64-72.
31. Bogner C, Dechow T, Ringshausen I, Wagner M, Oelsner M, Lutzny G, et al. Immunotoxin BL22 induces apoptosis in mantle cell lymphoma (MCL) cells dependent on Bcl-2 expression. Br J Haematol. 2010;148(1):99-109.
32. Jeon HJ, Kim CW, Yoshino T, Akagi T. Establishment and characterization of a mantle cell lymphoma cell line. Br J Haematol. 1998;102(5):1323-6.
33. O'Brien SM, Kantarjian H, Thomas DA, Giles FJ, Freireich EJ, Cortes J, et al. Rituximab dose-escalation trial in chronic lymphocytic leukemia. J Clin Oncol. 2001; 19(8):2165-70.
34. Wierda WG, Kipps TJ, Mayer J, Stilgenbauer S, Williams CD, Hellmann A, et al. Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. J Clin Oncol. 2010;28(10):1749-55.
35. Leonard JP, Coleman M, Ketas JC, Chadburn A, Ely S, Furman RR, et al. Phase I/II trial of epratuzumab (humanized anti-CD22 antibody) in indolent non-Hodgkin's lymphoma. J Clin Oncol. 2003;21(16):3051-9.
36. Kreitman RJ, Squires DR, Stetler-Stevenson M, Noel P, FitzGerald DJ, Wilson WH, et al. Phase I trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with B-cell malignancies. J Clin Oncol. 2005;23(27): 6719-29.
37. al-Katib A, Wang CY, McKenzie S, Clarkson BD, Koziner B. Phorbol ester-induced hairy cell features on chronic lymphocytic leukemia cells in vitro. Am J Hematol. 1992; 40(4):264-9.
38. Lee HW, Smith L, Pettit GR, Smith JB. Bryostatin 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts. Mol Pharmacol. 1997;51 (3):439-47.
39. Pavlick AC, Wu J, Roberts J, Rosenthal MA, Hamilton A, Wadler S, et al. Phase I study of bryostatin 1, a protein kinase C modulator, preceding cisplatin in patients with refractory non-hematologic tumors. Cancer Chemother Pharmacol. 2009;64(4):803-10.
40. Grant S, Roberts J, Poplin E, Tombes MB, Kyle B, Welch D, et al. Phase Ib trial of bryostatin 1 in patients with refractory malignancies. Clin Cancer Res. 1998;4(3): 611-8.
41. Du X, Youle RJ, FitzGerald DJ, Pastan I. Pseudomonas exotoxin A-mediated apoptosis is Bak dependent and preceded by the degradation of Mcl-1. Mol Cell Biol. 2010;30(14):3444-52.