Sepantronium Bromide (YM155) Enhances Response of Human B-Cell Non-Hodgkin Lymphoma to Rituximab

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ABSTRACT

In the treatment of B-cell non-Hodgkin lymphoma (B-NHL), rituximab improves long-term survival in combination with conventional chemotherapy. However, because the majority of patients with B-NHL eventually relapse, the development of more effective therapies is needed. Here, we evaluated the antitumor effects of a combination treatment involving sepantronium bromide (YM155), a first-in-class survivin suppressant, and rituximab in B-NHL xenograft mice models. To determine the efficacy of the combination treatment, YM155- and rituximab-treated B-NHL cell xenografted mice were monitored for tumor size and survival and subjected to 2\(^{18}\)F-fluoro-2\(^{18}\)F-fluoro-D-glucose (18F-FDG) and 3\(^{18}\)F-fluoro-3\(^{18}\)F-fluoro-3\(^{18}\)F-fluoro-3\(^{18}\)F-fluoro-3\(^{18}\)F-deoxythymidine (18F-FLT) positron emission tomography (PET) imaging. In addition, the cell proliferation status of excised tumors was examined by Ki-67 immunohistochemistry. In DB, WSU-DLCL-2, and Mino xenograft-bearing mice, the combination treatment of YM155 and rituximab induced significant tumor growth inhibition and tumor regression compared with either single agent. On day 3 after the initiation of treatment a significant decrease in both 18F-FDG and 18F-FLT tumor uptake from pretreatment levels was observed in combination treatment groups. The Ki-67 proliferation index was significantly decreased on day 3 in the xenograft models treated with combination treatment, suggesting that the combination of YM155 and rituximab reduced cell proliferation and glucose metabolism. Furthermore, compared with monotherapy, combination treatment prolonged survival times of severe combined immunodeficient mice with disseminated WSU-FSCCL and Jeko B-NHL tumors. Our findings demonstrate that YM155 and rituximab combination treatment enhances antitumor activity in B-NHL xenografts, and 18F-FLT and 18F-FDG PET imaging may allow the early functional evaluation of treatment responses in patients with B-NHL.

Introduction

B-cell non-Hodgkin lymphoma (B-NHL) is a heterogeneous group of B-cell malignancies that display considerable genetic diversity, which reflects the differentiation status of lymphocytes at the time of transformation (Küppers, 2005). The anti-CD20 antibody rituximab (Rituxan; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) is standard therapy for many types of CD20-positive B-cell lymphomas and improves long-term survival in combination with conventional chemotherapy (Cheson and Leonard, 2008). Because the majority of B-NHL patients eventually relapse (Coiffier, 2007), high-dose chemotherapy followed by autologous stem cell transplant is the accepted second-line treatment for diffuse large B-cell lymphoma (DLBCL), grade 3 follicular lymphoma, and mantle cell lymphoma. However, because many patients are not candidates for autologous stem cell transplant because of advanced age or comorbidities, a need exists for the development of novel therapeutic strategies to supplement current B-NHL treatment regimens.

In patients with B-NHL, increased expression levels of the antiapoptotic protein survivin are typically observed in malignant lymphocytes (Ambrosini et al., 1997). Survivin positivity in patients with B-NHL is also associated with increased chemo-resistance and worse clinical outcomes (Adida et al., 2000; Markovic et al., 2011). In addition, functional knockdown of survivin by short hairpin RNA induces cell apoptosis and growth inhibition of B-NHL cells (Ansell et al., 2004). Taken together, these findings suggest that survivin-targeted therapy would be suitable for the treatment of B-NHL.

ABBREVIATIONS: B-NHL, B-cell non-Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; 18F-FDG, 2\(^{18}\)F-deoxy-2\(^{18}\)F-fluoro-o-glucose; 18F-FLT, 3\(^{18}\)F-fluoro-3\(^{18}\)F-deoxythymidine; PET, positron emission tomography; SCID, severe combined immunodeficient; SUV, standardized uptake value; YM155, sepantronium bromide.
Sepantronium bromide (YM155) is a first-in-class compound that functions selectively to suppress survivin expression (Nakahara et al., 2007). We demonstrated previously that YM155 displays potent anticancer activity against a broad spectrum of human cancer cell lines and various human-derived tumor xenograft mouse models (Nakahara et al., 2011a; Yamanaka et al., 2011b). In aggressive B-NHL models, YM155 treatment resulted in potent and sustained antitumor activity (Kita et al., 2011). Immunohistochemical analyses of non-small cell lung cancer, melanoma, and B-NHL tumors have indicated that YM155-induced tumor regression is associated with the down-regulation of intratumoral survivin expression, growth inhibition, and apoptotic induction of tumor cells (Kita et al., 2011; Nakahara et al., 2011b; Yamanaka et al., 2011a). Therefore, we hypothesized that YM155 treatment in combination with rituximab would be effective against B-NHL, and we evaluated the efficacy of combination treatment by using human B-NHL-xenografted mouse models on tumor size and survival.

One powerful technique for evaluating the therapeutic efficacy of drugs in the clinical setting is positron emission tomography (PET) imaging, which allows noninvasive assessment of functional and metabolic alterations in tumor cells. The glucose analog 2-deoxy-2-18F-fluoro-D-glucose (18F-FDG) is the most commonly used PET tracer for diagnosis, staging, and response monitoring in patients with malignant lymphoma including DLBCL (Klabbers et al., 2003; Shankar et al., 2006). A second attractive PET tracer is 3'-18F-fluoro-3'-deoxythymidine (18F-FLT), which is a substrate of the DNA synthetic pathway and considered to be highly specific for proliferating tumor cells (Shields et al., 1998; Vesselle et al., 2002). 18F-FLT uptake correlates with the rate of tumor proliferation, as measured by Ki-67 tumor staining, in patients with non-small cell lung cancer (Vesselle et al., 2002). However, the potential utility of 18F-FDG and 18F-FLT PET for the assessment of tumor response to YM155 treatment has not been demonstrated. Here, we evaluated the antitumor activity of YM155 combined with rituximab against B-NHL by interim 18F-FDG and 18F-FLT PET imaging using human B-NHL-xenografted mouse models.

### Materials and Methods

**YM155 and Rituximab.** YM155 was synthesized in-house by Astellas Pharma, Inc. (Nakahara et al., 2007). For in vivo experiments, YM155 was dissolved and diluted in saline immediately before administration. Dose levels are expressed in terms of YM155, the cationic moiety of the drug substance. Rituximab was obtained from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan) and diluted in saline immediately before administration.

**Cell Lines.** The human DLBCL cell line DB and the human mantle cell line Mino were purchased from the American Type Culture Collection (Manassas, VA). The human DLBCL cell line WSU-DLCL-2, human follicular lymphoma cell line WSU-FSCCL, and human mantle cell lymphoma cell line Jeko were purchased from the German Resource Center for Biological Material (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) in a humidified incubator with an atmosphere containing 5% CO2 at 37°C.

**Animals for In Vivo Studies.** All animal experimental procedures were approved by the institutional animal care and use committee of Astellas Pharma, Inc., and Astellas Pharma, Inc., Tsukuba Research Center was awarded Accreditation Status by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were euthanized if tumor burden exceeded 10% of the host animal’s body weight.

**In Vivo Antitumor Activity in Subcutaneous Tumor Xenograft Models.** Five-week-old male BALB/c nude mice (CAnN.Cg-Foxn1nu/CrlCrlj) were obtained from Charles River Japan (Kanagawa, Japan). Cultured DB, WSU-DLCL-2, and Mino cells were harvested, suspended in a phosphate buffer solution, and then subcutaneously injected into the flanks of mice (3 × 106 cells/0.1 ml/mouse). Body weight and tumor volume were assessed twice weekly. Tumor volume was estimated by calculating tumor dimensions using calipers (length × width2 × 0.5). When tumor volumes reached >300 mm3, animals were randomly assigned into groups based on tumor volume. In the combination treatment study, the first day of drug treatment was designated as day 0, and observations continued until day 21, representing one full treatment cycle in the clinical study of YM155. YM155 was administered as a 7-day continuous infusion by using a micro-osmotic pump (Alzet models 1003D and 1007D; Durect Corporation, Cupertino, CA). Rituximab was administered intravenously at 50 mg/kg on days 0 and 2. Percentage of tumor regression was calculated by using the following formula: 100 × (1 − (mean tumor volume of each group on day 21/mean tumor volume of each group on day 0)).

**18F-FDG and 18F-FLT PET Imaging.** 18F-FDG was purchased from Nihon Medi-Physics (Hakui, Japan). 18F-FLT was synthesized in-house from its precursors. In brief, 18F was produced via an 18O(p,n)18F nuclear reaction by proton bombardment (12 MeV; 40 μA) of a 18O-water target by using a cyclotron-target system (OSCAR-12; JFE Plant and Service Corp., Yokohama, Japan). 18F-FLT was then synthesized by nucleophilic fluorination of 3-N-boc-5′-O-dimethoxytrityl-3′-O-nosyl-thymidine. For PET imaging studies, 5 × 106 cells/0.1 ml/mouse were inoculated subcutaneously in the right hind leg. 18F-FDG and 18F-FLT uptake were evaluated by using a small-animal Inveon PET scanner (Siemens, Knoxville, TN) on days 0 and 3 of treatment. Selected mice for 18F-FDG PET scans were fasted for more than 12 h before imaging. For imaging, 6- to 12-MBq aliquots of 18F-FDG or 18F-FLT were administered by tail vein injection to conscious animals, which were then maintained in cages for 1 h to allow for 18F-FDG and 18F-FLT uptake into tumors. Under 2.0 to 2.5% isoflurane gas anesthesia, mice were placed on the scanning stage, and a 5-min emission scan was performed by using the PET scanner. Images were reconstructed by OSEM3D and analyzed with ASIPro software (Seimens Medical Solutions, Malvern, PA). Regions of interest were drawn around the boundaries of tumors on axial slices, and the observed maximum pixel value was normalized to the injected dose and body weight to give the maximum standardized uptake value (SUVmax).

**Immunohistochemical Analysis of Lymphoma Xenografts.** Tumors were excised from mice, weighed, fixed in 4% paraformaldehyde, embedded in paraffin, and then sectioned at 5-μm thickness. The sections were dewaxed in xylene and then subjected to antigen retrieval by placing samples in 10 mM citrate buffer, followed by autoclaving for 15 min at 121°C. The sections were then incubated with mouse monoclonal anti-Ki-67 antibody (Dako North America, Inc., Carpenteria, CA) for 1 h. Antibody binding was visualized by using the Envision System (Madison, NJ). The sections were then incubated with diaminobenzidine and counterstained with hematoxylin. The Ki-67 proliferation index of each group was calculated as follows: Ki-67-positive cells (per 1000 cells) × tumor weight (g).

**Studies with Disseminated Xenografts.** Four-week-old female SCID mice (CB17/Scid-Prkdcscid/CrlCrlj) were purchased from Charles River Japan. WSU-FSCCL and Jeko cells were injected intravenously (4 × 106 cells/0.2 ml/mouse), and dissemination and tumor growth were allowed to proceed for 7 days. The mice were then divided into four comparable groups of 10 animals each based on body weight by using SAS software (SAS Institute, Cary, NC), and a clinical dose of either YM155 (2 mg/kg 7-day continuous infusion),
rituximab (50 mg/kg intravenously), or YM155 (2 mg/kg 7-day continuous infusion) plus rituximab (50 mg/kg intravenously) was administered once every 3 weeks for three courses in WSU-FSCCL and one course in Jeko models. Animals were monitored daily for the occurrence of mortality and euthanized while under diethyl ether anesthesia. Mice were scored as dead if any of the following signs of suffering were observed: cachexia, weakening, and difficulty in moving or eating.

Statistical Analysis. Differences in tumor volume on day 21 between the combination treatment group and each single-compound group were evaluated by using Student’s t test. Immunohistochemistry and PET imaging data were analyzed by using analysis of variance followed by post hoc Tukey’s test. For survival analysis, Kaplan-Meier curves were generated, and the differences between the control and drug-treated groups were assessed by using the log-rank test. All statistical analyses were performed by using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA), and P values less than 0.05 were considered to be significant. All values are expressed as means and S.E.M.

Results

YM155 Enhances Antitumor Activity of Rituximab against Subcutaneous Xenograft Tumors. We first evaluated the antitumor effects of YM155 in combination with rituximab in B-NHL xenograft-bearing mice models (Fig. 1). Because our preliminary studies demonstrated that a 2 mg/kg dose of YM155 induced tumor regression, including a complete response, a dose lower than 2 mg/kg was used in the present study. In each xenograft model, rituximab monotherapy showed slight antitumor activity. For YM155 monotherapy, although a low dose of YM155 (0.5 or 1 mg/kg/day) caused a delay in tumor growth, tumors began to regrow during the cessation of treatment, and none of the tumors showed complete regression by day 21. In contrast, combination treatment with YM155 and rituximab resulted in significant tumor regression (>100% inhibition). The percentage of tumor regression in the combination treatment group on day 21 was 76, 29, and 9% for the DB-, WSU-DLCL-2-, and Mino-xenografted mice models, respectively (Fig. 1). A complete response was achieved in two of five DB-xenografted mice. In addition, no animals in any group exhibited significant body weight loss at any time point during treatment (data not shown).

18F-FDG and 18F-FLT PET Imaging of WSU-DLCL-2 Xenograft-Bearing Mice. To assess the functional changes of tumor xenografts by the combination treatment, we performed 18F-FDG and 18F-FLT PET imaging as indicators of glucose metabolism and cell proliferation, respectively. Representative 18F-FDG (Fig. 2A) and 18F-FLT (Fig. 2B) images of WSU-DLCL-2 xenograft-bearing mice taken at baseline (before treatment, day 0) and 3 days post-treatment are shown. A marked decrease of tumoral FDG and FLT uptake 3 days after the initiation of combination treatment was observed (Fig. 2). In contrast, FDG and FLT uptake remained high in the control and rituximab monotherapy mice. Tumoral uptake of both FDG or FLT into the xenografts of all mice were analyzed, and the results are summarized in Table 1. Within 3 days of treatment, a decrease (42%) in 18F-FDG uptake was achieved in the combination treatment group (P < 0.05 versus control). YM155 monotherapy and combination treatment significantly reduced the tumor uptake of 18F-FLT by 32 and 39%, respectively (P < 0.05; P < 0.01 versus control). The combination treatment group also decreased 18F-FLT uptake compared with the rituximab monotherapy group (36%) (P < 0.05).

Fig. 1. Antitumor activity of YM155 against aggressive B-NHL xenografts. BALB/c nude mice bearing DB (A), WSU-DLCL-2 (B), and Mino (C) xenografts were treated with YM155 and rituximab. YM155 was given as a 7-day continuous subcutaneous infusion starting on day 0, and rituximab was administered intravenously by bolus injection on days 0 and 2. Tumor volume on day 21 was compared between each single-treatment group and the combination group by using Student’s t test. *, P < 0.05; **, P < 0.01 versus YM155 monotherapy group. ##, P < 0.01; ###, P < 0.001 versus rituximab monotherapy group. Mean, S.E.M. (n = 5 mice/group).
Histology and Ki-67 Immunohistochemistry. To examine the feasibility of PET analysis as a complementary technique to immunohistochemistry in routine clinical applications, we also analyzed tissue samples by using a standard Ki-67 immunostaining assay as an indicator of cell proliferation status. Representative sections from treated and untreated tumors are shown in Fig. 3. We found that sections obtained from untreated WSU-DLCL-2 xenotransplants showed high proliferative activity. In YM155 monotherapy (−46%) and rituximab monotherapy (−23%), and combination-treated animals (−56%), a significant decrease in the Ki-67 proliferation index was detected on day 3 (Table 2; \( P < 0.01 \), versus control). A significant decrease was observed in combination treatment tumors compared with rituximab monotherapy (\( P < 0.01 \)).

YM155 in Combination with Rituximab against Disseminated Aggressive B-NHL Xenografts. Advanced B-NHL commonly develops clinically as disseminated disease. To examine the survival benefit of YM155 and rituximab combination treatment, we evaluated the effects of the clinical dosage of YM155 (2 mg/kg/day) alone or in combination with rituximab in the disseminated SCID/WSU-FSCCL and SCID/Jeko lymphoma models (Fig. 4). The administration of either YM155 or rituximab alone extended the median survival from 35 to 60 and 42 days, respectively, in WSU-FSCCL-xenografted mice, and from 54 to 67 and 90 days, respectively, in Jeko-xenografted mice. It is noteworthy that the addition of YM155 to rituximab treatment significantly prolonged survival compared with the respective monotherapies in both examined models.

Discussion

The present study clearly shows that the survivin-suppressant YM155, in combination with rituximab, results in significant improvements in tumor regression and survival in B-NHL xenograft models. We also demonstrated that the antitumor effect of combined YM155 and rituximab treatment is accompanied by decreases in tumor glucose metabolism and cell proliferation, as assessed by small-animal \(^{18}\)F-FDG and \(^{18}\)F-FLT PET imaging.

In the DB, Mino, and WSU-DLCL-2 xenograft models, although rituximab alone exhibited slight antitumor activity, the combination of low doses of YM155 (0.5 or 1 mg/kg/day)
with rituximab significantly improved tumor response compared with the monotherapy groups (Fig. 1). Advanced B-NHL commonly develops clinically as disseminated disease. Here, the combination of YM155 and rituximab also significantly prolonged the survival of disseminated B-NHL models compared with either drug alone (Fig. 4). Because increased survivin dependence for tumor growth is reported to be associated with poor clinical outcomes, the combination of a survivin suppressant, YM155, and an anti-CD20 antibody may represent a potent strategy to treat B-NHL (Adida et al., 2000; Markovic et al., 2011).

A number of studies have suggested that the combination of rituximab with various agents potentially improves their therapeutic potential (Di Gaetano et al., 2001; Ghetie et al., 2001; Alas et al., 2002; Chow et al., 2002; Emmanouilides et al., 2002; Boye et al., 2003; Jazirehi et al., 2003; Ackler et al., 2010). The mechanisms of action of rituximab are not fully understood and remain a matter of debate. Two immune-mediated mechanisms, antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, have attention as possible processes linked to rituximab action. To investigate whether natural killer-dependent antibody-dependent cell-mediated cytotoxicity activity is important for enhancing the effectiveness of YM155 treatment in vivo, we evaluated the combination regimen in lymphoma xenografts established in NOD/SCID mice, which have impaired T/B cell lymphocyte development and natural killer cell function (Shultz et al., 1995). Combined rituximab and YM155 treatment resulted in a significant enhancement of antitumor activity over rituximab alone, a finding that is consistent with the results in BALB/c nude mice (data not shown). This result suggests that the therapeutic interaction between these two agents does not seem to arise from the antibody-mediated effector functions of rituximab, and the rituximab-mediated inhibition of intracellular survival pathways is important for enhancing the effectiveness of YM155 treatment in vivo. Mechanistically, rituximab has been reported to abrogate the intracellular signal transduction of survival pathways involving p38, mitogen-associated protein kinase, and nuclear factor-κB, resulting in decreased Bcl-2 expression and sensitization to drug-induced apoptosis. Rituximab also up-regulates Raf kinase inhibitor protein, thus decreasing the activity of the extracellular signal-regulated kinase 1/2 pathway, leading to reduced Bcl-xL expression (Bonavida, 2007; Vega et al., 2011). Previously, we have demonstrated that concomitant treatment with YM155 enhanced chemosensitivity to Bcl-2 inhibitor in DLBCL (data not shown). We postulate that the anticancer effects of YM155 and rituximab observed here result from the simultaneous inhibition of different cell survival pathways, the survivin pathway by YM155, and the Bcl-2/Bcl-xL pathway by rituximab. However, Western blot analysis revealed that rituximab did not suppress the expression of Bcl-2/Bcl-xL in the DLBCL cell line (data not shown). Thus, further investigations are required to identify the signaling pathways that are affected by the combined exposure to YM155 and rituximab.

We explored whether 18F-FDG and 18F-FLT accumulation are sufficiently large to be quantitatively imaged with PET early in the treatment. The uptake of 18F-FDG and 18F-FLT was significantly decreased in the combination-treated group by day 3; on the other hand, no apparent decrease was observed in the rituximab monotherapy group (Fig. 2). Both PET imaging techniques could detect early responses to treatment based on tumor activity, with results that were consistent with tumor regression determined by caliper measurements (Figs. 1 and 2). Recent animal and preliminary clinical studies have suggested that 18F-FLT may be more sensitive than 18F-FDG for assessing tumor response (Troost et al., 2010; Kahraman et al., 2011). Our present findings indicate that 18F-FDG and 18F-FLT have comparable sensitivities for detecting early changes in tumor cell activity after combined YM155 and rituximab treatment in a B-NHL xenograft model (Fig. 2), indicating that the combination treatment induces suppression of glucose metabolism and cell proliferation. It is noteworthy that the significant decrease in 18F-FLT uptake observed here in combination-treated mice was also consistent with a decrease in Ki-67 immunostaining (Fig. 3). Thus, the similar trends observed between PET output metrics and the standard histological marker Ki-67 suggest that PET imaging can be used to monitor cellular proliferation in tumors. These results suggest that PET imaging with 18F-FDG or 18F-FLT could evaluate the response of tumors to YM155 and rituximab treatment within several days of initiating combined treatment, because this would allow nonresponding tumors to be identified.

In conclusion, combined YM155 and rituximab treatment has shown promising antitumor effects in B-NHL xenograft mouse models. In addition, PET imaging data suggest that tumor responses to combination treatment of YM155 and rituximab can be monitored by using the tracer 18F-FLT with a similar sensitivity to that of 18F-FDG-PET. Based on the positive preclinical results of YM155 in B-NHL, a phase II
open-label study of YM155 in combination with rituximab in relapsed patients with CD20-positive B-NHL was initiated and is currently underway.

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Authorship Contributions

Participated in research design: Kita, Mitsuoka, Miyoshi, and Sasamata.

Conducted experiments: Kita, Mitsuoka, Kaneko, Nakata, Jitsuoka, and Miyoshi.

Contributed new reagents or analytic tools: Jitsuoka.

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