Prenyltransferases Regulate CD20 Protein Levels and Influence Anti-CD20 Monoclonal Antibody-mediated Activation of Complement-dependent Cytotoxicity*§

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Background: The influence of farnesyltransferase inhibitors (FTIs) on CD20 levels is unknown.

Results: FTIs increase CD20 expression and improve rituximab-mediated activation of complement-dependent cytotoxicity. FTIs sensitize tumor cells to anti-CD20 mAbs. The combination of FTIs with anti-CD20 mAbs seems to be a reasonable therapeutic approach worth to be tested in patients with B-cell tumors.

Conclusion: FTIs increase CD20 expression and improve rituximab-mediated activation of complement-dependent cytotoxicity. The influence of farnesyltransferase inhibitors (FTIs) on CD20 levels is unknown. FTIs increase CD20 expression and improve rituximab-mediated activation of complement-dependent cytotoxicity.

Significance: The combination of FTIs with anti-CD20 mAbs seems to be a reasonable therapeutic approach worth to be tested in patients with B-cell tumors.

Anti-CD20 monoclonal antibodies (mAbs) such as chimeric rituximab and completely human ofatumumab are used in the treatment of both indolent and aggressive non-Hodgkin lymphomas (NHLs)1 and have revealed a significant activity in patients with chronic lymphocytic leukemia (CLL) (1). These antibodies can trigger indirect effector mechanisms of the immune system such as activation of complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and immunophagocytosis (1, 2). Although the antitumor activity of anti-CD20 mAbs in NHL is well established, the mechanisms of this resistance are still incompletely understood. In some in vitro studies with cells derived from patients suffering from various B-cell malignancies, a positive correlation between CD20 levels and rituximab sensitivity was found (5, 6). van Meerten et al. (7) have demon-

Anti-CD20 monoclonal antibodies (mAbs) such as chimeric rituximab and completely human ofatumumab are used in the treatment of both indolent and aggressive non-Hodgkin lymphomas (NHLs)2 and have revealed a significant activity in patients with chronic lymphocytic leukemia (CLL) (1). These antibodies can trigger indirect effector mechanisms of the immune system such as activation of complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and immunophagocytosis (1, 2). The mechanisms of this resistance are still incompletely understood. In some in vitro studies with cells derived from patients suffering from various B-cell malignancies, a positive correlation between CD20 levels and rituximab sensitivity was found (5, 6). van Meerten et al. (7) have demon-

The abbreviations used are: NHL, non-Hodgkin lymphoma; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; FNTB, farnesyltransferase subunit β; FTL, farnesyltransferase inhibitor; GGTL, geranylgeranyltransferase inhibitor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; R-CDC, rituximab-mediated complement-dependent cytotoxicity; PI, propidium iodide; CFSE, carboxyfluorescein succinimidyl ester; shRNA, short hairpin RNA.
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strated a sigmoidal correlation between CD20 expression level and rituximab-mediated CDC (R-CDC) but not ADCC. In this in vitro experimental model, the level of CD20 expression was the only variable, and it was clearly shown that reduced CD20 expression leads to impaired CDC. A direct correlation between R-CDC and the number of CD20 molecules in primary NHL cells was also found by Bellosillo et al. (6). Therefore, strategies that lead to up-regulation of CD20 expression may improve R-CDC against low CD20-expressing cells and provide a rationale for overcoming rituximab resistance.

Accumulating evidence indicates that CD20 can be modulated at transcriptional, posttranscriptional, and even post-translational levels. Several case or retrospective studies reported that rituximab treatment may result in CD20-negative relapses (8–15), although their prevalence and duration are currently unknown. A number of mechanisms that account for the modulation of CD20 levels have been proposed. Most likely their occurrence and significance vary depending on the type of malignancy. In CLL, rituximab-mediated down-modulation of CD20 is associated with reduced levels of CD20 mRNA both in vitro (16) and in vivo (17), indicating regulation at the level of transcription. For example, activated Flt3 signaling cascade has been reported to inhibit expression of PU.1, a transcription factor involved in the expression of CD20 gene (18). Down-regulation of CD20 mRNA has been also observed in CD20-negative cells obtained from patients after relapse of rituximab-treated B-cell malignancies (15). Several studies revealed that CD20 can undergo “shaving” (19) or lysosomal internalization (20) following rituximab exposure. Epigenetic mechanisms also play an emerging role in the regulation of CD20 levels (15, 21, 22).

We have observed previously that statins impair detection of CD20 in NHL cells and impair R-CDC and ADCC (23). Statins are inhibitors of cholesterol synthesis and decrease production of prenyl groups (farnesyl and geranylgeranyl pyrophosphates), which are necessary for posttranslational modification of ~1% of cellular proteins. In experiments aimed at elucidation of the molecular mechanisms of statin-mediated modulation of CD20, we observed that neither geranylgeranyltransferase (GGTI) nor farnesyltransferase (FTI) inhibitors could mimic the effect of statins. On the contrary, prenyltransferase inhibitors improved R-CDC. FTIs were initially developed to target tumors with Ras mutation (24). However, subsequent studies revealed their activity in tumors with normal Ras that seems to result from inhibition of prosurvival signaling mediated by other prenylation-dependent pathways. Importantly, tipifarnib, a farnesyltransferase inhibitor, was recently shown to exert some therapeutic activity in patients with relapsed and refractory lymphomas (25). Therefore, we decided to investigate in more detail the influence of prenyltransferase inhibitors on antitumor activity of anti-CD20 mAbs.

EXPERIMENTAL PROCEDURES

Cell Culture—Human Burkitt lymphoma (Raji and Ramos) and human follicular lymphoma (DoHH2) cell lines (purchased from American Tissue Culture Collection), HEK-293T cells (purchased from DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 units/ml penicillin, and 250 ng/ml amphotericin B (Invitrogen). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ and passaged approximately every other day.

Leukocyte Isolation from Blood and in Vitro Culture—Primary cells from patients with B-cell tumors (NHL and CLL) were isolated from full blood using Histopaque-1077 (Sigma-Aldrich) as described elsewhere (23). Cells were cultured with increasing concentrations of L-744,832 for 48 h in Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, 100 units/ml penicillin, and 250 ng/ml amphotericin B (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO₂. Approval for the study was obtained from the Institutional Review Board of the Medical University of Warsaw and was conducted according to the Declaration of Helsinki. Each patient gave a written informed consent for the procedures.

Reagents—Rituximab, a chimeric IgG1, was purchased from Roche Applied Science. Ofatumumab (2F2; HuMax-CD20) and FITC-conjugated ofatumumab were generous gifts from Genmab A/S (Utrecht, The Netherlands). Farnesyltransferase inhibitors (FTI-276 and FTI-277) and geranylgeranyltransferase inhibitors (GGTI-286, GGTI-298, and GGTI-2133) were from Calbiochem (Merck LGaA). L-744,832 was purchased from Enzo Life Sciences (Plymouth Meeting, PA). FTI-277 was dissolved in water, whereas other inhibitors were dissolved in dimethyl sulfoxide. Bortezomib obtained from Millenium Pharmaceuticals was dissolved in 0.9% NaCl. Cycloheximide (Sigma-Aldrich) was freshly dissolved before each experiment in water. Propidium iodide (PI) was purchased from Sigma and dissolved in water at a concentration of 10 mg/ml.

Cytotoxicity Assays—Complement-dependent cytotoxicity was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay or propidium iodide staining. An MTT assay on control or drug-treated cells was performed as described elsewhere (23). Cell viability was expressed as a relative viability of tumor cells (percentage of control cultures incubated with medium and serum). For PI staining, control or drug-treated cells were rinsed with PBS and seeded into a 96-well flat bottom plate in FBS-free RPMI 1640 medium as described above. Rituximab/ofatumumab and human AB serum (10% final concentration) were added to a final volume of 100 μl/well. After a 1-h incubation, 100 μl of PI (4 μg/ml) was added to each well. Cells (alive, PI-negative; dead, PI-positive) were analyzed for red fluorescence on a flow cytometer (FACScan, BD Biosciences).

ADCC—Raji cells were resuspended in PBS, 0.1% BSA (6 X 10⁶ cells/ml) and incubated with 1 μl of CFSE (5 mM stock in DMSO; Molecular Probes) for 10 min at 37 °C in the dark. Cells were washed twice with 10 ml of ice-cold PBS to avoid excessive staining. After a 48-h incubation with L-744,832, CFSE-stained cells were washed with PBS and seeded at a density of 2 X 10⁵ cells/50 μl into a 96-well round bottom microtiter plate. Then rituximab (100 μg/ml) or a control medium was added for 15 min followed by addition of natural killer cells (at an effector: target ratio of 5:1) isolated from peripheral blood mononuclear
cells of healthy donors. Magnetic natural killer cell isolation was performed with an EasySep® Human CD56 Positive Selection kit (STEMCELL Technologies Inc.). After incubation (4 h at 37 °C), 100 μl of PI (final concentration, 4 μg/ml) was added. Raji cells were analyzed for green and red fluorescence by flow cytometry (FACScan, BD Biosciences). At least 1 × 10⁶ CFSE-stained events were analyzed in each sample. The percentage of cellular cytotoxicity was calculated in the CFSE-positive population using the following formula: % specific lysis = (% of PI-negative cells in samples with rituximab and PBMC/%) of PI-negative cells in control samples) × 100.

Flow Cytometry Studies—A suspension (100 μl) of control or drug-treated cells at a density of 2 × 10⁶/ml was incubated with saturating amounts of fluorochrome-conjugated antibodies for 30 min at room temperature in the dark. The following FITC-conjugated antibodies were used: IgG1 (isotypic control, clone X40; BD Biosciences), anti-CD20 (clone L27; BD Biosciences), anti-CD55 (clone IA10; BD Pharmingen), anti-CD46 (clone E4.3; BD Pharmingen), anti-CD21 (Gen Trak), anti-CD45RA (clone L48; BD Biosciences), anti-CD19 (clone J3-119; Beckman Coulter), anti-CD54 (clone 84H10; Immunotech), anti-HLA-DR (clone L243; BD Biosciences), ofatumumab (Genmab A/S), and polyclonal rabbit anti-human IgG (Dako). The following unconjugated antibody was used: rituximab (Roche Applied Science). Prior to analysis, the cells were washed with PBS and resuspended in PBS supplemented with PI (2 μg/ml). The results were analyzed using CellQuest Pro Software Version 5.2. The mean fluorescence intensity of PI-negative cells served as a measure for antibody binding on a per cell basis.

Reverse Transcription PCR (RT-PCR) and Real Time PCR—Cells were washed with PBS, pelleted, and resuspended in 1 ml of TRIzol reagent (Invitrogen) to extract total RNA according to the manufacturer’s protocol. The first strand cDNA synthesis utilizing 0.5 to the manufacturer’s protocol. The chromatin was eluted from the beads with proteinase K for 2 h at 65 °C. Subsequently, DNA was purified using ChIP Grade Protein G Magnetic Beads (Cell Signaling Technology) according to the manufacturer’s protocol. The chromatin was eluted from the beads by adding elution buffer and incubation at 65 °C for 30 min followed by digestion with proteinase K for 2 h at 65 °C. Subsequently, DNA was purified using spin columns (Qiagen).

Quantitative Real Time PCR for ChIP Samples—Real-time PCR was performed using LightCycler Fast Start DNA Master PLUS SYBR Green I (Roche Applied Science) and LightCycler Probes Master (Roche Applied Science) and probes labeled with Fam and Dabcyl. The amplification of cDNA was performed using a LightCycler 480 II device (Roche Applied Science) according to the manufacturer’s recommendations for a final volume of 20 μl. In each PCR run, the samples were measured in duplicates to estimate their reproducibility. The specific primers used for reactions with LightCycler Fast Start DNA Master PLUS SYBR Green I as well as primers and probes used for reactions with LightCycler Probes Master (Roche Applied Science) are shown in the supplemental Methods. Samples in which the cDNA was omitted were used as negative controls. The results were analyzed after 40 cycles of amplification using LightCycler 480 Software 1.5 and normalized for the content of the housekeeping genes (actin for quantitative real time PCR using SYBR Green and β₂-microglobulin for quantitative real time PCR using hydrolysis probes). The -fold change for each gene was calculated using a user-non-influent, second derivative method. The specificity of the reactions was confirmed by analysis of the PCR product melting profile.

Western Blotting—Control or drug-treated cells were washed twice with PBS, lysed, and separated on a 10% SDS-polyacrylamide gel as described elsewhere (23). The following antibodies (at 1:1000 dilution) were used for the overnight incubation: anti-CD20 (polyclonal; Abcam), anti-ICAM-1 (clone H-108; Santa Cruz Biotechnology), and anti-ubiquitin (clone P4D1; Santa Cruz Biotechnology). After washing with TBST (Tris-buffered saline, Tween 20), the membranes were incubated for 45 min with corresponding horseradish peroxidase (HRP)-coupled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). The chemiluminescence reaction for horseradish peroxidase was developed using self-made chemiluminescence reagent (luminol/coumaric acid/hydrogen peroxide) and visualized with a Stella 8300 imager (Raytest, Straubenhardt, Germany). The blots were stripped with 0.1 M glycine (pH 2.6) and reprobed with anti-tubulin mouse mAb (clone DM1A; Calbiochem). Densitometric analysis was performed using Aida Image Analyzer Software Version 4.26.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed using a SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology) according to the manufacturer’s protocol. The cells were fixed and cross-linked with 1% formaldehyde. After cell lysis, chromatin was digested with microwellular nuclease and sonicated (UP100H; Hielsher) to obtain DNA fragments of approximately 150–900-bp size and subsequently used for immunoprecipitation to enrich Oct-2 (1:100; clone EP543Y), TFE3 (1:20; clone H-300), and PU.1 (1:50; clone 9G7) complexes using specific antibodies (Abcam, Santa Cruz Biotechnology, and Cell Signaling Technology, respectively). Normal rabbit IgG (1:100) and anti-histone H3 (1:50) antibodies (Cell Signaling Technology) served as negative and positive controls, respectively. For each immunoprecipitation, 20 μg of cross-linked chromatin preparation diluted in ChIP buffer to a final volume of 0.5 ml was used. The samples were incubated for 4 h at room temperature (TFE3) or overnight at 4 °C (other antibodies) with rotation. Immunoprecipitation with 30 μl of ChIP Grade Protein G Magnetic Beads (Cell Signaling Technology) was performed according to the manufacturer’s protocol. The chromatin was eluted from the beads by adding elution buffer and incubation at 65 °C for 30 min followed by digestion with proteinase K for 2 h at 65 °C. Subsequently, DNA was purified using spin columns (Qiagen).
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Plasmids—A CD20 coding sequence was amplified by PCR from cDNA from Raji cells and cloned into pLVX-IRES-Puro vector (Clontech). The sequence of the construct (pLVX-CD20-IRES-Puro) was confirmed by DNA sequencing. pBSK-pCD20 containing bp -431/+52 of CD20 promoter was synthesized by Epoch Life Science (Missouri City, TX). A truncated CD20 promoter deprived of binding sites for NF-κB, Oct-1, and Oct-2 (BAT box); PU.1/Pip (PU box); and TFE3 and upstream stimulatory factor (E-box) comprising bp -39/+52 of CD20 promoter was created by PCR amplification from the wild type promoter and subcloned into luciferase expression vector pLVX-Tight-Puro-Luc (Clontech). The tPrt promoter of pLVX was replaced with CD20 promoter by ligation into BamHI and XhoI sites. GIPZ lentiviral shRNAmir (Open Biosystems, represented by Thermo Fisher Scientific, Huntsville, AL) was used for the knockdown of farnesyltransferase subunit β (FNTB) in Raji cells. The V3LHS_396971 vector encoding FNTB-targeting shRNAmir (71-2) was used for the transfection of HEK-293T cells and lentivirus production (according to the procedure described below).

Lentiviral Vector Production and Transduction of Target Cells—HEK-293T cells were seeded into 6-well plates at a density of 5 × 10⁵ cells/well in RPMI 1640 culture medium enriched with 10% heat-inactivated FBS and a mixture of antibiotics/antimycotics. 24 h later, the cells were co-transfected with 1–3 μg of gene of interest-containing vector and components of the second generation of packaging vectors, namely 1.5 μg of psPAX2 packaging vector and 1 μg of pMD2.G envelope vector using Genejuice® transfection reagent (Novagen, EMD4Biosciences Inc., Gibbstown, NJ) according to the manufacturer's protocol. pMD2.G and psPAX2 plasmids were generous gifts from Prof. Didier Trono (École polytechnique fédérale de Lausanne, Switzerland). 72 h posttransfection, the lentivirus-containing medium was collected, centrifuged at 500 × g for 10 min at room temperature, and added to the culture of target cells (Raji or HEK-293T) at a 2:1 (lentivirus-containing medium:culture medium) ratio. Puromycin (2 μg/ml) was used to select gene of interest-expressing cells.

Luciferase Assay—For luciferase assay, 3 × 10⁵ transduced Raji cells incubated for the indicated time periods with the tested compounds (cycloheximide and L-744,832) were pelleted, washed with PBS, and lysed with Passive Lysis Buffer (Promega) for 15 min. Then the lysates were centrifuged for 10 min at 13,000 × g at 4 °C, and supernatants were assayed for firefly luciferase activity using home-made luciferase substrate (0.05 mM Tris, pH 7.8, 0.01 mM MgCl₂, 0.01 mM DTT, 0.2 mM ATP, 0.5 mM luciferin, 0.25 mM CoA) in a GloMax®-Multi Detection System (Promega). Protein concentration in supernatants was estimated using the Bradford protein assay (Bio-Rad). The results were calculated as luminescence/μg of total protein and expressed as a percentage of corresponding controls.

Statistical Analysis—Data were calculated using Microsoft Excel 2007. Differences were analyzed for significance by Student’s t test. Significance was defined as a two-sided p value less than 0.05. All the experiments using cell lines were performed independently at least three times.

RESULTS

Influence of Farnesyltransferase and Geranylgeranyltransferase Inhibitors on the Activity of Anti-CD20 mAbs—In the initial experiments, the influence of non-toxic concentrations of FTI and GGTI on R-CDC was examined. Raji cells were preincubated with L-744,832 (5–20 μM), FTI-276 (5–20 μM), FTI-277 (5–20 μM), GGTI-286 (2–10 μM), GGTI-298 (1–4 μM), or GGTI-2133 (1–5 μM). After a 48-h preincubation, Raji cells were collected, and equal numbers of live cells were cultured with rituximab (1–100 μg/ml) in 10% human AB serum as a complement source to induce CDC. Survival was assessed in an MTT assay after a 1-h incubation with antibody and serum. These experiments revealed that all of the studied FTIs potentiated R-CDC (Fig. 1A). Incubation of Raji cells with GGTI resulted in mixed responses: whereas GGTI-286 slightly increased R-CDC, GGTI-298 did not affect it, and GGTI-2133 antagonized R-CDC when used at the highest (5 μM) concentration. Flow cytometry measurements of relative CD20 expression in Raji cells preincubated with FTI and GGTI revealed a correlation between increased sensitivity/resistance to R-CDC and the levels of surface CD20 (Fig. 1B). Among all FTIs and GGTIs studied, L-744,832 seemed to demonstrate the most significant influence on both R-CDC and CD20 levels. L-744,832 also significantly increased R-CDC in three of five primary tumor cells isolated from patients with NHL (Patients A–D) or CLL (Patient E) (Fig. 1C). Therefore, L-744,832 was selected for further systematic studies using Raji, Ramos, and DoHH2 cells. After a 48-h incubation with increasing concentrations of L-744,832, equal numbers of live cells were cultured with rituximab or ofatumumab (0.01–100 μg/ml depending on the cell line studied). Survival of tumor cells was measured by flow cytometry with PI staining. These experiments confirmed that L-744,832 significantly improved R-CDC against Raji and DoHH2 cells (Fig. 2, A and C). For example, survival of Raji cells dropped from 72% in controls (rituximab alone) to 42, 30, and 21% when the cells were preincubated with 5, 10, and 20 μM concentrations of L-744,832, respectively, and cultured in the presence of 1 μg/ml of rituximab and 10% human AB serum (supplemental Fig. 1A). Similarly, L-744,832 significantly potentiated ofatumumab-mediated CDC against Raji and DoHH2 cells (Fig. 2, A and C). However, L-744,832 did not sensitize Ramos cells to anti-CD20-mediated cytotoxicity (Fig. 2B). Preincubation of Raji cells with L-744,832 only slightly improved rituximab-mediated ADCC (Fig. 2D). This observation can be explained by the fact that ADCC depends to a lesser extent on the level of target antigen expression as compared with CDC.

L-744,832 Modulates CD20 Levels in B-cell Tumors—To evaluate the influence of L-744,832 on surface CD20 expression, flow cytometry was performed using a FITC-conjugated anti-CD20 mAb (L27), FITC-conjugated ofatumumab, or rituximab followed by staining with a secondary FITC-conjugated IgG. These studies revealed a significantly increased binding of anti-CD20 mAb to Raji cells incubated with a range of L-744,832 concentrations (from 5 to 20 μM) (Fig. 3, A and B, and supplemental Fig. 1B). A slightly increased binding of L27 was also observed in DoHH2 but not in Ramos cells (Fig. 3C).
Flow cytometry analysis of primary CLL cells revealed that L-744,832 increased CD20 expression by at least 20% in 11 of 18 (61.1%) cases (Fig. 4). A 20% increase in CD20 levels consistently improved R-CDC in Raji cells. Staining for other B-cell surface molecules (CD19, CD21, CD54, and HLA-DR) in L-744,832-treated Raji cells was slightly increased but to a lesser degree as compared with CD20 especially when L-744,832 was used at the highest (20 μM) concentration (Fig. 3D). Changes in the expression of complement regulatory proteins in cells preincubated with L-744,832 were relatively modest with only a 5–17% increase in the CD46 level and an 11–22% decrease in CD55 level (Fig. 3D). CD59 was...
**FIGURE 2.** The influence of L-744,832 on anti-CD20 mAb-mediated cytotoxicity (CDC and ADCC) in human Burkitt lymphoma and human follicular lymphoma cell lines. Raji (A), Ramos (B), and DoHH2 (C) cells were preincubated for 48 h with increasing concentrations of L-744,832. After washing, cells were incubated for 60 min with increasing concentrations of rituximab or ofatumumab and 10% human AB serum. Cell viability was measured with PI staining using flow cytometry. *, p < 0.05 versus controls in Student’s t test. D, CFSE-stained Raji cells preincubated for 48 h with L-744,832 cells were incubated for 4 h with rituximab (100 μg/ml) and natural killer cells at an effector:target ratio of 5:1. After addition of PI, cells were analyzed for green and red fluorescence by flow cytometry. *, p < 0.05 versus controls in Student’s t test. Error bars represent S.D.

**FIGURE 3.** The influence of L-744,832 on the expression of CD20 and other antigens in B-cell lymphoma cell lines. Raji cells (A and B) and Ramos and DoHH2 cells (C) preincubated for 48 h with increasing concentrations of L-744,832 were incubated with saturating amounts of FITC-conjugated anti-CD20 mAb (L27), FITC-conjugated ofatumumab, or rituximab followed by secondary FITC-conjugated anti-human IgG. D, Raji cells preincubated for 48 h with increasing concentrations of L-744,832 were incubated with saturating amounts of fluorochrome-conjugated mAbs (anti-CD19, anti-CD21, anti-CD54, anti-HLA-DR, anti-CD46, and anti-CD55) for 30 min at room temperature in the dark. Cells were analyzed using flow cytometry. Results are presented as mean fluorescence intensity ± S.D. after background subtraction of PI-negative cells. *, p < 0.05 versus controls in Student’s t test. Error bars represent S.D.
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FIGURE 4. The influence of L-744,832 on CD20 expression in primary CD20-positive leukemia/lymphoma cells. Primary human CD20-positive B-CLL cells were incubated for 48 h with increasing concentrations of L-744,832. After washing, cells were incubated with a saturating amount of FITC-conjugated anti-CD20 mAb (L27) for 30 min at room temperature in the dark. Prior to flow cytometry analysis, cells were washed with PBS and resuspended in PBS with PI. Results are presented as mean fluorescence intensity ± S.D. after background subtraction of PI-negative cells. *, p < 0.05 versus controls in Student’s t test. Error bars represent S.D.

not detectable in controls or in L-744,832-preincubated Raji cells (not shown).

Modulation of CD20 Expression by L-744,832 Occurs at a Transcriptional Level—To get insight into the mechanisms of L-744,832-induced up-regulation of CD20 levels in Raji cells, RT-PCR (Fig. 5A) and quantitative real time PCR analyses were performed. The results of SYBR Green quantitative real time PCR experiments revealed that CD20 is up-regulated at the mRNA level already 4 h after incubation of Raji cells with L-744,832 (Fig. 5B), and these increased levels persisted for up to 24 h (Fig. 5B). A nearly 3-fold induction of CD20 mRNA was also observed in quantitative real time PCR experiments with hydrolysis probes after 24-h incubation with increasing concentrations of L-744,832 (Fig. 5C). Quantitative real time PCR studies with SYBR Green and hydrolysis probes were performed with various reference genes (actin and β2-microglobulin, respectively) and revealed an induction of CD20 mRNA levels. Interestingly, preincubation of Raji cells with L-744,832 also increased mRNA levels for 24 h (Fig. 5A), a novel splice variant of mRNA that encodes a truncated, non-anchored CD20 protein. ΔCD20 selectively expressed in malignant but not resting B-cells was recently isolated from healthy donors (26). This protein associates with intracellular domains of normal CD20, and its levels increase in rituximab-resistant cells. It was suggested that association with ΔCD20 can modulate levels of CD20 in the plasma membrane or may affect its translocation to lipid rafts (26). However, our observations indicate that despite increased mRNA levels for ΔCD20 the potentiated sensitivity to anti-CD20-mediated CDC in Raji cells preincubated with L-744,832 is maintained. Western blotting studies revealed that CD20 is also up-regulated at the protein level in total cellular lysates of Raji cells pretreated with increasing concentrations of L-744,832 for 48 h. Densitometric analysis indicated that the amount of CD20 protein increased more than 2.5-fold at 20 μM L-744,832 (Fig. 5D). To verify whether modulation of CD20 levels by L-744,832 results from specific inhibition of farnesyltransferase or is an off-target effect of this compound, Raji cells were transduced with a GIPZ lentiviral shRNAmir (71-2) targeting FNTB or a scrambled shRNAmir (control). Transductants were checked for FNTB knockdown (Fig. 5E). Western blotting experiments revealed that shRNAmir-mediated FNTB knockdown increases CD20 levels (Fig. 5F). Moreover, incubation of Raji cells for 24 h with a translation inhibitor, cycloheximide, completely prevented the L-744,832-mediated increase of CD20 levels in Western blotting (Fig. 5G).

Modulation of CD20 Expression by L-744,832 Requires CD20 Promoter—To see whether L-744,832 requires an endogenous promoter of CD20 gene to mediate its effects, HEK-293T cells were stably transduced with lentiviral vector containing CD20 gene driven by a constitutive CMV promoter. Although preincubation of Raji cells with L-744,832 increased CD20 levels, a concomitant preincubation of HEK-293T cells with L-744,832 did not modulate expression of CD20 as measured by flow cytometry and Western blotting (Fig. 6, A and B).

To get more detailed insight into the transcriptional regulation of the CD20 promoter, a lentiviral luciferase reporter construct was created containing bp −431/+52 of CD20 promoter driving the expression of firefly luciferase (pLVX WT promCD20 Puro Luc). Raji cells stably transduced with this construct (Raji WT promCD20 Luc) were incubated with 10 μM L-744,832 for 1–24 h. The activity of luciferase started to increase 3 h after incubation with L-744,832 and reached the maximum at 24 h (Fig. 6C). Cycloheximide completely abolished L-744,832-mediated up-regulation of luciferase activity (Fig. 6D). Incubation with L-744,832 also increased luciferase gene transcription in RT-PCR experiments (Fig. 6E), indicating that transcriptional regulation of luciferase gene depends on the activity of CD20 promoter and does not result from increased stability of firefly luciferase.

To further elucidate the mechanism of CD20 promoter activation, a construct with truncated CD20 promoter deprived of
binding sites for NF-κB, Oct-1, Oct-2, PU.1/Pip, TFE3, and upstream stimulatory factor (Fig. 6F) was generated (trunc promCD20 Puro Luc) and stably introduced into Raji cells (Raji trunc promCD20 Luc). Incubation for 24 h with increasing concentrations of L-744,832 strongly activated the WT CD20 promoter (4–5-fold induction), whereas the increase of activity of truncated CD20 promoter was relatively modest (2-fold induction) and concentration-independent (Fig. 6G). A ChIP assay revealed that L-744,832 promotes binding of PU.1 and Oct-2 but not TFE3 to target DNA sequences within CD20 promoter in Raji cells (Fig. 6H).

L-744,832 Neither Influences CD20 Degradation by Proteasome nor Has an Impact on CD20 Distribution—Recent studies indicate that CD20 can be ubiquitinated, and proteasome inhibitors bimodally regulate surface CD20 levels (27). To verify the possibility that L-744,832 inhibits proteasome activity, Raji cells were preincubated with 5, 10, and 20 μM L-744,832, and the levels of polyubiquitinated proteins were determined using Western blotting. The results of these experiments revealed that although L-744,832 increased CD20 levels in total cellular lysates it did not lead to a build-up of polyubiquitinated proteins (supplemental Fig. 2A). Moreover, preincubation of Raji cells with L-744,832 did not affect chymotrypsin-like activity of the proteasome (supplemental Fig. 2B). Bortezomib used as a positive control in these experiments diminished the activity of proteasomes by >80% when used at a 20 nM concentration (supplemental Fig. 2B). Because endocytosis and trafficking of intracellular vesicles are regulated by prenylated proteins, we quantified the effect of L-744,832 on internalization of CD20 molecules using a reversible biotinylation assay. These experiments revealed that CD20 undergoes a slow rate of endocytosis, which was not inhibited by L-744,832 (supplemental Fig. 2, C and E). Confocal microscopy of control and L-744,832-preincubated Raji cells did not show any changes in the distribution of CD20 protein (supplemental Fig. 2, D and F).

**DISCUSSION**

Transcriptional regulation of CD20 expression is mainly reported in the context of CD20 antigen loss and down-regulation of CD20 mRNA. There are only a few reports demonstrat-
ing a cytokine (IL-4, GM-CSF, or IFN-α)-mediated increase in CD20 surface expression (28, 29). Transcriptional up-regulation of CD20 dependent on ERK phosphorylation was reported by Wojciechowski et al. (30) in lymphoma and primary CLL cells treated with bryostatin-1. An increase in CD20 transcription was also shown to be triggered by CpG independently of PU.1 transcription factor in CLL cells (31). Epigenetic therapy also was reported to restore CD20 mRNA expression, a phenomenon observed in CD20-negative cells (15).

The results of our studies indicate that L-744,832-induced inhibition of farnesyltransferase activity leads to up-regulation of CD20 expression and to improved antitumor activity of anti-CD20 mAbs. PCR studies revealed an FTI-mediated induction of CD20 mRNA that was accompanied by activation of the CD20 promoter-luciferase construct incubated with L-744,832 for 24 h with increasing concentrations of L-744,832 (C). Raji cells stably transduced with wild type CD20 promoter-luciferase construct were incubated with L-744,832 for 24 h with increasing concentrations of L-744,832, lysed, and assayed for firefly luciferase activity. Luciferase activities were normalized for protein concentration. *, p < 0.05 versus controls in Student’s t test. E, RNA from Raji WT promCD20 Luc incubated for 24 h with L-744,832 was isolated with TRizol and used for first strand cDNA synthesis and subsequent PCR amplification of firefly luciferase (LUC) and GAPDH products. F, schemes of wild type and truncated CD20 promoters used in the studies. G, Raji cells stably transduced with WT or truncated CD20 promoter-luciferase construct were incubated for 24 h with increasing concentrations of L-744,832, lysed, and assayed for firefly luciferase activity. Luciferase activities were normalized for protein concentration. *, p < 0.05 versus controls in Student’s t test; **, p < 0.05 versus corresponding groups of Raji cells transduced with WT and truncated CD20 promoter in Student’s t test. H, formaldehyde-fixed chromatin from Raji cells incubated for 24 h with L-744,832 was used for immunoprecipitation to enrich Oct-2, TFE3, and PU.1 complexes using specific antibodies. To assess the amount of transcription factor (TF) bound to CD20 promoter, a quantitative real time PCR (with LightCycler Fast Start DNA Master PLUS SYBR Green I) was performed with primers specific to the part of CD20 promoter that surrounds each transcription factor binding site (ChIP assay). Results are presented as a -fold change in promoter occupancy in immunoprecipitated relative to input samples. *, p < 0.05 versus controls in Student’s t test. Error bars represent S.D.
Prenyltransferases Regulate CD20 Levels

promoter. FTI-induced modulation of CD20 levels did not occur in HEK cells stably transduced with pLVX-CD20-IRES-Puro construct in which CD20 expression is regulated by a constitutive CMV promoter. A kinetic study with cycloheximide confirmed that transcriptional regulation of luciferase gene depends on the activity of CD20 promoter and is not a result of increased stability of firefly luciferase. Cycloheximide failed to block the induction of the CD20 promoter but prevented CD20 up-regulation at the protein level, indicating that L-744,832-mediated up-regulation requires de novo protein synthesis. Therefore, it seems that FTI-mediated effects result from a transcriptional activation of the CD20 promoter. To further elucidate the mechanism of CD20 modulation, a chromatin immunoprecipitation assay was performed and revealed an increased binding of PU.1 and Oct-2 transcription factors to their corresponding binding sites within the CD20 promoter in FTI-pretreated Raji cells. FTI-mediated activation of CD20 transcription seems to be complex with more than one transcription factor or signaling pathway involved. Because FTIs were reported to affect various cellular processing pathways such as protein degradation and endocytosis, a series of experiments was designed to check the possibility of an L-744,832-mediated effect on CD20 protein at the posttranscriptional level. NHL cells chronically exposed to rituximab acquire a resistant phenotype associated with reduced surface CD20 levels and exhibit up-regulation of the components of the ubiquitin-proteasome system (32). Proteasome inhibition partially reverses rituximab resistance (32), indicating that CD20 might be a substrate for intracellular proteolytic degradation systems. Because L-744,832 is a peptidomimetic compound based on a CAAAX motif present in farnesylated Ras family members, it can be hypothesized that it might competitively target the proteasome, delaying degradation of CD20. Isoprenoid inhibitors (FTI-277 and GGTL-298) were shown by others to inhibit chymotrypsin-like activity of the proteasome in breast cancer cell lines (33). However, the results of our experiments indicate that L-744,832-dependent CD20 up-regulation is not mediated by proteasome inhibition.

Several previous studies indicated potential mechanisms involved in surface CD20 modulation. For example, 1F5 (34, 35) as well as rituximab (16, 36) can be internalized by malignant cells on ligand binding. Stimulation of normal B-cells through CD40 has been shown to down-regulate CD20 expression, which occurs by protein kinase C-dependent endocytosis (37). Internalization of CD20 can be also induced by lenalidomide (38). In a reversible biotinylation assay, we observed a slow rate of CD20 endocytosis, which was not inhibited by L-744,832 treatment. A recent study revealed several mechanisms that affect CD20 expression in rituximab-resistant cell lines. Although CD20 was down-regulated at the transcriptional level, a forced expression induced by IL-4 restored cytoplasmic but not surface CD20 (39). Our studies indicate that FTI can increase CD20 expression, leading to higher surface levels of this molecule and increased susceptibility to mAb-mediated CDC.

Altogether, our observations indicate for the first time that CD20 expression can be modulated by prenyltransferase inhibitors. Although inhibition of farnesyltransferase activity significantly up-regulates expression of CD20, the effect of geranylgeranyltransferase inhibitors on this protein is more complex and requires further studies. The influence of L-744,832 on CD20 levels is independent from its posttranslational regulation.

Because some patients do not respond to initial anti-CD20 mAb-mediated therapy and down-regulation of CD20 is observed in patients with rituximab-resistant relapse, increasing CD20 expression seems to be a reasonable therapeutic approach to increase the rate of responses. Importantly, farnesyltransferase inhibitors evaluated in clinical trials revealed excellent safety profiles (25, 40, 41). Moreover, a farnesyltransferase inhibitor, tipifarnib, was recently shown to have a therapeutic activity in patients with relapsed and refractory lymphomas (25). In light of our studies, the combination of anti-CD20 mAbs with FTI seems to be a promising strategy worth testing in patients with NHL or CLL.

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