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Drug-free Macromolecular Therapeutics Induce Apoptosis in Cells Isolated from Patients with B Cell Malignancies with Enhanced Apoptosis Induction by Pretreatment with Gemcitabine

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Conflict of interest J.Y. and J.K. are co-inventors on two pending US patent application (PCT/US2014/023784 and PCT/US2017/37736 assigned to the University of Utah) related to this work. J.K. is Chief Scientific Advisor, J.Y. Scientific Advisor, and PJS Chief Medical Advisor for Bastion Biologics. J.K., J.Y., and P.J.S. are stockholders in Bastion Biologics.

Informed consent. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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Abstract

Drug-free macromolecular therapeutics (DFMT) is a new paradigm for the treatment of B cell malignancies. Apoptosis is initiated by the biorecognition of complementary oligonucleotide motifs at the cell surface resulting in crosslinking of CD20 receptors. DFMT is composed from two nanoconjugates: 1) bispecific engager, Fab'-MORF1 (anti-CD20 Fab' fragment conjugated with morpholino oligonucleotide), and 2) a crosslinking (effector) component P-(MORF2)_X (N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer grafted with multiple copies of complementary morpholino oligonucleotide). We evaluated this concept in 44 samples isolated from patients diagnosed with various subtypes of B cell malignancies. Apoptosis was observed in 65.9% of the samples tested. Pretreatment of cells with gemcitabine (GEM) or polymer-gemcitabine conjugate (2P-GEM) enhanced CD20 expression levels thus increasing apoptosis induced by DFMT. These positive results demonstrated that DFMT has remarkable therapeutic potential in various subtypes of B cell malignancies.

Graphical abstract

Drug-free macromolecular therapeutics (DFMT) is a new paradigm for the treatment of B cell malignancies. Apoptosis is initiated by crosslinking of CD20 receptors triggered by the biorecognition of complementary oligonucleotide motifs at the cell surface. The therapeutic efficacy of DFMT on 44 primary patient samples with various B-cell malignancies has been evaluated. Pretreatment of cells with gemcitabine or polymer-gemcitabine conjugate enhanced CD20 expression and increased levels of apoptosis. The results indicate that DFMT has significant translational potential.

Keywords

Drug-free macromolecular therapeutics; B cell lymphoma; CD20; apoptosis; nanomedicine

Background

Treatment of Non-Hodgkin lymphoma is challenging as the disease comprises over 35 different subtypes with the most prevalent types being diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL) and mantle cell lymphoma (ML).¹ The majority of NHLs (85%) are of B cell origin, and more than 95% of B cell lymphomas bear the cell surface antigen CD20, one of the most reliable biomarkers and therapeutic targets for B cells.² Rituximab (RTX), the first anti-CD20 monoclonal antibody approved by FDA for clinical use in 1997, initiated a new era for treatment of B cell NHLs.³ Its combination with chemotherapy can further enhance the therapeutic activity and still remains mainstay of therapy in young and fit patients.⁴ With the development of new, more active modern chemotherapy protocols and targeted therapies, much progress has been made over the past two decades.⁵ Unfortunately, relapsed or resistant disease is still a major cause of treatment failure.⁶

Recently we have developed a new paradigm for the treatment of B cell malignancies, drugfree macromolecular therapeutics (DFMT),^{7–13} which initiates apoptosis by the biorecognition of a pair of complementary motifs at the cell surface resulting in crosslinking of CD20 receptors. As no toxin or cytotoxic agent is involved, DFMT does not induce the side effects of traditional chemotherapy. The effectiveness of this innovative crosslinking CD20 approach has been proven *in vitro*^{8–11} and *in vivo*.^{8,9,12} Preliminary results with patient cells have been also published,^{12,13} in which Fab' fragment of 1F5 anti-CD20 monoclonal antibody (mAb) conjugated with a 25-base pair morpholino oligonucleotide (Fab_{1F5}'-MORF1) was administered to patient cells as bispecific engager, then a crosslinking effector composed of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer grafted with multiple copies of complementary morpholino oligonucleotide P-(MORF2)x was used to crosslink CD20 *via* hybridization of MORF1/MORF2 and trigger B cell apoptosis. Human serum albumin conjugated with MORF2 is also under investigation to perform as a crosslinking effector.¹⁴

Chronic lymphocytic leukemia (CLL), the most common form of adult leukemia is usually a slowly progressive disease of autoreactive mature B cells. Normally, these tumor cells have low sensitivity to anti-CD20 mAbs.¹⁵ Although many CLL cases have an indolent course, there is considerable heterogeneity and some patients rapidly transform into aggressive lymphomas.¹⁶ Various somatic mutations and the mutational status of immunoglobulin heavy chain variable genes (IGHV) have been associated with outcome. Non-mutated IGHV is associated with a worse outcome. Genomic aberrations often observed are 17p deletion (17p-), 11q deletion (11q), trisomy 12 (+12), 13q deletion (13q-), and others.¹⁷ Mutations of NOTCH1 and SF3B1 are also observed with relatively high frequency in CLL patients.¹⁸ Currently, standard first-line therapies for CLL include chemoimmunotherapy that incorporate anti-CD20 mAb, or targeted therapies, depending on risk factors. We have previously shown that that DFMT-based CD20 receptor crosslinking system is cytotoxic to CLL cells and has advantages over current cytotoxic therapies and immunotherapies.¹³ Moreover, we found that pre-incubation of CD20-low expression RTX-resistant B cells with gemcitabine upregulated CD20 level and enhanced apoptosis.^{9,19} Interestingly, the CD20 enhancement appeared to be time-dependent but not concentration-dependent. Consequently,

we hypothesized that *in vivo* a long circulating HPMa copolymer – gemcitabine conjugate (2P-GEM)²⁰ should be more efficient than free gemcitabine. Indeed, we validated this hypothesis in an animal model of NHL.⁹

Herein we evaluated the efficacy of DFMT on a large group of primary tumor cells from patients with various B cell lymphomas (N=9) or CLL (N=35), including a variety of genotypes. We also tested whether combined gemcitabine with DFMT will enhance therapeutic efficacy in the patient cells with low CD20 expression. Our results suggest that DFMT has significant activity against CLL cells with a spectrum of somatic mutations and that gemcitabine pretreatment may enhance efficacy of CD20-targeted therapies in B-cell malignancies with low CD20 expression.

Methods

Patient samples

Malignant B cells were isolated from patients diagnosed by the hematologic malignancies service at the Huntsman Cancer Institute. All patients provided informed consent to a research protocol approved by the University of Utah Institutional Review Board (IRB#45880). Samples P1 – P9 were cryopreserved and samples P10 – P44 were fresh.

Nanoconjugates

Fab'-MORF1, Cy5-labeled Fab'-MORF1, P-(MORF2)_{9.5}, and Cy3-labeled P-(MORF2)_{9.5} nanoconjugates were synthesized and characterized as previously reported.^{8,10} Two types of Fab'-MORF1 were used, derived from 1F5 (chimera mouse anti-CD20 mAb) and RTX, respectively.

Determination of CD20 Expression

Cell surface CD20 expression was determined by flow cytometry. Cells (2×10^5) were treated with 0.5 μ M rituximab for 1 h at 4 °C followed by goat-anti-human (GAH) secondary antibody conjugated with AlexaFluor-488 (1:500 dilution). Incubation time was 20 min at room temperature. Cells were washed and re-suspended in PBS prior to flow cytometry analysis. Cells treated only with fluorescence-labeled secondary antibody were used as negative controls.

Induction of Apoptosis

Cells (2×10^5) were suspended in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Incubation was carried out at 37 °C in a humidified atmosphere with 5% CO₂.

Cells were treated with Fab'-MORF1 and P-MORF2 at 1 μ M equivalent MORF1/MORF2 concentration consecutively (1 h interval). For samples P1–P6, Fab' derived from 1F5 mAb (chimera mouse anti-CD20 mAb) was used. In this case, 1F5 and goat-anti-mouse (GAM) secondary antibody (KPL, Gaithersburg, MD) were used for comparison. For samples P7–P44 Fab' from RTX was used. For comparison, 0.5 μ M RTX (equal Fab' concentration with Fab'-MORF1) hyper-crosslinked with 0.25 μ M goat-anti-human (GAH) secondary antibody

(KPL, Gaithersburg, MD) was used. Cells treated with PBS were used as negative controls. Cells were incubated with treatment at 37 °C for 20 h. All experiments were conducted in duplicate or triplicate, depending on the number of cells available.

Annexin V/propidium iodide (PI) staining

Cells (2×10^5) were suspended in 0.4 mL medium for different treatments as indicated. Harvested cells were washed twice with PBS prior to staining with propidium iodide (PI) and FITC-conjugated Annexin V (Oncogene Research Products, Boston, MA) for 15 min at room temperature, protected from light, followed by flow cytometry analysis. For gemcitabine pretreatment, cells were pretreated with 500 ng/mL gemcitabine (or gemcitabine equivalent in 2P-GEM) in RPMI-1640 medium for 48 h at 37 °C and washed with PBS before treatment as described above.

Caspase-3 Activity Measurement

Cells (2×10^5) were suspended in 0.4 mL medium for DFMT or RTX/GAH treatment. Cells were incubated at 37 °C with 5% CO₂ for indicated time periods. Harvested cells were washed twice with PBS prior to resuspension in 50 µL caspase 3 solution (PhiPhiLux kit, OncoImmunin, Gaithersburg, MD) and incubation at 37 °C for 1 h. Caspase 3 activity was measured according to the manufacturer's protocol.

Cell Cycle Analysis

Cells were washed twice with PBS after the indicated treatment and permeabilized in 70% ethanol overnight. Cells were stained with excess PI in PBS, and DNA content was determined by flow cytometry.

Mitochondrial Membrane Potential Analysis

CLL patient cells (2×10^5) were used for different treatments as described. Cells were washed with PBS and re-suspended in 100 µL PBS. Two µL of 200 µM JC-1 (1H-benzimidazolium, 5,6-dichloro-2-[3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2H-benzimidazol-2-ylidene)-1-propenyl]-1,3-diethyl-, iodide) was added and incubated with cells at 37 °C for 30 min. JC-1 is a mitochondrial membrane potential sensor (Thermo Scientific). As a positive control, 1 µL of 50 mM CCCP (carbonyl cyanide *m*-chlorophenylhydrazine) was added and incubated simultaneously with JC-1 for 30 min. Cells were then washed and re-suspended in PBS followed by flow cytometry analysis using 488 nm excitation with 520/30 nm and 585/42 band-pass filters.

Bcl-2 Expression Detection

CLL patient cells (2×10^5) were used for different treatments as indicated. After treatment, cells were fixed with 4% paraformaldehyde for 15 min at room temperature followed by permeabilization with 90% methanol for 30 min on ice. Anti-Bcl-2 mAb labeled with AlexaFluor 488 (1:500, Santa Cruz Biotechnology) was used for cell immunostaining in 0.5 % bovine serum albumin (BSA) buffer for 1 h at room temperature. After washing with PBS, fluorescence was quantified using flow cytometry analysis.

Confocal Microscopy

Patient CLL cells (2×10^5) were treated with 1 μ M Fab'-MORF1-Cy5 for 1 h. Cells were then washed with PBS and further incubated with 1 μ M P-MORF2-Cy3 for 1 h. Afterwards cells were washed with PBS and re-suspended in PBS in 4-well chambers (MatTek Corporation) prior to confocal visualization (Olympus laser scanning confocal microscope, FV1000). For the control group, cells were pretreated with 10-fold excess of RTX for 1 h before Fab'-MORF1/P-MORF2 treatment described in previous publication⁸.

Statistical analysis

All experiments were conducted in duplicate or triplicate, depending on the number of cells available. Data are presented as mean $\pm$ standard deviation (SD). Differences were considered significant for *P* values < 0.05 using the Student's *t* test.

Results

The efficacy of drug-free macromolecular therapeutics (DFMT) was assessed in 44 samples including 35 CLL along with other B cell lymphomas such as diffuse large B cell lymphoma (DLBCL), marginal zone lymphoma (MZL), follicular lymphoma (FL), mantle cell lymphoma (MCL) etc (Table 1). The isolated cells were treated with Fab'-MORF1 and P-(MORF2)_{9.5} (9.5 MORF2 per chain) consecutively with a 1 h interval. Rituximab (RTX) or 1F5 were used to produce Fab' fragments for Fab'-MORF1 synthesis. In all treatments, conjugates were added at a concentration of 1 μ M Fab' equivalent (0.5 μ M of mAb). Results of apoptosis assays are summarized in Table 2. P1–P6 cells were treated with Fab'-(1F5)-MORF1/P-(MORF2)_{9.5} while P7–P44 cells were treated with Fab'-(RTX)-MORF1/P-(MORF2)_{9.5}. Patients P1–P9 had various B cell lymphomas while patients P10–P44 had chronic lymphocytic leukemia (CLL). In samples P1–P9, DFMT showed significant efficacy in inducing apoptosis compared to controls in all samples except for samples P5 and P6. In samples P2, P4, P7 and P9, DFMT showed superior efficacy at apoptosis induction compared to RTX treatment. P4 and P9 were MCL samples. P1, P3 and P5 were DLBCL samples. P6 and P8 were MZL samples, while P2 was a FL sample. All MCL samples responded to DFMT treatment while 2 of 3 samples of DLBCL responded to DFMT treatment. One MZL sample and one FL responded to DFMT treatment. These results demonstrated that DFMT strategy works on different types of B-cell lymphoma tested here.

Regarding CLL samples, a variety of chromosomal abnormalities, including 17p13 deletion (N=4), 11q22 deletion (N=3), and 13q14 deletion (N=25) have been observed. Altogether Fab'-(RTX)-MORF1/P-(MORF2)_{9.5} induced cell apoptosis effectively in 65.9% of samples (Table 2).

When sufficient cells were available, a detailed evaluation of apoptosis induction by DFMT was performed. A typical example such an analysis is shown in Figure 1 (Sample P12). In that analysis, we evaluated surface CD20 expression (Fig. 1A), plasma membrane rupture (Fig. 1B), caspase 3 activation (Fig. 1C), genomic DNA fragmentation (Fig. 1D), mitochondrial membrane permeabilization (Fig. 1E), and Bcl-2 inhibition (Fig. 1F). Also, confocal imaging was used to document specific biorecognition at the cell surface, first

biorecognition of Fab'-MORF1 by CD20 followed by biorecognition/hybridization of MORF1/MORF2 (Fig 1G). The results showed that DFMT was effective on inducing apoptosis (Fig. 1B–F). The high CD20 expression in sample P12 was associated with a substantially increased response to DFMT treatment (Table 2). As shown in Fig. 1B, cell population shifted from left lower zone to right lower zone tremendously demonstrating significantly increased cell apoptosis. Cell cycle assay (Fig. 1D) also showed that with DFMT and RTX/GAH treatment, more cells were trapped in Sub G1 phase indicating increased amount of apoptotic cells. Especially in the group treated with DFMT, the number of apoptotic cells was nearly twice higher than that treated with RTX/GAH. Decreased expression of anti-apoptotic protein Bcl-2 was observed with treatment of DFMT and RTX/GAH, respectively (Fig. 1F), showing that inhibition of Bcl-2 is involved in the apoptosis induction mechanism of DFMT treatment. Confocal imaging (Fig. 1G) showed biorecognition events and colocalization of the two nanoconjugates at the cell surface. Specificity of binding to CD20 was demonstrated by blocking self-assembly at the cell using pre-treatment with excess RTX.

Our results suggest that DFMT has therapeutic potential for different types of B-cell malignancies. Statistical analysis shows that the level of surface CD20 expression is an important factor affecting therapeutic efficacy. Additional work with a larger set of patient samples from various diagnoses is needed to identify other critical features that are important in the response to CD20 targeting and to identify subsets of patients suitable for DFMT treatment.

A promising strategy to further improve the therapeutic efficacy of DFMT is to use gemcitabine pretreatment. GEM is used to treat different types of malignancies and can lead to accumulation of cells in S phase.²² Importantly, GEM was shown to increase CD20 expression level in B cells.¹⁹ As CD20 expression level is a critical factor affecting the therapeutic efficacy of antiCD20 therapy, combination of CD20-targeting therapy with GEM could potentially increase the efficacy of the DFMT. We pre-treated cells from samples P40, P42 and P43 for 48 h with GEM at 500 ng/mL as these samples had low CD20 expression levels. Cells were then washed and treated with DFMT as well as RTX/GAH. GEM pretreatment significantly increased CD20 expression compared to controls without GEM pretreatment (Fig. 2A, 2C). Representative results of flow cytometry showed increased apoptosis (Fig. 2D). Correspondingly, statistical analysis showed increased apoptosis level cells with GEM pretreatment (Fig. 2B). Alternatively, P26 cells were pretreated with a long-circulating HPMA copolymer-GEM conjugate (2P-GEM). Cells pretreated with 2P-GEM showed higher CD20 expression as well as increased apoptosis levels (Fig. 2E, 2F).

Discussion

B cell malignancies affect millions of people worldwide with significant morbidity and mortality. The DFMT B cell depletion strategy is able to avoid the adverse effects induced by rituximab treatment with promising therapeutic efficacy. Data presented here validate that DFMT treatment has therapeutic potential against a wide variety of B cell lymphoma subtypes. In many samples, DFMT showed statistically significant increases in apoptosis when compared to RTX/GAH, indicating that the proposed treatment could have better

therapeutic efficacy, at least in certain cases. However, in several samples, DFMT showed only comparable or even or lower efficacy. Different factors may be at play, including differences in the level of expression of CD20. Expression level of CD20 was the dominant strength in determination of therapeutic potential of treatments. Patient samples with high CD20 expression levels were more likely to show increased apoptosis following treatment with DFMT. The higher the CD20 expression level, the better the therapeutic efficacy of the treatment as shown in Figure 3.

Genomic aberration is also a factor affecting traditional therapies. The most common cytogenetic mutation in CLL is deletion of 13q14, detected in around 50% of CLL patients.²³ It might not be sufficient to be considered an adverse prognostic factor.²⁴ However, 13q14 deletion is a critical genomic aberration for increased apoptotic resistance. The 13q14 region is usually described as a negative regulator of the anti-apoptotic protein Bcl-2.²⁴ Mutation of this region increases the expression of Bcl-2, allowing CLL cells to survive, resulting in lower apoptosis levels induced by treatment. The incidence of 17p deletion in CLL patients is close to 10% and is associated with an inferior prognosis.²³ The 17p13 region is involved in important regulatory functions. Its deletion is believed to be a strong independent marker in evaluating death caused by disease. p53 tumor suppressor gene is deleted in patients with 17p13 deletion. P53 deficiency may allow cells to survive from therapeutic treatment as p53-dependent apoptosis pathway is interrupted.²³ Deletion of 11q occurs in 10–20% of CLL patients.²⁵ The 11q22 region is involved in activation of p53-dependent apoptosis pathway thus its loss leads to reduced cell death.²³ Combination of deletions of 17p and 11q are considered as significantly poorer prognoses.²⁶ We evaluated 25 primary patient samples with 13q14 deletion, 4 samples with 17p13 deletion and 3 samples with 11q22 deletion. Previously, we had shown that DFMT induced efficient CD20 crosslinking-mediated apoptosis in patient cells with 17p13 deletion.¹³ International prognostic index (IPI) is used in clinics to predict outcome of individual patients based on patient's clinical characteristics including genomic aberrations. Higher IPI values indicate more problems in treatment. Based on the results summarized in Table 2 with patient information in Table 1, therapeutic efficacy of our DFMT treatment on CLL primary patient samples varied regarding to similar genomic aberrations. Seven samples with different mutations were selected for evaluation (Figs. 3A and 3B). Fig. 3A shows that the higher the CD20 expression level, the higher the DFMT mediated apoptosis induction. Fig. 3B demonstrates that the value of IPI does not hamper the efficacy of DFMT. For example, both samples P18 and P31 have 13q14 and 17p dual deletions. IPI of P18 is 4 while IPI of P31 is 7, indicating a worse outcome prediction of sample P31. However, DFMT exhibited superior apoptosis induction in P31 sample over P18 sample, demonstrating that CD20 expression level is the determinant factor affecting therapeutic efficacy of DFMT. Similar phenomena were observed in P39 and P43 patient samples with IPI score of 3. With the same IPI score, DFMT showed much higher efficiency in apoptosis induction in P39 compared to P43 as P39 possessed a higher CD20 expression level. It seems that CD20 expression level could overcome the anti-apoptotic effect involved with critical deletions.

It appears that it is essential to increase CD20 expression level to improve therapeutic efficacy of DFMT treatment. Exposure to gemcitabine was reported to increase CD20 expression level.²² The increase in CD20 expression resulted from gemcitabine-induced NF-

κ B activation as NF- κ B binds to the CD20 promoter to induce CD20 transcriptional activation.^{9,15} With patient samples P26, P40, P42 and P43, cells were pretreated with GEM or 2P-GEM. Figure 2 shows that pretreatment with GEM or polymer conjugated GEM (2P-GEM) increases expression level of CD20 receptor significantly, thus increasing the efficacy of DFMT in inducing apoptosis. These results support the observation that CD20 expression level is the key factor for DFMT treatment efficacy. Combination therapy of DFMT with GEM would be a promising strategy to improve its therapeutic efficacy in insensitive samples. More work is needed to confirm the potential increased efficacy of the combination of GEM with CD20-targeted therapy. In addition, comprehensive studies will be conducted with GEM and 2P-GEM pretreatment on patient cells, CD20+ B cell lines, and on animal models to assure the feasibility of combination strategy.

In summary, apoptosis induction by DFMT in primary cells from patients with various B cell malignancies was evaluated. DFMT effectively increased apoptosis of tumor cells from patients with a variety of B cell malignancies, irrespective of genomic aberrations. In CLL samples, the CD20 expression level is positively correlated with susceptibility to DFMT-induced apoptosis. Pretreatment with GEM or HPMA copolymer-GEM conjugate improved DFMT therapeutic efficacy in poorly responding samples, likely due to increased CD20 expression. With increased CD20 expression, DFMT treatment is able to overcome the anti-apoptotic effect involved in critical genomic aberrations. The results suggest that DFMT is a promising modality for the treatment of different B cell malignancies. Especially with RTX-resistant B cell malignancies, combination therapy of DFMT with GEM provides a prospective solution to overcome drug resistance. In addition, crosslinking of receptors as a design principle for novel therapeutic approaches is applicable to other receptors such as CD79b,²⁷ prostate stem cell antigen,²⁸ and DR5.²⁹

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Abbreviations

DFMT	drug-free macromolecular therapeutics
MORF	morpholino oligonucleotide
HPMA	<i>N</i> -(2-hydroxypropyl)methacrylamide
GAH	goat-anti-human
GAM	goat-anti-mouse
2P-GEM	backbone degradable HPMA polymer-gemcitabine conjugate

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Figure 1.

Drug-free macromolecular therapeutics induced apoptosis of CLL cells from P12. *Negative control*, cells treated with PBS; *DFMT*, Fab'-MORF1 (1 μ M) followed (1 h later) by P-(MORF2)_{9,5} (1 μ M; MORF2 eqv); *RTX/GAH*, RTX (0.5 μ M) followed (1 h later) by GAH (0.25 μ M) (A) Cell surface CD20 expression as determined by fluorescent-labeled antibody. *Negative control*, cells treated with only GAHAlexaFluor488; *RTX/GAH-AlexaFluor488*, RTX (0.5 μ M) followed (1 h later) by GAH-AlexaFluor488 (1:200). Incubation time was 20 min. (B) Representative flow cytometry analysis of cells exposed to different treatments (incubation time 20 h) and stained by PI and FITC-labeled Annexin V. (C) Representative flow cytometry analysis of cells exposed to different treatments (incubation time 6 h) and stained by FITC-labeled anti-active Caspase-3 Ab. (D) Cell cycle analysis. After exposed to different treatments (incubation time 6 h), cells were permeabilized and stained by PI to measure DNA content. SubG1 phase represents apoptotic cells. (E) Mitochondrial membrane potential determined by JC-1 fluorescence. Incubation time was 6 h. After treatment, 4 μ M JC-1 was added to all samples. (F) Bcl-2 expression as determined by Alexa-fluor488-labeled rabbit anti-Bcl-2 Ab. Incubation time was 6 h. After treatment, Alexa-fluor488-labeled anti-Bcl-2 Ab was added to all samples (1:50). (G) Confocal imaging. *RTX block*, cells were treated with RTX (10 μ M) for 1 h followed by regular

DFMT treatment. Fab'-MORF1 was labeled by Cy5 while P-(MORF2)_{9.5} was labeled by Cy3. (***) $p < 0.001$, *ns*, no significant difference; Student's *t* test).

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Figure 2.

Pretreatment with GEM or 2P-GEM increases apoptosis efficacy. (A-D) P40; (E-F) P26.

Pretreatment with GEM or 2P-GEM: Cells were treated with 500 ng/ml GEM or GEM equivalent at 37°C for 48 h before DFMT treatment; *Negative control*, cells treated with PBS; *DFMT*, Fab'-MORF1 (1 μ M) followed (1 h later) by P-(MORF2)_{9,5} (1 μ M; MORF2 eqv); *RTX/GAH*, RTX (0.5 μ M) followed (1 h later) by GAH (0.25 μ M) (A) Cell surface CD20 expression as determined by fluorescence-labeled antibody. *Negative control*, cells treated with GAH-AlexaFluor488 only; *RTX/GAH*, RTX (0.5 μ M) followed (1 h later) by GAH-AlexaFluor488 (1:200). Incubation time was 20 min. (B) Apoptosis efficacy was increased in all the treatment groups after GEM pretreatment. Incubation was 24 h. (C) Representative flow cytometry analysis of CD20 expression on cell surface with or without GEM pretreatment. (D) Representative flow cytometry analysis of cells treated by DFMT therapy with or without GEM pretreatment. (E) Cell surface CD20 expression with or

without 2P-GEM pretreatment. **(F)** Apoptosis efficacy increased in all treatment groups after 2P-GEM pretreatment. (** $p < 0.001$, *ns*, no significant difference; Student's *t* test).

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Figure 3.

Correlations between apoptosis level (%) and CD20 expression level / International Prognostic Index (IPI). **(A & B)** Selected samples with various genomic aberrations. * Samples with 17p deletion. **(A)** Relationship between level of apoptosis induction and CD20 expression. **(B)** IPI score does not affect efficacy of DFMT in samples with genomic aberrations. **(C)** Correlation between increased apoptosis level (%) and CD20 expression level in P10-P44 samples.

Table 1.

Clinical Characteristics of Lymphoma Patients

Patient	^a Gender	Age	^b Diagnosis	^c IPI score	13q14	11q22	17p	^d Pretreat
P1	F	66	BCL ₂ BETWEEN					
P2	F	53	FL					
P3	F	53	LBCL					
P4	M	67	MCL					
P5	F	21	DLBCL					Frozen Samples
P6	F	70	SMZL					
P7	N/A	N/A	N/A					
P8	F	81	SMZL					
P9	M	67	MCL					
P10	F	87	CLL	4	Yes	No	No	Chlorambucil + Rituximab
P11	M	80	CLL	6	N/A	N/A	No	None
P12	N/A	N/A	CLL	N/A	N/A	N/A	N/A	None
P13	M	59	CLL	0	Yes	No	No	None
P14	F	66	CLL	1	Yes	No	No	None
P15	M	78	CLL	4–6	Yes	No	No	None
P16	M	66	CLL	4	Yes	No	No	None
P17	F	58	CLL	0	Yes	No	No	None
P18	M	60	CLL	4	Yes	No	Yes	None
P19	M	72	CLL	4	Yes	No	No	None
P20	F	63	CLL	3–5	Yes	No	No	None
P21	M	85	CLL	6	Yes	Yes	No	None
P22	M	60	CLL	5	Yes	No	No	BR-Ibrutinib
P23	M	69	CLL	2	N/A	N/A	No	FCR
P24	M	59	CLL	0	Yes	No	No	None
P25	M	56	CLL	0	N/A	N/A	No	None
P26	F	48	CLL	1	Yes	No	No	None
P27	M	74	CLL	1	Yes	No	No	None
P28	F	55	CLL	1	Yes	No	No	None
P29	M	65	CLL	2	No	No	No	None
P30	M	79	CLL	1	Yes	No	No	None
P31	F	65	CLL	7	Yes	No	Yes	Ibrutinib-Obino-CVP-Idelalisib-Venetoclax-BI836826
P32	M	80	CLL	6	No	No	No	None
P33	M	79	CLL	4–6	Yes	No	No	None
P34	M	58	CLL	1	N/A	N/A	No	None
P35	M	70	CLL	4	Yes	No	No	Ibrutinib
P36	F	45	CLL	1	No	No	No	None
P37	M	62	CLL	7	Yes	Yes	Yes	None
P38	F	63	CLL	0	Yes	No	No	None

Patient	^a Gender	Age	^b Diagnosis	^c IPI score	13q14	11q22	17p	^d Pretreat
P39	F	77	CLL	1–3	Yes	No	No	None
P40	F	73	CLL	1–3	Yes	No	No	None
P41	F	52	CLL	2	N/A	N/A	No	None
P42	F	63	CLL	5	N/A	N/A	Yes	None
P43	F	65	CLL	3	Yes	Yes	No	None
P44	M	71	CLL	2	Yes	Yes	No	None

^a. M male; F female

^b. Abbreviations: BCL (B-Cell Lymphoma Unclassifiable), DLBCL (Diffuse Large B-Cell lymphoma), BL (Burkett Lymphoma), FL (Follicular Lymphoma), LBCL (Large B-Cell Lymphoma), MCL (Mantle Cell Lymphoma), SMZL (Splenic Marginal Zone Lymphoma), CLL (Chronic Lymphocytic Leukemia)

^c. IPI: International Prognostic Index (IPI). Low risk (score 0–1); Intermediate risk (score 2–3); High risk (score 4–6); Very-high risk (score 7–10)²¹

^d. Abbreviations: BR (Bendamustine and rituximab); FCR (Cyclophosphamide, fludarabine and rituximab); FR (Fludarabine and rituximab); CVP (Cyclophosphamide, vincristine sulfate and prednisone)

Table 2.

Experimental Results of Apoptosis Assays

Patient #	CD20 expression	Pretreatment	Increased Apoptosis% [#]	
			DFMT ^{#1}	RTX/GAH ^{#2}
P1		-	31.09 [*]	36.97 [*]
P2		-	4.37 ^{**}	1.67 [*]
P3		-	16.56 [*]	18.13 [*]
P4		-	11.26 ^{**}	2.99
P5	N/A	-	1.27	-3.17
P6		-	10.24	8.9
P7		-	9.09 ^{**}	6.09 [*]
P8		-	12.17 [*]	15.24 [*]
P9		-	17 ^{**}	5.1 [*]
P37	1.05	-	0.78	11.68 [*]
P28	1.10	-	4.56 [*]	18.23 [*]
P35	1.14	-	-3.36	-4.59
P13	1.18	-	-1.33	0.44
P27	1.22	-	3.12 [*]	15.4 [*]
P38	1.24	-	-2.88	-1.15
P42	1.26	-	4.79 [*]	10.96 [*]
	1.51	Pretreat with GEM	11.94 [*]	12.6 [*]
P29	1.32	-	5.2 [*]	15.84 [*]
P34	1.55	-	3.82 [*]	14.71 [*]
P22	1.62	-	-0.16	0.62
P17	1.81	-	8.8 [*]	15.87 [*]
P14	1.87	-	7.64 [*]	9.72 [*]
P30	2.12	-	1.45	-0.05
P40	2.20	-	1.67 [*]	-0.4
	3.30	Pretreat with GEM	17.97 ^{**}	11.57 [*]
P19	2.24	-	-0.42	22.94 [*]
P33	2.28	-	2.5	0.12
P36	2.31	-	3.37 [*]	12.29 [*]
P10	2.39	-	6.38	21.4 [*]
P18	2.43	-	-2.93	1.75
P16	2.45	-	3.2	1.34
P43	2.63	-	3.89 [*]	34.23 [*]
	3.05	Pretreat with GEM	23.47 [*]	40.77 [*]

Patient #	CD20 expression	Pretreatment	Increased Apoptosis% [#]	
			DFMT ^{#1}	RTX/GAH ^{#2}
P20	2.64	-	-4.09	27.94 [*]
P24	2.73	-	-0.99	25.79 [*]
P11	3.00	-	4.63 [*]	17.11 [*]
P15	3.16	-	15.55 ^{**}	9.67 [*]
P23	3.17	-	-0.36	4.12 [*]
P31	3.23	-	13.1 [*]	13.96 [*]
P39	3.63	-	18.95 ^{**}	-6.42
P21	3.89	-	5.97 [*]	21.88 [*]
P44	4.18	-	49 ^{**}	39.5 [*]
P41	5.67	-	19.07 ^{**}	4.78 [*]
P26	6.80	-	10.11 ^{**}	2.94 [*]
	15.60	Pretreat with 2P-GEM	21.61 ^{**}	10.97 [*]
P25	7.55	-	10.4 ^{**}	2.97 [*]
P12	13.31	-	38.13 ^{**}	28.35 [*]
P32	N/A	-	22.51 [*]	25.07 [*]

GEM: Gemcitabine

2P-GEM: Backbone degradable HPMA copolymer-gemcitabine conjugate

P10-P44 were sorted by CD20 expression levels

All samples were tested with Annexin V assay (except samples P4 and P40 that were tested tested with Caspase 3 assay)

[#] Increased Apoptosis%: Apoptosis level achieved after subtraction of value of negative control group

^{#1} DFMT: samples were treated by Fab'-MORF1 followed by P-MORF2

^{#2} RTX/GAH: samples were treated by RTX followed by goat-anti-human 2nd antibody

^{*} Significantly higher than Negative Control (P<0.05; Student's t test)

^{**} Significantly higher than Negative Control and RTX/GAH (P<0.05; Student's t test)