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Targeted drug delivery and cross-linking induced apoptosis with anti-CD37 based dual-ligand immunoliposomes in B chronic lymphocytic leukemia cells

Bo Yu [a,b,1], Yicheng Mao [b,c,1], Yuan Yuan [b,d], Chaofang Yue [a,b], Xinmei Wang [b], Xiaokui Mo [e], David Jarjoura [e], Michael E. Paulaitis [a,b], Robert J. Lee [b,c], John C. Byrd [f,g], L. James Lee [a,b,**,2], Natarajan Muthusamy [g,*,2]

[a] Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH, USA
[b] NSF, Nanoscale Science and Engineering Center (NSEC), The Ohio State University, Columbus, OH, USA
[c] Division of Pharmaceutics and Pharmaceutical Chemistry, College of Pharmacy, The Ohio State University, Columbus, OH, USA
[d] The State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, PR China
[e] Center for Biostatistics, The Ohio State University, Columbus, OH, USA
[f] Division of Medicinal Chemistry, College of Pharmacy, The Ohio State University, Columbus, OH, USA
[g] Division of Hematology, Department of Internal Medicine, The Ohio State University, 455E, OSUCCC, 410, West 12th Avenue, Columbus, OH 43210, USA

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ABSTRACT

Despite advances in chemo and immunotherapeutic agents for B chronic lymphocytic leukemia (B-CLL), the undesirable adverse side effects due to non-specific cellular uptake remain to be addressed. We identified anti-CD37 monoclonal antibody immunoliposomes (ILs) as vehicles for targeted delivery to B chronic lymphocytic leukemia cells. To achieve maximal benefits for all patients, a new strategy of dual-ligand immunoliposomes (dILs) was developed. A combinatorial antibody microarray technology was adapted to quickly identify optimal antibody combinations for individual patient cells. For proof-of-concept, a B-cell specific antibody, either anti-CD19 or anti-CD20, was combined with anti-CD37 to construct dILs with enhanced selectivity and efficacy. Consistent with data from the antibody microarray, these dILs provided highly specific targeting to both leukemia cell lines and B-CLL patient cells. Compared with the single antibody ILs, the anti-CD19/CD37 dILs clearly demonstrated superior delivery efficiency and apoptosis induction to B-CLL patient cells, whereas the anti-CD20/anti-CD37 dILs were found to be the most efficient for delivery to leukemia cell lines. In addition, it was observed that anti-CD37 ILs without payload drug mediated effective CD37 cross-linking and induced potent apoptosis induction. The anti-CD19/CD20 dILs showed the improved cell apoptosis induction compared to either anti-CD19 ILs or anti-CD20 ILs. Our findings suggest that the dual-ligand ILs may provide a preferred strategy of personalized nanomedicine for the treatment of B-cell malignancies.

1. Introduction

B-CLL is a common type of adult leukemia for which current treatments are not curative. Alkylating agents and purine nucleoside analogs have been considered as the drugs of choice for treatment of CLL for many years. The chemotherapeutic agent fludarabine used by itself or in combination with alkylator-based agents is effective in a subset of patients but non-specific effects of these drugs on bystander cells are problematic [1]. Undesirable side effects associated with these therapies include prolonged immune suppression resulting from direct apoptosis induction to normal immune effector cells [1–3].

The introduction of the anti-CD20 monoclonal antibody rituximab (RIT) [4–6] has substantially impacted CLL therapy [4,7,8]. RIT, when given in combination with fludarabine and cyclophosphamide, has been shown to extend survival in symptomatic CLL [4,7,9]. In addition to rituximab, alemtuzumab that targets CD52, an

* Corresponding author. Tel.: +1 614 292 8135; fax: +1 614 292 3312.
** Corresponding author. Engineering Nanoscale Science and Engineering Center, 1012 Smith Laboratory, 174 W, 18th Avenue, Columbus, OH 43210, USA. Tel.: +1 614 292 2408; fax: +1 614 292 3769.
E-mail addresses: lee.31@osu.edu (L.J. Lee), raj.muthusamy@osumc.edu (N. Muthusamy).
[1] These authors contributed equally to the work.
[2] These two senior authors contributed equally to this work.

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antigen expressed on normal lymphocytes as well as many T- and B-cell neoplasms has been used for first-line treatment for CLL [5,6]. The immunosuppressive effects of alemtuzumab caused by T and NK cell depletion, however, impose limit to its use in aged patients. New antibodies against CD19, CD40, CD23, CD37, and CD74 are in early clinical trials for the treatment of CLL [10–13]. Recently, CD37 antigen has been identified as a potential target for therapy in B-cell malignancies [13–15]. CD37, a 40–52 kDa glycoprotein, is highly expressed on B cells and has limited or no expression on other hematopoietic cells such as T cells and NK cells [16,17]. In particular, CD37 on B-CLL cells is uniformly present and relatively elevated [13,15].

B-cell lymphomas and leukemias often involve multiple, different pathological factors and pathways. Therapeutic efficacy of most of the antibodies in clinical use is attributed to their interaction with a single target. Simultaneous blockade of multiple targets either via the combination of two antibodies (Abs) or by a bispecific antibody (BsAb) may provide better clinical efficacy and/or reach a broader patient population [18–20]. In fact, improved therapeutic efficacy of combining milatuzumab and RIT monoclonal antibodies (mAbs) has already been demonstrated in the preclinical model of mantle cell lymphoma (MCL) [21]. In addition, the bispecific anti-CD20/CD22 and anti-CD20/CD74 antibodies have demonstrated enhanced efficacy for B-cell lymphomas and leukemias [18,22].

Specific and efficient *in vivo* delivery of therapeutic agents to target B-CLL cells remains a major challenge in the clinic. To address these issues, monoclonal antibody conjugated nanocarriers such as immunoliposomes (IL) have been increasingly recognized as a promising strategy for selective delivery of anti-cancer drugs to B-CLL cells [11,23,24]. In addition, recent efforts on dual-ligand mediated delivery approaches offer the potential to improve selectivity and efficiency over single-ligand approaches [25–29]. Dual Ab targeted ILs have shown improved therapeutic effects of anti-cancer drugs in B-cell malignancies [30,31]. However, dual-ligand ILs against antigens co-expressed on the same cells have not been investigated in CLL.

Creation of multivalent antibody constructs using liposomes or gold nanoparticles has recently been shown to have enhanced efficacy compared to free, bivalent antibody [11,32–35]. Because of the extensive cross-linking of the target/antibody complex via the multivalent antibody constructs, various cellular responses such as inhibition of cell growth, induction of apoptosis, or internalization of the surface molecules, can be significantly enhanced. For example, RIT-coated liposomes (devoid of encapsulated drug) have displayed much higher efficacies than equal amounts of free monomeric RIT [33,34]. Our recent work also indicates that anti-CD74 ILs mediate potent cell killing of B-CLL cells even without an anti-cancer drug payload [11]. Nevertheless, the multivalency of nanoparticle-based antibody constructs has only been focused so far on single therapeutic antibodies.

Based on the above rationale, we sought to achieve high selectivity and targeting efficacy to B-CLL cells through ILs. In this work, we developed liposomal nanoconstructs that are simultaneously surface modified with two types of antibody ligands having specificity and high affinity to B-CLL cells. To fulfill the purposes of screening for the proper Ab combination for individual CLL patient cells, a combinatorial antibody microarray technology was used to quantitatively characterize binding efficiencies of single and dual antibodies in a systematic and high throughput fashion. Anti-CD37 ILs were first demonstrated to provide highly specific targeting to both leukemia cell lines and B-CLL patient cells. Combination of anti-CD37 Ab with either anti-CD19 or anti-CD20 Ab on the same ILs was used to further improve the targeting of B-CLL cells. The multivalent effects of dual antibody immunoliposomes (dILs) on B-CLL patient cells were examined by in vitro apoptosis induction assay. Finally, a small molecule drug, FTY720, as a model of a therapeutic payload, was encapsulated in the dILs and evaluated for therapy efficacy in B-CLL cells.

2. Material and methods

2.1. Reagents

Egg phosphatidylcholine (Egg PC) and methoxy-polyethylene glycol (M_w ~ 2000 Da)–distearoyl phosphatidylethanolamine (PEG2000–DSPE) were obtained from Lipoid (Newark, NJ). Maleimide–PEG2000–DSPE (Mal–PEG2000-DSPE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol (Chol), calcein, 2-iminothiolane (Traut's reagent) and other chemicals were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Rituxan (Rituximab) was obtained from Genentech, Inc. (South San Francisco, CA). Purified anti-human CD37 and anti-human CD19 monoclonal murine antibodies were purchased from BD Biosciences (San Diego, CA). Alexa 488 labeled anti-mouse secondary antibody was from Invitrogen (Carlsbad, CA). FTY720 was purchased from ChemieTek (Indianapolis, IN).

2.2. Cell culture

Raji Burkitt's lymphoma, Daudi, Romas, RS11846 and Jurkat cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and penicillin (100 U/ml)/streptomycin (100 μg/ml; Sigma–Aldrich, St. Louis) at 37 °C in an atmosphere of 5% CO_2. Blood was obtained from patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the institutional review board (IRB) of The Ohio State University (Columbus, OH). All patients examined in this series had immunophenotypically defined CLL as outlined by the modified 96 National Cancer Institute criteria. B CLL cells were isolated from freshly donated blood using Ficoll density gradient centrifugation (Ficoll–Paque Plus, Amersham Biosciences, Piscataway, NJ). Enriched CLL fractions were prepared by using the "Rosette-Sep" kit from Stem Cell Technologies (Vancouver, British Columbia, Canada) according to the manufacturer's instructions.

2.3. Immunostaining and flow cytometry

Cell lines (0.5×10^5/ml) or B-CLL cells (1.0×10^5/ml) were incubated with PE-labeled anti-CD20, anti-CD19, and anti-CD37 as well as mouse IgG_1 isotype control antibody (BD Biosciences, San Diego, CA), at 4 °C for 30 min. The cells were then spun down at 300× *g* for 10 min and rinsed twice with cold phosphate-buffered saline (PBS, pH = 7.4) and then analyzed by flow cytometry on a Beckman Coulter EPICS XL (Beckman Coulter) to determine antigen expression levels. A minimum of 10,000 events were collected under the LIST mode for each assay. The data was analyzed using WinMDI software.

For surface staining, PBMC cells were incubated with PE labeled anti-CD19 (as a B cell marker) or PE labeled anti-CD3 (as a T cell marker) on ice for 30 min. The cells were washed twice with cold PBS (pH = 7.4) and analyzed by flow cytometry. To study B-cell selectivity, PBMC cells were incubated with FITC labeled anti-CD37 on ice for 30 min. The cells were then spun down and rinsed twice with cold phosphate-buffered saline (PBS, pH = 7.4). The treated cells were further stained with PE-labeled anti-CD19 or anti-CD3 antibodies to identify B and T cell populations respectively. After another washing and spin-down, the cells were analyzed by flow cytometry. The data was analyzed using WinMDI 2.8 software.

2.4. Antibody internalization assay

Cells were incubated with PE-labeled antibodies (PE-anti-CD20, PE-anti-CD19 and PE-anti-CD37) at 37 °C for 30, 60, 120 and 240 min. The antibody control was added at 0 min on ice to ensure that internalization did not occur till temperature was raised to 37 °C. After incubation, cell surface bound antibodies were removed with stripping buffer (100 mM glycine, 100 mM NaCl (pH = 2.5)) thus allowing detection of only internalized fluorochrome labeled Ab by flow cytometry. Appropriate IgG isotypes were used as negative controls. Internalization is defined as time-dependent increase in the Mean Fluorescent Intensity (MFI) after acidic washing by stripping buffer.

2.5. Combinatorial antibody microarray

The Ab microarrays were constructed by printing antibodies against CD19, CD20, CD37 and their dual combinations onto a surface-modified glass slide. Three pure (anti-CD19, anti-CD20, and anti-CD37) and dual antibody combinations were printed onto Nexterion® slide H using a non-contact piezoelectric arrayer (Perkin Elmer, Waltham, MA), at the same total antibody concentration (0.5 mg/ml). Each sample was arrayed in triplicate with a spot center-to-center distance of 400 μm into a subarray of 6.8 mm × 6.8 mm. Cells, from B-CLL patients, were labeled with CFSE