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Drug-free macromolecular therapeutics: Impact of structure on induction of apoptosis in Raji B cells

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ABSTRACT

Recently, we developed a new paradigm in macromolecular therapeutics that avoids the use of low molecular weight drugs. The activity of the "drug-free macromolecular therapeutics" is based on the biorecognition of complementary motifs at cell surface resulting in receptor crosslinking and apoptosis induction. The system is composed of two nanoconjugates: (1) a single-stranded morpholino oligonucleotide (MORF1) attached to an anti-CD20 Fab′ fragment (Fab′-MORF1); (2) multiple copies of complementary oligonucleotide MORF2 grafted to a linear polymer of *N*-(2-hydroxypropyl)methacrylamide (HPMA) – P-$(MORF2)_x$. The two conjugates crosslink CD20 antigens via MORF1-MORF2 hybridization at the surface of $CD20^+$ malignant B-cells and induce apoptosis. Preclinical studies in a murine model of human non-Hodgkin's lymphoma showed cancer cells eradication and long-term survivors. The aim of this study was to determine the relationship between the detailed structure of the nanoconjugates and apoptosis induction in Raji cells to allow system optimization. The factors studied include the length of the MORF sequence, the valence of P-$(MORF2)_x$ (varying x), molecular weight of P-$(MORF2)_x$, incorporation of a miniPEG spacer between Fab′ and MORF1 and between polymer backbone and pendant MORF2, and comparison of two Fab′ fragments, one from 1F5 antibody (Fab'_{1F5}), the other from Rituximab (Fab'_{RTX}). The results of apoptosis induction in human Burkitt's B-cell non-Hodgkin's lymphoma (NHL) Raji cells as determined using three apoptotic assays (Annexin V, Caspase 3, and TUNEL) indicated that: a) An improvement of apoptotic activity was observed for a 28 base pair MORF sequence when compared to MORFs composed of 20 and 25 base pairs. The differences depended on type of assay, concentration and exposure schedule (consecutive vs. premixed). b) The higher the valence of P-$(MORF2)_x$ the higher the levels of apoptosis. c) Higher molecular weight of P-$(MORF2)_x$ induced higher levels of apoptosis. d) A $miniPEG_8$ spacer was effective in enhancing apoptotic levels in contrast to a $miniPEG_2$ spacer. e) There was not a statistically significant difference when comparing Fab'_{1F5}-MORF1 with Fab'_{RTX}-MORF1.

1. Introduction

Molecular biorecognition is at the center of all biological processes. Many life activities are based on self-assembly and specific interaction between macromolecules, e.g. coiled-coil peptide/protein recognition, antibody-antigen binding and DNA hybridization. The high-fidelity self-assembling nature motifs and their mimics can be employed to label/functionalize cell surfaces [1], influence immune response [2], control cell signaling pathways [3–5], manipulate cell fate [6–7], and trigger cellular events [3,8–17]. Biorecognition forms the basis for the design of precisely defined smart systems, including targeted therapeutics [10,12], imaging agents, stimuli-sensitive and self-assembled biomaterials [18], and biosensors [19]. Incorporation of native biorecognition motifs as grafts attached to synthetic polymer chains results in hybrid macromolecules that have the potential for self-assembly. For example, two *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing grafts of complementary coiled-coil forming peptides [20] or HPMA copolymers grafted with peptide nucleic acids self-assemble into 3D hydrogels [21]. Similarly, two poly(*N*,*N*-dimethylacrylamide)s grafted with complementary oligonucleotides (oligoT10 and oligoA10) self-assemble into hydrogels [22].

Cell surface receptor clustering (crosslinking) as a result of multivalent ligand biorecognition is a driving force of various cellular events such as growth factor signaling [23], immune system function [24,25], neuronal cell communication [26], hormone uptake [27], cell adhesion [28], activation [29], and apoptosis [30,31]. For example, EGF receptor crosslinking appears to be a necessary and sufficient signal for induction of DNA synthesis [32]. Crosslinking a variety of human plasmacytoid dendritic cell surface receptors leads to the regulation of interferon-α

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production [33]. Insulin-receptor crosslinking may enhance the biological activity of insulin [27]. When bound to antibodies CD20 receptors are hyper-crosslinked via Fc receptor-expressing immune effector cells (e.g., macrophages, natural killer cells), resulting in apoptosis induction in B-cells [34].

Conventional polymeric nanomedicines utilize polymers as delivery vehicles to modify the biodistribution of anticancer drugs. Recent designs of nanomedicines add another function – to trigger or improve therapeutic effects through natural biological responses [35]. Macromolecular therapeutics employ biomimetic strategies to stimulate or control specific cellular activities [36,37]. The research presented here is based on a novel paradigm in the nanomedicine research area – drug-free macromolecular therapeutics [9]. The basic idea is to induce apoptosis by crosslinking of cell-surface (slowly internalizing) receptors mediated by the biorecognition of high-fidelity natural binding motifs, such as antiparallel coiled-coil peptides [10] or complementary oligonucleotides [12].

Non-Hodgkin's lymphoma (NHL) is a prevalent cancer in the United States with around 72,000 new cases in 2015 [38]. About 85% NHL originate from B-cells the remaining diseases are mostly of T-cell origin [39]. CD20 is one of the most reliable biomarkers of B-lymphocytes. It is highly expressed on the surfaces of most malignant B-cells, as well as normal B-cells; however, it is not expressed on stem cells or progenitor cells and mature or activated plasma cells [40]. Clustering of CD20 receptors at the surface of B-cells mediates the interaction of CD20 with Src-family kinases and triggers apoptotic signaling [41]. The therapeutic efficacy of anti-CD20 mAb (Rituximab) is ascribed to three cellular events: antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and CD20-mediated apoptosis [30,34]. All of these mechanisms require immune effector cells to function [42]. The clinical non-responsiveness and adverse effects of Rituximab or other therapeutic mAb has been partly attributed to the Fc fragment-related biological events [43,44].

In contrast, drug-free macromolecular therapeutics trigger direct and specific apoptosis of B-cell lymphomas without the help of effector cells [10]. This is achieved by the design of synthetic effectors that reproduce the function of immune effector cells. Two nanoconjugates are being used: the first is an anti-CD20 Fab′ fragment conjugated to one natural motif (peptide 1, oligonucleotide 1). B cells exposed to this nanoconjugate are decorated with the natural motif. Since the CD20 receptor is very slowly internalizing a new biorecognition site is created. Further exposure of decorated cells to an HPMA copolymer grafted with multiple copies of the complementary motif (peptide 2, oligonucleotide 2) results in crosslinking of CD20 receptors and initiation of apoptosis [9]. This design was validated using a complementary pair of coiled-coil forming peptides CCE and CCK [20]. Apoptosis was initiated by CD20 receptor crosslinking both in vitro [10,17,45] and in vivo [11].

Our recent studies use a pair of complementary morpholino oligonucleotides (MORF1, MORF2) due to their fast hybridization, excellent binding affinity, and stability in plasma as well as water-solubility. The system is composed of two nanoconjugates: (1) a single-stranded morpholino oligonucleotide MORF1 attached to an anti-CD20 Fab′ fragment, (2) multiple copies of complementary oligonucleotide MORF2 grafted to a linear HPMA copolymer – P-$(MORF2)_x$. The two conjugates crosslink CD20 antigens via MORF1-MORF2 hybridization at the surface of $CD20^+$ malignant B-cells and induce apoptosis [12–17]. Preclinical studies in a murine model of human non-Hodgkin's lymphoma showed cancer cells eradication and long-term survivors [12,14].

The aim of this study is to determine the relationship between the detailed structure of nanoconjugates and apoptosis induction in Raji cells to allow system optimization. The factors studied include the length of the MORF sequence, the valence of P-$(MORF2)_x$ (varying x), molecular weight of P-$(MORF2)_x$, incorporation of a miniPEG spacer between Fab′ and MORF1 and between polymer and MORF2, and comparison of two Fab′ fragments, one from 1F5 antibody (Fab'_{1F5}), the other from Rituximab (Fab'_{RTX}).

2. Materials and methods

2.1. Materials

N-(3-Aminopropyl)methacrylamide hydrochloride (APMA) was purchased from Polysciences (Warrington, PA). 2,2′-Azobis(2,4-dimethylvaleronitrile) (V-65), 2,2′-azobis[2-(2-imidazolin-2-yl) propane]dihydrochloride (VA-044), and 4,4-azobis(4-cyanopentanoic acid) (V-501) were from Wako Chemicals (Richmond, VA). Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was from Soltec Ventures (Beverly, MA), crosslinkers $SM(PEG)_2$ (succinimidyl-[(*N*-maleimidopropionamido)-diethyleneglycol] ester) and $SM(PEG)_8$ (succinimidyl-[(*N*-maleimidopropionamido)-octaethyleneglycol] ester), were from Thermo Fisher Scientific. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) [46] and 4-cyanopentanoic acid dithiobenzoate (CPDB) [47] were prepared as previously described. Pepsin, 1-amino-2-propanol, and cysteamine were from Sigma-Aldrich and tris(2-carboxyethyl)phosphine (TCEP) from Thermo Scientific. All solvents were obtained from Sigma-Aldrich as the highest purity available.

The complementary 3′-amine-derivatized (20, 25 and 28)-mer phosphorodiamidate morpholino oligonucleotides and 3′-disulfide amide 25-mer phosphorodiamidate morpholino oligomers (MORF2-SSR) were from Gene Tools (Philomath, OR). MORF1 (20 bp, Mw = 6926 Da; 25 bp, Mw = 8631 Da; 28 bp, Mw = 9639 Da); MORF2-SSR (25 bp, Mw = 8585); MORF2, (20 bp, Mw = 6792 Da; 25 bp, Mw = 8437 Da; 28 bp, Mw = 9438 Da). For the design of base sequences, a sequence scrambling software (http://www.sirnawizard.com/scrambled.php) and a sequence analysis software (http://www.basic.northwestern.edu/biotools/oligocalc.html) were used [12].

2.2. Methods

2.2.1. Synthesis of Fab'_{1F5}-MORF1, Fab'_{1F5}-PEG_2-MORF1 and Fab'_{RTX}-MORF1 conjugates

The 1F5 mAb (IgG2a) was prepared from a mouse hybridoma cell subclone 1F5 (ATCC) in a CellMax® bioreactor (Spectrum Laboratories) and purified on a Protein G Sepharose 4 Fast Flow column (GE Healthcare). Rituximab (RTX) was purchased from Biogen Idec and Genentech USA. Preparation of Fab'_{1F5} (or Fab'_{RTX}) followed a previously reported procedure [12,14]. Briefly, antibodies were digested into $F(ab')_2$ with 10% (w/w) pepsin in citric buffer (pH 4.0). Immediately before conjugation, $F(ab')_2$ was reduced to Fab′-SH by 10 mM TCEP.

The Fab'_{1F5}-MORF1, Fab'_{1F5}-PEG_2-MORF1 and Fab'_{RTX}-MORF1 conjugates were synthesized similarly as previously described [12,14]. A typical procedure (Scheme 1) was as follows: First, 200 nmol MORF1-NH_2 (20, 25 or 28 bps) was reacted with 1.34 mg (4 μmol) SMCC (or $SM(PEG)_2$) in 200 μL DMSO to produce the MORF1-mal (containing a 3′-maleimide). The reaction was performed at room temperature (RT) for 24 h. The excess of SMCC (or $SM(PEG)_2$) was removed by ultrafiltration (3000 Da cut-off) with PBS (pH 6.5) four times to yield MORF1-mal.

Second, the MORF1-mal was mixed with 60 nmol (3 mg) freshly reduced Fab′-SH in 600 μL PBS (pH 6.5). The reaction was performed at 4 °C for 24 h. Finally, the Fab′-MORF1 conjugate was purified using size exclusion chromatography (SEC) to remove free, unconjugated Fab′ and MORF1. An ÄKTA FPLC system equipped with Sephacryl S-100 HR16/60 column (GE Healthcare) eluted with PBS (pH 7.2) was used. To determine Fab′ equivalent concentration of the Fab′-MORF1 conjugate, a bicinchoninic acid (BCA) protein assay (Pierce) was used. UV–visible spectroscopy (Varian Cary 400, Agilent Technologies) was used for quantification of the MORF1 equivalent concentration; the molar absorptivity of MORF1-20 bp, MORF1-25 bp and MORF1-28 bp were 220,470, 278,000, and 309,980 M^{-1} cm^{-1}, respectively (at 265 nm, in 0.1 N HCl).