RESEARCH ARTICLE

Editorial Process: Submission:12/05/2022 Acceptance:01/26/2026 Published:02/05/2026

Synthesis of Immunotoxin of Anti-CD3 Monoclonal Antibody-Bongkrekic Acid and Cytotoxic Effect Against CD3+ T Cells in Peripheral Blood Mononuclear Cells

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Abstract

Introduction: Cancer is a major health concern worldwide. Common cancer treatments such as surgery, chemotherapy, and radiotherapy have not been able to completely reduce the rate of cancer-related death. Monoclonal antibodies (MAb) have been widely used for cancer treatment in the form of immunotherapy or targeted therapy. Immunotoxins are a form of targeted therapy based on monoclonal antibody-toxin conjugates. Antibodies deliver toxins to specific cancer cells and induce cell death. **Objective:** This study aimed to synthesize a conjugate of bongkrekic acid (BKA), a potent mitochondrial toxin, with anti-CD3 MAb and evaluate its specificity in peripheral blood mononuclear cells (PBMC).**Methods:** In silico assays were performed to predict conjugation sites and interactions between BKA and MAb. The synthesis of the conjugate was carried out chemically using EDC-HCl/Sulfo-NHS zero-length crosslinker and confirmed using a UV-Vis spectrophotometer. PBMC were used as a specificity test model. The conjugate exhibited selective cytotoxic toward CD3+ T cells without affecting other cells in PBMCs.**Results:** In silico assays using molecular docking showed conjugation sites in the cavity of the CH2-CH3 Fc IgG2a domain and covalent interactions with lysine, asparagine, and glutamine amino acids. Measurements of absorption at wavelengths of 280 and 260 nm indicated the presence of protein and BKA in the synthesized conjugate. Incubation of PBMC with BKA and anti-CD3 MAb resulted in a significantly lower average number of cells ($p< 0.05$) than that observed in the group treated with the conjugate. **Conclusion:** In silico assays revealed an interaction between the carboxylic groups of BKA and the primary amine group of antibodies. The Conjugates exhibited lower cytotoxicity compared to BKA and anti-CD3 Mab individually. In vitro assays have not been able to show the specificity of the conjugate due to the anti-CD3 MAb alone exhibiting cytotoxic properties in PBMCs.

Keywords: Immunotoxin, anti-CD3 MAb-BKA, CD3+ T cells.

Asian Pac J Cancer Prev, **27 (2)**, 443-451

Introduction

Cancer is one of the diseases that is a major health problem in the world. In 2020, there were an estimated 19.3 million new cases, with approximately 10 million cancer deaths [1]. In the development of cancer treatment modalities, surgery, chemotherapy, and radiotherapy are the most common therapies carried out as single or combination therapy [2]. However, the treatment has not been able to completely reduce the rate of death from cancer. Surgery is effective for removing solid tumors; however, the presence of residual tumors supports recurrence and induces metastasis [3]. Treatment with chemotherapy although it can reduce the level of morbidity and mortality due to cancer, there are cytotoxic effects caused by chemotherapeutic agents that are systemic and cause resistance [4]. While radiotherapy hurts normal cells [5]. Targeted therapy is one of the focuses that continues to be developed to produce more effective cancer therapies by increasing specificity and minimizing the off-target side effects caused to normal tissues [6].

Immunotoxins are monoclonal antibody-based targeted therapy [7]. Monoclonal antibodies (MAb) are conjugated with toxins using chemical reactions or recombinant technology. The structure of immunotoxins is a conjugate between Mab and toxins derived from bacteria, plants, or mammalian proteins [8]. MAb in immunotoxins acts as "magic bullets" that recognize specific antigens in cancer cells and will deliver toxin compounds to cancer cells [9], so that the toxin can carry out killing activity in cancer cells.

Toxins used in the synthesis of immunotoxins are

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proteins derived from bacteria (diphtheria toxin (DT), Pseudomonas exotoxin A (PE), and Anthrax toxin), plant toxins (ricin, gelonin, saporin, and pokeweed), and mammalian proteins (Granzim and RNase) [8]. The main mechanism of toxins in inducing cancer cell death includes inhibiting protein synthesis through ADP ribosylation in eukaryotic protein Elongation Factor 2 (eEF2) (DT and PE). Meanwhile, ricin and gelonin toxin proteins inhibit protein synthesis by causing N-glycosylation of rRNA [8]. In this study, bongkrekic acid (BKA) which is a toxin derived from the bacterium Burkholderia cocovenenans was conjugated with anti-CD3 MAb and wanted to know its potential as an immunotoxin candidate. BKA is a well-known mitochondrial toxin that acts as a specific ligand and an inhibitor of the enzyme adenine nucleotide translocase (ANT) or ATP/ADP translocator [10].

In this study, a carbodiimide zero-crosslinker EDC. HCl/sulfo-NHS was used in the chemical synthesis of immunotoxins. The water-soluble carbodiimide EDC may be used to form active ester functionalities with carboxylate groups using the water-soluble compound, NHS (sulfo-NHS) [11]. EDC.HCl will activate the tricarboxylic group of BKA with the help of sulfo-NHS to form a stable BKA-NHS ester. The ester was reactive against the nucleophilic primary amine group of antibodies from the anti-CD3-MAb-BKA conjugate. However, some active groups of BKA allow the formation of several ester structures that have different reactivities to the primary amine group of the antibody and affect the product of the conjugation reaction. Therefore, in silico studies were conducted to determine the optimal conditions for the conjugation reaction.

The target specificity and internalization ability of antibodies are major problems in the selective intracellular delivery of toxins. In this study, a specificity model was carried out in vitro using PBMC. PBMC contain various components such as lymphocyte cells namely T cells (70-85%), B cells (5-10%), NK cells (5-20%), monocytes, and dendritic cells [12]. T cells are mainly composed of two large components: CD4+ and CD8+ T cells. Both T cell types have a common CD3 protein marker [13]. In this study, anti-CD3 Mab-BKA was chemically synthesized to be selectively targeted at CD3 glycoproteins on the surface of lymphocyte cells. The PBMC specificity model aims to prove that the synthesized immunotoxins are cytotoxic in one target cell type, but not cytotoxic to normal cells. Anti-CD3 Mab-BKA is expected to be specifically cytotoxic to CD3+ T cells compared with other cell populations in PBMC.

Materials and Methods

Materials

Bongkrekic Acid (Fermentek); Anti-CD3 Monoclonal antibody (OKT3) (Biolegend); EDC.HCl (Sigma Aldrich); Sulfo-NHS (Sigma Aldrich); MES Hydrate (Sigma Aldrich); Dialysis tubing cellulose membrane (Sigma Aldrich); Histopaque 1077 (Sigma Aldrich), RPMI 1640 Medium (Thermo); PBS steril pH 7,4 (Thermo); trypan blue (Thermo).

Methods

Preparation of Anti-CD3 MAb-BKA

The conjugation reaction of anti-CD3 MAb with BKA was performed using EDC.HCl/Sulfo-NHS chemistry. EDC.HCl activates the carboxylate group of BKA such that it is more reactive to the primary amine group of the antibody. The protocol consisted of two steps: the activation reaction of the carboxylic group of BKA into a more stable ester form, and the conjugation reaction (Figure 1). The activation step began by adding 40 µL of EDC.HCl solution (2 mg/mL) and 45 µL of sulfo-NHS solution (2 mg/mL) to 100 µL of BKA in MES buffer (1 mg/mL). The solution was homogenized and allowed to react for 30 min at room temperature. For conjugation, 100 µL anti-CD3 MAb in MES buffer (1 mg/mL) was added to the activated BKA solution (molar ratio 1:1). The samples were then mixed and incubated at 4 °C for 24 h [11]. Finally, the synthesized conjugate was dialyzed against 50 mM phosphate buffer for 24 h at 4 °C to remove any unreacted compounds. The conjugate concentration was determined using UV–Vis spectrophotometry. The concentration of synthesized conjugate was determined as a protein compound at a wavelength of 280 using albumin protein as a standard [14].

Conjugate Confirmation

The formation of anti-CD3 MAb-BKA was confirmed using UV-Vis spectrophotometry. The absorbance of the synthesized conjugate was measured at a wavelength of 280 nm as a protein compound and 260 nm as the maximum wavelength of BKA, compared to the absorbance of BKA and anti-CD3 Mab [14]. Previously, the maximum wavelength of BKA has been measured in the range of 190-320 nm.

Molecular Docking Studies

BKA structure consists of three carboxylate groups (Figure 2). These reactive groups allow the variation of BKA-NHS esters with different reactivities to anti-CD3 MAb and influence the conjugation ability. In this study, seven BKA-NHS ester structures were created in a two-dimensional manner to obtain the most stable structure with the highest affinity for anti-CD3 MAb. Molecular docking was conducted between BKA, BKA-NHS esters, and crosslinkers (EDC.HCl and Sulfo-NHS) against the Fc of anti-CD3 MAb (IgG2a) using Molecular Operating Environment (MOE) [15]. The Fc of anti-CD3 MAb used was a crystal structure of Fc IGg2a downloaded from the Protein Data Bank (https://www.rcsb.org/). BKA, BKA-NHS esters, EDC.HCl, dan Sulfo-NHS were created in 2D using ChemSketch [16]. Then, structural optimization of the protein and ligands was carried out using MOE software. The results of molecular docking were analyzed in terms of the binding site, binding free energy (ΔG), and ligand-protein interaction, as seen from 2D visualizations.

PBMC Isolation

Conjugate specificity tests were performed using PBMC isolated from blood by the gradient centrifugation method using Histopaque reagents. Peripheral blood samples from one volunteer were obtained using five

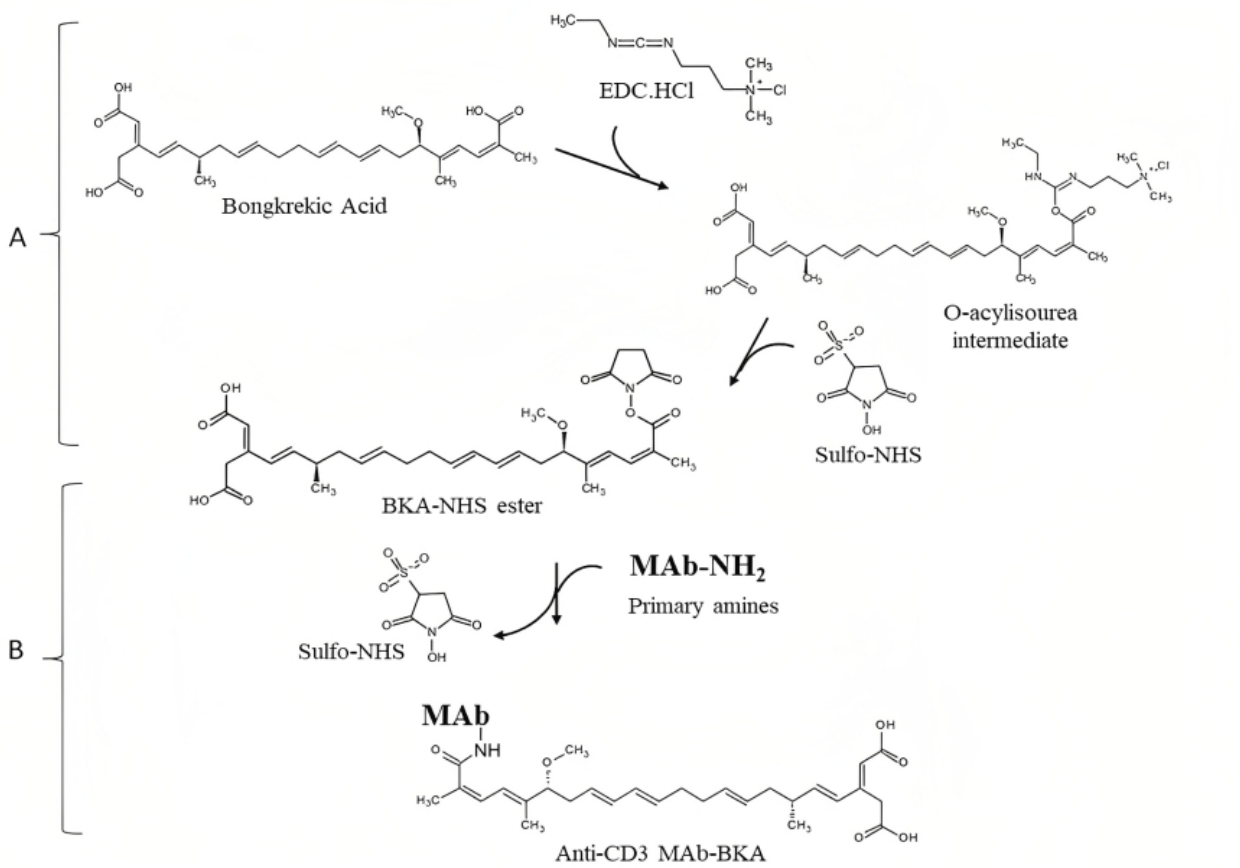

Figure 1. Two Steps Conjugation Reaction. Activation of carboxylic functional groups of BKA by EDC.HCl/Sulfo-NHS forms BKA-NHS ester (A). BKA-NHS ester reacts with primary amines of antibody form anti-CD3-MAb-BKA conjugate (B).

4 mL heparinized vacutainers. A total of 20 mL of blood from one healthy subject was diluted with PBS (volume ratio 1:1). The diluted blood was slowly flowed into the Histopaque solution (volume ratio 1:1) into a 50 mL centrifuge tube and centrifuged at 400 x g for 30 min. A layer of the buffy coat was washed twice by adding PBS and centrifuged at 250 x g for 10 min [17]. The centrifuged pellets were suspended in RPMI medium without supplementation and cell calculations were carried out using a hemocytometer. The number of cells per milliliter was calculated by the formula:

$$\text{Cell viability (\%)}: \frac{amounts\ of\ viable\ cells}{amounts\ of\ total\ cells} \text{ x } 100\%$$

In-vitro Anti-CD3 MAb-BKA Specificity Evaluation

The specificity of the conjugate was expressed by the number of live PBMC in the group administered the conjugate compared to the BKA and anti-CD3 MAb groups at the same concentration. In this study, it was expected that conjugate administration would produce the highest number of living cells because it was only cytotoxic to CD3+ T cells in the PBMC. The viability of PBMC was determined using the trypan blue excursion method by examining the number of live cells. The cell suspension was seeded into a 96-well culture plate (1×10^6 cells/well). Cells in the complete medium are given test compounds with the following group:

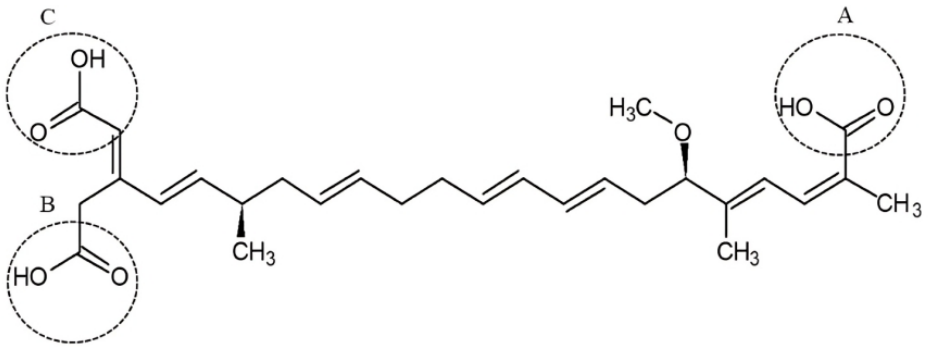

Figure 2. Chemical Structure of Bongkrekic Acid with Three Carboxylate Functional Groups (MW: 486.6 g/mol).

Group A: PBMC + BKA
Group B: PBMC + Anti-CD3 MAb
Group C: PBMC + Anti-CD3 MAb-BKA
Group D: PBMC + Control

The test compounds were prepared at concentrations of 10, 20, 40, 60, and 80 µL/mL and were incubated at 37oC and 5% CO2 for 24 h. After incubation, the cells were transferred to microtubes and centrifuged at 250 × g for 10 min. Pellets were suspended with RPMI without supplementation and cell calculation was carried out.

The number of live cells: Average number of cells x dilution factor x 10^4

Statistical Analysis

The number of live cells between the test groups was analyzed using a one-way analysis of variance with parametric statistics. If the data were not normally distributed, data analysis was performed using the Kruskal–Wallis non-parametric test. Significant differences between test groups were obtained when $p<0.05$.

Results

Preparation of Anti-CD3 MAb-BKA

After dialysis, the concentration of anti-CD3 MAb-BKA was determined based on the concentration of conjugated antibody proteins and measured at a wavelength of 280 nm. The absorbance of the conjugate was then inserted into the standard albumin curve equation, resulting in a concentration of 3.46 mg/mL. The maximum wavelength of the previously measured BKA was 260 nm, as shown in Figure 3. Confirmation of the formation of anti-CD3 MAb-BKA was carried out by comparing the absorbance of anti-CD3 MAb, BKA, and the conjugate at 280 and 260 nm, resulting in the comparison of the wavelengths shown in Table 1. At a wavelength of 280 nm, the absorbance of the conjugate approached that of anti-CD3 MAb at the same concentration (20 µg/mL). This indicates the protein content of the conjugate compound. Whereas at a wavelength of 260 nm, the absorbance of conjugate when compared to the absorbance of BKA at the same concentration (20 µg/mL), shows a lower absorbance compared to BKA.

Molecular docking studies

The Fc structure of anti-CD3 MAb used was a crystal structure of the non-FCRN-binding Fc Fragment of rat IgG2a (PDB Code:1I1C). Then, one of the CH2-CH3 domains was used to perform molecular docking of the ligands. Molecular docking results are presented in Table 2. The lowest binding energy value was produced by BKA, followed by BKA-NHS 1C, and sulfo-NHS. The three test ligands with the highest affinities had stable interactions with the binding site. The 3D conformation and surface of the CH2-CH3 domain with the ligand-binding site are shown in Figure 4. These ligands tended to be localized in the cavity between the CH2 and CH3 domains. A 2D visualization of the docking results is shown in Figure 5. shows the type of amino acid and the type of bond that occurs between the ligand and amino acid at the binding site. In BKA, there are seven hydrogen bonds between lysine, glutamine, and asparagine amino acids, with oxygen atoms on the carboxylic and ether functional groups. In BKA-NHS 1C, there were four hydrogen bonds between the same amino acid and oxygen atoms in the carboxylic and ether groups. Sulfo-NHS ligands undergo hydrogen bonds on oxygen atoms in sulfate and carbonyl groups with the lysine. Briefly, lysine, glutamine, and asparagine are amino acids that interact with ligands. Among the three types of amino acids, the

Table 1. The Absorbance of Conjugate Compared to the Anti-CD3 MAb and BKA at the Wavelength of 280 nm and 260 nm

Test Solution	Wavelength (nm)	
	280	260
Anti-CD3 MAb	0.234	0.085
BKA	0.110	0.573
Anti-CD3 MAb-BKA	0.175	0.176

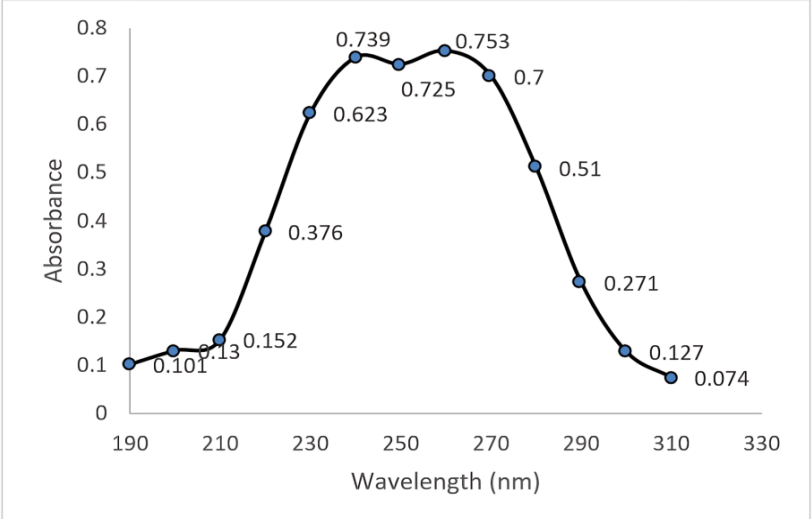

Figure 3. Maximum Wavelength of Bongkrekic Acid Measured by Spectrophotometer UV-Vis at 260 nm.

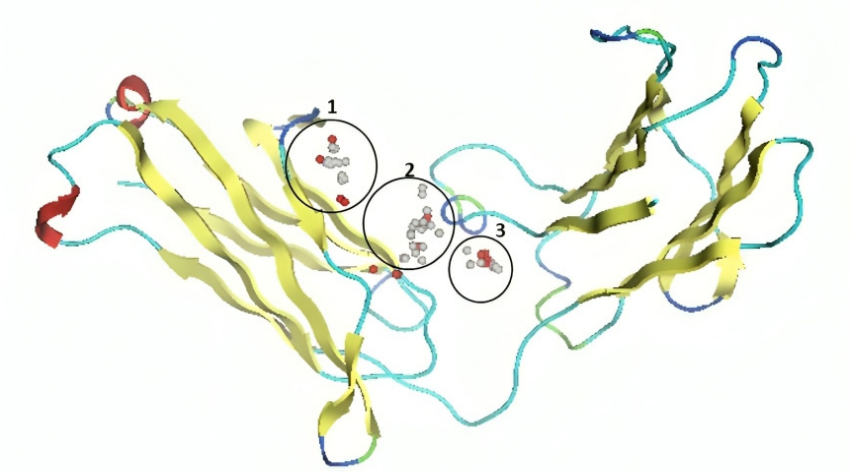

Figure 4. Sulfo-NHS (1), BKA-NHS 1C (2), and BKA (3) Binding Sites on CH2-CH3 Domain of 1I1C Chain.

amino acid lysine most interacts with ligands.

In-vitro Anti-CD3 MAb-BKA Specificity Evaluation

Results of PBMC 24-hours incubation with BKA, anti-CD3 MAb, and the conjugate are shown in Figure 6. Viability was expressed as the number of living cells against concentration. All groups showed a significant reduction in the number of live cells compared with the control at all concentrations ($p<0.05$). The number of living cells decreased significantly with an increase in BKA and conjugate concentrations ($p<0.05$). However, the administration of anti-CD3 MAb did not result in a significant decrease in the number of living cells. A comparison of the number of living cells after treatment with BKA, anti-CD3 MAb, and the conjugate is shown in Figure 6D. PBMC-administered BKA resulted in a lower number of live cells than the conjugate group ($p<0.05$). Meanwhile, the number of living cells in the anti-CD3 MAb group was between that in the BKA and conjugate groups.

Discussion

The synthesis of anti-CD3 MAb-BKA was carried

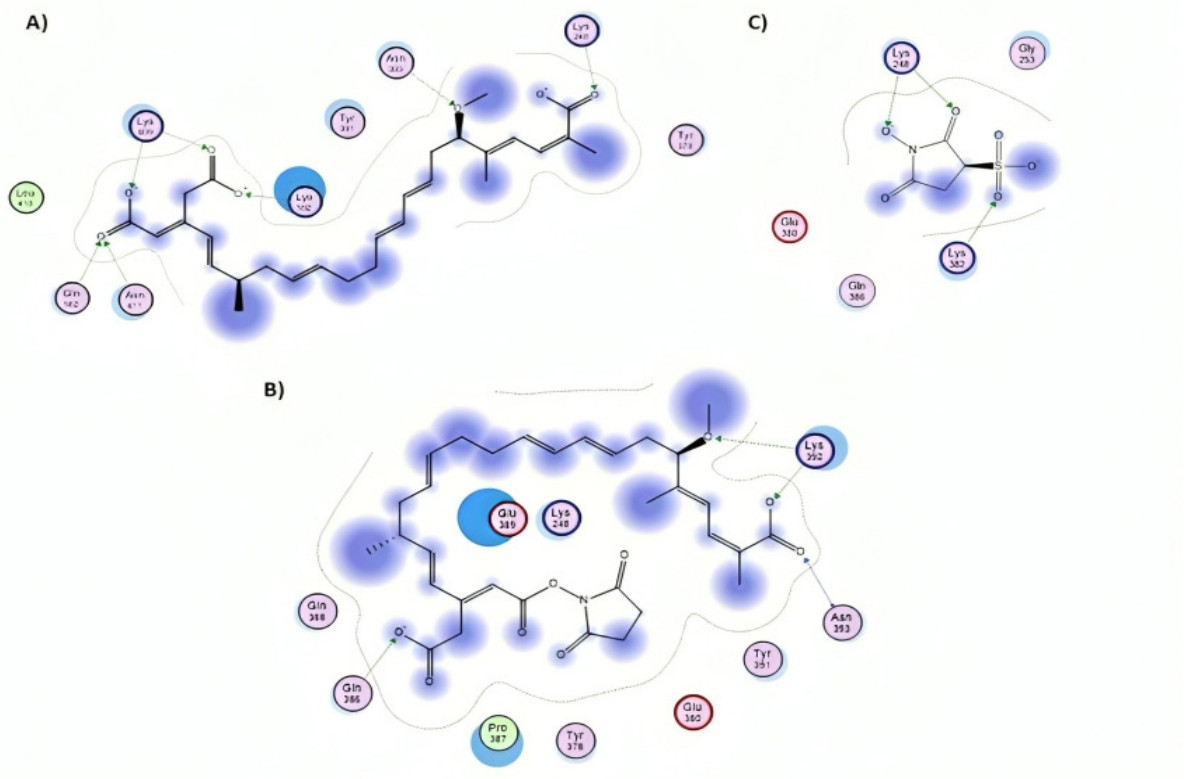

Figure 5. The 2D Visualization of Ligand Interaction: A) BKA, B) BKA-NHS 1C, and C) sulfo-NHS. Lysine, glutamine, and asparagine are amino acids interacting via hydrogen bonds with all three ligands. Among the three types of amino acids, the amino acid lysine most interacts with ligands.

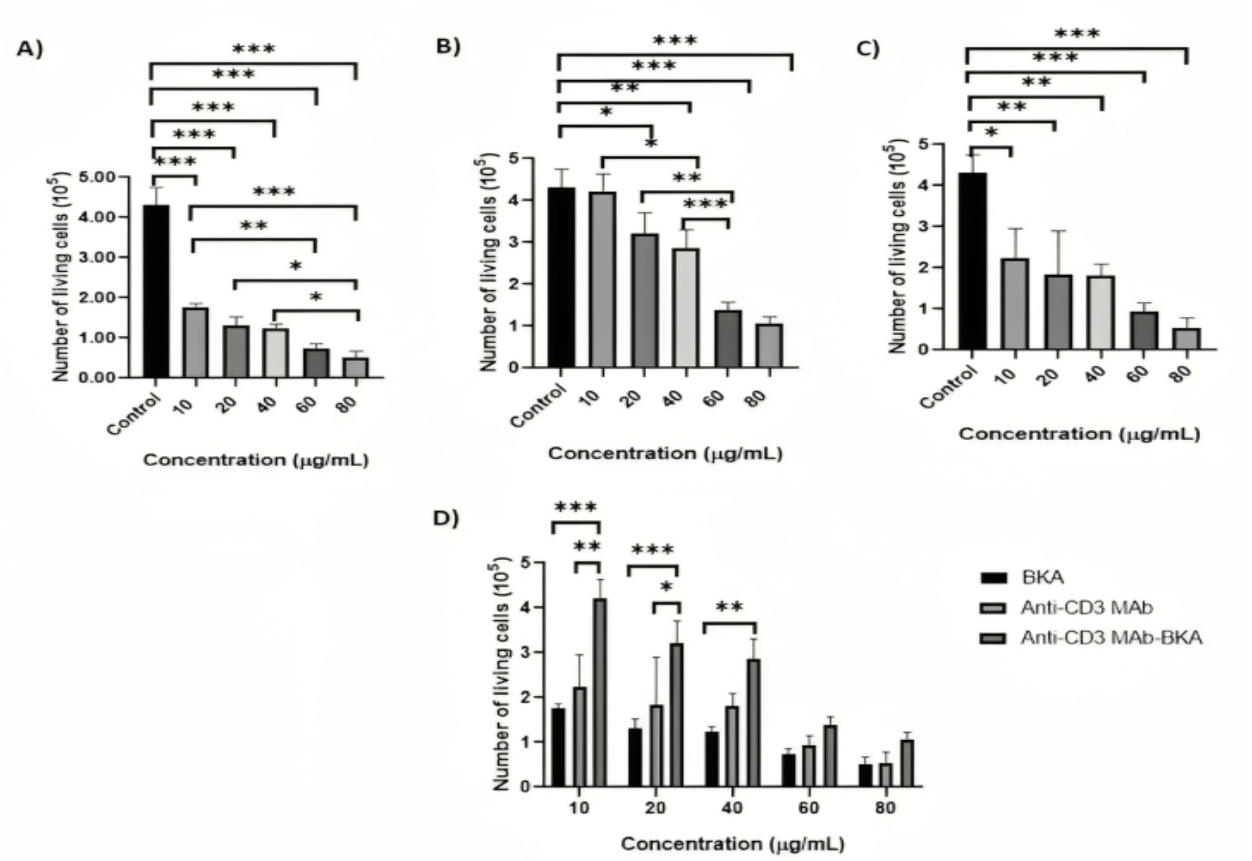

Figure 6. Live Cells Number After 24-hours Incubation of Treatment: BKA (A), conjugate (B), anti-CD3 MAb (C). All groups showed a significant reduction of live cells number compared to control in every concentration ($p<0.05$). The number of living cells decreased significantly with an increase in BKA and conjugate concentrations ($p<0.05$) (A, B). Comparison of the number of living cells on the administration of BKA, conjugate, and anti-CD3 MAb in PBMC. PBMC-administered BKA resulted in lower number of live cells compared to conjugate group ($p<0.05$) (D)

Table 2. Results of Molecular Docking of Ligands with CH2-CH3 Domain of 1I1C Chain

Ligands	Parameters		
	ΔG	Pki	H Binding
BKA	-14.4737	13.183	LYS 248, GLN 362, LYS 392, ASN 393, LYS 409, LYS 409, ASN 411
EDC.HCl	-6.6618	5.326	-
BKA-NHS 1A*	-11.5482	6.356	LYS 246, LYS 409
BKA-NHS 1B	-10.128	9.534	LYS 246, LYS 246, LYS 258, LYS 258
BKA-NHS 1C	-12.5641	8.244	GLN 386, LYS 392, LYS 392, ASN 393
BKA -NHS 2A	-8.0097	5.652	LYS 248, ASN 243
BKA-NHS 2B	-10.4015	5.569	TYR 391
BKA-NHS 2C	-11.8011	7.32	LYS 409, ASN 411,
BKA-NHS 3	-9.2538	4.264	LYS 392
Sulfo-NHS	-12.2801	8.107	LYS 248, LYS 248, LYS 382, GLN, 386, GLN 386

* 1, number of carboxylate functional group activated into ester; A, position of ester groups according to Figure 2.

out through a chemical reaction using water-soluble carbodiimides EDC.HCl combined with sulfo-NHS. EDC-HCl reacts with the carboxyl groups of BKA to form o-acylisourea intermediates that react with amine group antibodies. However, the nature of o-acylisourea is easily hydrolyzed before reacting with primary amines on antibodies [11]. Sulfo-NHS is used in activation step to generate stable BKA-NHS ester (Figure 1A). In the presence of amine nucleophiles, the sulfo-NHS ester is rapidly hydrolyzed, allowing the formation of an anti-CD3-MAb-BKA conjugate with an amide bond (Figure 1B).

EDC.HCl/sulfo-NHS-based approaches for the preparation of conjugates is influenced by scalability and the ratio of the amount of each reactant in the conjugation reaction [18]. In this experiment, a mole ratio was used

between BKA, EDC.HCl, and sulfo-NHS are 1:2:2. The number of moles of crosslinker was increased in order for the esterification reaction to occur in one or more carboxylic groups of BKA. The conjugation reaction was carried out with a molar ratio of 1:1 between BKA and anti-CD3 antibodies to avoid the binding of BKA at multiple conjugation sites on antibodies. Primary amine groups such as the amino acids lysine, asparagine, arginine and glutamine, and N-terminal antibodies, enable conjugation reactions at multiple binding sites and produce heterogeneous conjugate compounds [19].

Purification of conjugate compounds by dialysis was performed to remove unreacted BKA, EDC-HCl, and sulfo-NHS. The excess of EDC.HCl has to be removed as it will otherwise induce intramolecular cross-linking of the antibodies [18]. Spectrophotometric method was used to confirm the formation of conjugate according to protein and BKA absorption of conjugate.

The chemical structure of BKA, consisting of several reactive groups, leads to the possibility of the formation of various ester and conjugate product structures. Therefore, in molecular docking, we created a possible structure of BKA-NHS esters, as well as free BKA and crosslinker, and docked them with the Fc portion of IgG2a. The Fc region was used because of the possibility of conjugation sites occurring with the primary amine group at the N-terminus of the antibodies. However, conjugation reactions can also occur in the side chains of the immunoglobulin amino acid residues in Fc and Fab. IgG2a isotypes have primary amine groups on the amino acids lysine (K), arginine (R), asparagine (N), and glutamine (Q) [20]. Therefore, in next study, molecular docking between ligands and Fab IgG2a also needs to be performed to determine possible conjugation sites on Fab that may affect the interaction of antibodies with CD3 antigens.

Among the ten structures docked with Fc IgG2a, three structures had the lowest binding energy (ΔG), namely BKA, BKA-NHS 1C, and sulfo-NHS. A low ΔG value indicates a strong interaction between ligands and proteins [21]. The binding energy of BKA was lower than that of its ester form due to the presence of more hydrogen bonds in BKA than in its ester form. Of the seven ester structures, BKA-NHS 1C, with one activated carboxylate group, had a higher affinity for Fc IgG2a than BKA, with two or three activated groups. This relates to the mole ratio between BKA and crosslinker at the activation reaction stage. Sulfo-NHS also showed reactivity toward the amine groups. Therefore, BKA and sulfo-NHS must be purified for successful conjugation. 3D visualization of molecular docking showed amino acids interacting with the ligands. The amino acids lysine, glutamine, and asparagine interact the most with the ligands. Lysine, glutamine, and asparagine are amino acids with primary amine groups and amide side chains. The primary amine group on the amino acid acts as a nucleophile and can react with BKA-NHS ester to form conjugate compounds with amide bonds.

Target specificity is a major problem for selective intracellular delivery of the toxin to target cells. Anti-CD3 Mab, specifically target the structure of CD3 glycoproteins on the cell surface of T lymphocytes, in this study were used as a specific antibody model that deliver BKA to CD3+ T cells in the PBMC fraction. Conjugate compounds containing toxins kill CD3+ T cells, leaving other cells in PBMC alive. Incubation of PBMC with BKA solution resulted in a reduction in live cell number with increasing concentration of BKA, and the lowest average number of living cells compared to the administration of anti-CD3 MAb and conjugate. This is because BKA, which acts as a cytotoxic agent, does not selectively kill cells. BKA, which has a known role as an inhibitor of the ANT enzyme in the inner membrane of mitochondria, can trigger cell death by inhibiting ATP formation [10]. However, in this study, incubation of PBMC with BKA did not kill all cells at any concentration. The in-vitro toxicity of BKA to cells is influenced by various factors such as cell type, number of cells per well, incubation time, and concentration of BKA used [22]. Several studies describe the relationship between the role of BKA as an inhibitor of the ANT enzyme with metabolic activity and energy use by cancer and normal cells. At increasing concentrations of BKA, there was no significant decrease in viability or amount of ATP in normal cells [22]. While the results in cancer cells showed a significant decrease in viability and amount of ATP, due to high energy use and metabolic activity in cancer cells [23]. BKA uses energy sources for cell survival to rely on glucose when ATP is suppressed within the mitochondria. BKA inhibits the formation of ATP at the oxidation state of phosphorylation, so that cells meet the ATP needs through glycolysis. BKA is also known to induce cell death in a medium that is low in glucose [24]. Therefore, in-vitro testing of conjugate compounds in PBMC requires additional incubation time and medium conditions to further determine the effect of BKA.

Treatment of PBMC with the conjugate showed a higher average number of living cells compared to BKA administration. This indicates that the conjugate has a lower cytotoxic ability to PBMC compared to BKA, and has the possibility of being directed only at CD3+ T cells in PBMC. However, incubation of PBMC with anti-CD3 antibodies also resulted in a lower number of live PBMC cells compared to conjugate compounds at the same concentration. Anti-CD3 antibodies have a killing effect on T cells that is greater than the conjugate compound. This suggests that anti-CD3 MAb may also play a role in the cytotoxic properties of the conjugate, and not specifically due to the BKA mechanism.

Anti-CD3 Mab-BKA interacts with the CD3 antigen in the epsilon subunit located on the extracellular side and causes the occurrence of several cellular mechanisms [25]. The interaction between the Fab anti-CD3 antibody and the CD3 antigen causes internalization of the CD3/TCR-antibody anti-CD3 complex in lymphocyte cells [26]. The binding of anti-CD3 antibodies leads to internalization of the antibody along with the CD3/TCR complex and degradation of lysosomes [27]. The CD3/TCR-antibody complex is internalized via the clathrin/AP2 protein-dependent endocytosis pathway [28]. This mechanism has the potential to deliver conjugates to the lysosomal acid environment and protease activity hydrolyze amide bonds producing free BKA toxins in the cytosolic environment. Free BKA then enters the mitochondria to perform its

cytotoxic functions.

In addition to triggering endocytosis activity, anti-CD3 antibodies can stimulate lymphocyte activation and proliferation [20, 29]. Anti-CD3 MAb can also increase PBMC proliferation with an incubation time of more than 24 hours [21]. Anti-CD3 MAb on the other hand can induce T cell elimination through complement-mediated effector mechanisms, ADCC, cell lysis, and apoptosis in vivo and in-vitro [30]. Monocytes have the potential to cause ADCC. FcγR in monocytes interacts with the Fc portion of antibodies and induces the release of lytic enzymes and kills lymphocyte cells through phagocytosis [31]. This mechanism may be the cause of the decrease in the number of living cells due to the administration of anti-CD3 MAb. Meanwhile, administration of anti-CD3 MAb that have been conjugated with BKA, especially at the N-terminal of the Fc antibody, has great potential in blocking the interaction of Fc with the Fc receptor of monocyte cells. This probably leads to reduced function of monocyte effectors, and results in a higher number of living cells in the conjugate compared to the administration of anti-CD3 MAb.

This study has several limitations. PBMCs, consisting of cells with specific markers, have the potential to model the specificity of the conjugate. However, based on the results of the in vitro tests, this model could not been able to fully prove the specific target of the synthesized conjugate. The diverse mechanisms by which anti-CD3 MAb is administered require evaluation. In the administration of anti-CD3 Mab, it is necessary to use pure lymphocyte cultures by eliminating the presence of monocyte cells, which is compared to the administration of conjugate and BKA. At the synthesis stage of the conjugate, the reaction conditions include the ratio of reactant concentration, temperature, and pH. In this study, no purification was performed after the BKA activation stage to remove the remaining reactants before reacting with the antibodies. Confirmation of the formation of conjugate compounds in this study has not used other methods to confirm conjugate compounds have formed.

Conclusion

In silico tests revealed an interaction between the carboxylic group of BKA and the primary amine group of the antibodies. Confirmation of conjugate formation by UV-Vis spectrophotometry revealed the uptake of BKA and proteins in the conjugate. In vitro assays of conjugates showed lower cytotoxic effects than those of BKA and anti-CD3 MAb. This study was not able to fully demonstrate the specificity of conjugate compounds due to the anti-CD3 MAb which also shows cytotoxic properties in PBMC. However, this study could be the first research into the development of BKA as a toxin candidate for immunotoxin synthesis. BKA has potential as a cytotoxic agent in immunotoxins and as a target for cancer therapy. Mitochondria are an important part of the cell, have the potential to be targeted by cytotoxic agents, and require further investigation to become effective candidates for therapeutic targets in cancer.

Author Contribution Statement

Khafsah Sangadah contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript. Mohamad Sadikin and Sri Widia A. Jusman devised the project, the main conceptual ideas, and the design of the research, as well as analyzed and discussed the results.

Acknowledgements

Funding Statement

Financial supports were provided by PUTI Pascasarjana 2022 (Publikasi Terindeks International) – Universitas Indonesia. Authors would like to thank to Bioinformatics Core Facilities Cluster, Indonesian Medical Education and Research Institute (IMERI) FMUI, for provide advice and assistance during in-silico study in this research.

Approval from Scientific Body

This study was a part of an approved student thesis by Master's Programme in Biomedical Sciences, Faculty of Medicine University of Indonesia (FMUI).

Ethical Issue

Ethical approval for this study was obtained from Komite Etik Penelitian Kesehatan, Faculty of Medicine University of Indonesia (KEPK FKUI). Written informed consent was obtained from legally authorized representatives before the study.

Statement Conflict of Interest

The authors declare that there is no conflict of interest.

Abbreviation List

BKA: Bongkrekic Acid

BKA-NHS: Bongkrekic Acid-N-Hydroxysuccinimide Ester

CD3: Cluster of Differentiation 3

EDC-HCl: N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide Hydrochloride

FcRn: Neonatal Crystallizable Fragment Receptor

MAb: Monoclonal Antibody

MES: 2-(N-morpholino)ethanesulfonic acid

NHS: N-Hydroxysuccinimide

PBMC: Peripheral Blood Mononuclear Cell

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209-49. https://doi.org/10.3322/caac.21660.
2. Debela DT, Muzazu SG, Heraro KD, Ndalama MT, Mesele BW, Haile DC, et al. New approaches and procedures for cancer treatment: Current perspectives. SAGE Open Med. 2021;9:20503121211034366. https://doi.org/10.1177/20503121211034366.
3. Tohme S, Simmons RL, Tsung A. Surgery for cancer: A trigger for metastases. Cancer Res. 2017;77(7):1548-52. https://doi.org/10.1158/0008-5472.Can-16-1536.

4. Amjad MT, Chidharla A, Kasi A. Cancer chemotherapy. Statpearls.Treasure Island (FL): StatPearls Publishing Copyright © 2025, StatPearls Publishing LLC; 2025.
5. Majeed H, Gupta V. Adverse Effects of Radiation Therapy. StatPearls; 2022. Available from: https://www.ncbi.nlm.nih.gov/books/NBK563259/
6. Zahavi D, Weiner L. Monoclonal antibodies in cancer therapy. Antibodies (Basel). 2020;9(3). https://doi.org/10.3390/antib9030034.
7. Becker N, Benhar I. Antibody-based immunotoxins for the treatment of cancer. Antibodies. 2012;1(1):39-69 https://doi.org/10.3390/antib1010039.
8. Akbari B, Farajnia S, Ahdi Khosroshahi S, Safari F, Yousefi M, Dariushnejad H, et al. Immunotoxins in cancer therapy: Review and update. Int Rev Immunol. 2017;36(4):207-19. https://doi.org/10.1080/08830185.2017.1284211.
9. Dosio F, Brusa P, Cattel L. Immunotoxins and anticancer drug conjugate assemblies: The role of the linkage between components. Toxins (Basel). 2011;3(7):848-83. https://doi.org/10.3390/toxins3070848.
10. Anwar M, Kasper A, Steck AR, Schier JG. Bongkrekic acid-a review of a lesser-known mitochondrial toxin. J Med Toxicol. 2017;13(2):173-9. https://doi.org/10.1007/s13181-016-0577-1.
11. Greg H. Bioconjugate Chemistry (2nd ed.); 2008. Available from: https://www.ptonline.com/articles/how- to-get-better-mfi-results
12. Verhoeckx K, Cotter P, López-Expósito I, Kleiveland C, Lea T, Mackie A, et al. The impact of food bioactives on health: in vitro and ex vivo models.Cham (CH): Springer Copyright; 2015.
13. Alarcón B, Berkhout B, Breitmeyer JB, Terhorst C. Assembly of the human t cell receptor-cd3 complex takes place in the endoplasmic reticulum and involves intermediary complexes between the cd3-gamma.Delta.Epsilon core and single t cell receptor alpha or beta chains. J Biol Chem. 1988;263 6:2953-61. https://doi.org/10.1016/S0021-9258(18)69161-6.
14. Puspitasari D, Wanandi SI, Sadikin M. Conjugation of cetuximab - puromycin and its target-specific effect on triple negative breast cancer cell lines. Asian Pac J Cancer Prev. 2022;23(5):1803-12. https://doi.org/10.31557/apjcp.2022.23.5.1803.
15. ChemicalComputingGroup M. Molecular operating environment. Chemical Computing Group ULC; 2022.
16. ChemSketch V. 2, Advanced Chemistry Development, Inc. (ACD/Labs), Toronto, ON, Canada [Internet]. 2022
17. Sheet pi, description p, instructions p (n.D.). Histopaque® -1077, 10771, 3–5.
18. Suleiman E, Mayer J, Lehner E, Kohlhauser B, Katholnig A, Batzoni M, et al. Conjugation of native-like hiv-1 envelope trimers onto liposomes using edc/sulfo-nhs chemistry: Requirements and limitations. Pharmaceutics. 2020;12(10). https://doi.org/10.3390/pharmaceutics12100979.
19. McCombs JR, Owen SC. Antibody drug conjugates: Design and selection of linker, payload and conjugation chemistry. Aaps j. 2015;17(2):339-51. https://doi.org/10.1208/s12248-014-9710-8.
20. Van Wauwe JP, Goossens JG. The mitogenic activity of okt3 and anti-leu 4 monoclonal antibodies: A comparative study. Cell Immunol. 1983;77(1):23-9. https://doi.org/10.1016/0008-8749(83)90003-5.
21. Resetkova E, Arreaza G, Yoshikawa N, Morita T, Kim H, Carayon P, et al. Study of induction of activation of human peripheral blood mononuclear cells with a non-activating form of anti-cd3 moab in autoimmune thyroid disease (aitd). Clin Exp Immunol. 1993;91(3):397-403. https://doi.org/10.1111/j.1365-2249.1993.tb05915.x.
22. Kano A, Kamita M, Iwasaki T, Shindo M. Bongkrekic acid induces selective cytotoxicity in tumor cells, revealed by cck-8. Evergreen. 2017;4:23-7. https://doi.org/10.5109/1929661.
23. Takeda S, Okazaki H, Kudo T, Kakizoe K, Himeno T, Matsumoto K, et al. Bongkrekic acid as a warburg effect modulator in long-term estradiol-deprived mcf-7 breast cancer cells. Anticancer Res. 2016;36(10):5171-82. https://doi.org/10.21873/anticanres.11087.
24. Kano A, Iwasaki T, Shindo M. Bongkrekic acid facilitates glycolysis in cultured cells and induces cell death under low glucose conditions. Biochem Biophys Rep. 2019;20:100683. https://doi.org/10.1016/j.bbrep.2019.100683
25. Adair JR, Athwal DS, Bodmer MW, Bright SM, Collins AM, Pulito VL, Rao PE, Reedman R, Rothermel AL, Xu D, Zivin RA. Humanization of the murine anti-human CD3 monoclonal antibody OKT3. Human Antibodies. 1994 Feb;5(1-2):41-7. https://doi.org/10.3233/HAB-1994-51-206
26. Kuhn C, Weiner HL. Therapeutic anti-cd3 monoclonal antibodies: From bench to bedside. Immunotherapy. 2016;8(8):889-906. https://doi.org/10.2217/imt-2016-0049.
27. Schaffar L, Dallanegra A, Breittmayer JP, Carrel S, Fehlmann M. Monoclonal antibody internalization and degradation during modulation of the cd3/t-cell receptor complex. Cell Immunol. 1988;116(1):52-9. https://doi.org/10.1016/0008-8749(88)90209-2.
28. Onnis A, Baldari CT. Orchestration of immunological synapse assembly by vesicular trafficking. Front Cell Dev Biol. 2019;7:110. https://doi.org/10.3389/fcell.2019.00110.
29. Wood KJ, Shankar S, Hester J, Issa F. Concepts and challenges in organ transplantation: rejection, immunosuppression, and tolerance. InClinical Immunology; London: Elsevier; 2019. p. 1097-114.e1.
30. Chatenoud L. Cd3-specific antibody-induced active tolerance: From bench to bedside. Nat Rev Immunol. 2003;3(2):123-32. https://doi.org/10.1038/nri1000.
31. Ashraf MA. Poverty alleviation and conventional MFIs: Challenges and prospects. Nova Science Publishers, Incorporated; 2019.

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