Bioconjugate Chemistry

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Long-Acting Human Interleukin 2 Bioconjugate Modified with Fatty Acids by Sortase A

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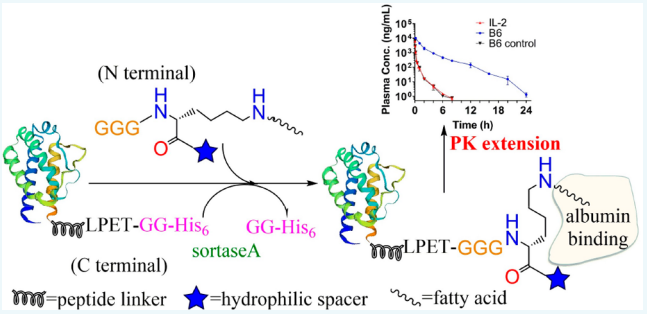
ABSTRACT: Human Interleukin 2 (IL-2) has already achieved impressive results as a therapeutic agent for cancer and autoimmune diseases. However, one of the limitations associated with the clinical application of IL-2 is its short half-life owing to rapid clearance by the kidneys. Modification with fatty acids, as an albumin noncovalent ligand with the advantage of deep penetration into tissues and high activity-to-mass ratio, is a commonly used approach to improve the half-life of native peptides and proteins. In this investigation, we attempted to extend the half-life of IL-2 through conjugation with a fatty acid using sortase A (srtA). We initially designed and optimized three IL-2 analogues with different peptide linkers between the C-terminus of IL-2 and srtA recognition sequence (LPETG). Among these, analogue A3 was validated as the optimal IL-2 analogue for further modification. Next, six fatty acid moieties with the same fatty acid and different hydrophilic spacers were conjugated to A3 through srtA. The six bioconjugates generated were screened for *in vitro* biological activity, among which bioconjugate B6 was identified as near-optimal to IL-2. Additionally, B6 could effectively bind albumin through the conjugated fatty acid, which contributed to a significant improvement in its pharmacokinetic properties *in vivo*. In summary, we have developed a novel IL-2 bioconjugate, B6, modified with fatty acids using srtA, which may effectively serve as a new-generation long-acting IL-2 immunotherapeutic agent.



INTRODUCTION

Human interleukin 2 (IL-2) was originally identified more than 40 years ago as an immunoregulatory factor produced by activated helper T cells.[1] IL-2 can maintain T lymphocytes and natural killer (NK) cell growth *in vitro* and stimulate the proliferation and activation of T cells *in vivo*, by activating Janus kinase (JAK)-signal transducer and activator of transcription 5 (STAT5) signaling pathway through interactions with IL-2 receptor complexes containing alpha (IL-2Rα, CD25), beta (IL-2Rβ, CD122), and common gamma chain receptors (γc, CD132).[2−5] Based on the significant efficacy of high-dose IL-2 as tumor immunotherapy in clinical trials in the 1980s, a high-dose recombinant IL-2 formulation (Aldesleukin, 125-L-serine-2-133-interleukin 2) was approved by the U.S. Food and Drug Administration (FDA) for metastatic renal cancer in 1992 and metastatic melanoma in 1998.[6] Recent studies have also validated the significant potential of low-dose IL-2 as a therapeutic agent for autoimmune diseases, such as systemic lupus erythematosus (SLE), type 1 diabetes (T1D), and chronic graft-versus-host disease (GvHD).[7−10]

Although IL-2 has impressive results as a therapeutic agent for cancer and autoimmune disease, the short half-life of IL-2 (13 to 85 min in human) limit the clinical usefulness of this protein.[11] Due to the extensive renal clearance and lack of recycling processes mediated by the neonatal fragment crystallizable receptor (FcRn), maintaining drug concentrations within the therapeutically effective range is difficult.[12] Consequently, IL-2 needs to be frequently administered, which has a significant adverse effect on overall treatment adherence of patients.[13]

Several attempts have been made to generate long-acting IL-2 derivatives. For instance, poly(ethylene glycol) (PEG) conjugation has been used to extend the circulating half-life of IL-2 since 1987, but causing several low-bioavailability, immunogenicity, and nonbiodegradability issues.[2,14−17] Genetic fusion and covalent conjugation of human serum albumin (HSA) and fragment crystallizable (Fc) region have been additionally employed for generation of long-acting modified IL-2.[11,18−20] However, reduced expression, low biological activity, complications of the process, and high cost remain major drawbacks.[21]

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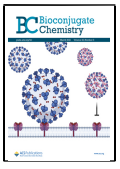

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Modification with long-chain fatty acids, as an albumin noncovalent ligand, is another commonly used approach to improve the half-life of native peptides and proteins; it has never been used on IL-2.[22] Compared with direct conjugation of HSA, conjugation with fatty acids has several advantages including a shorter production cycle, lower production cost, deeper penetration into tissues, and higher activity-to-mass ratio, showing great promise to prolong the half-life of therapeutic peptides and proteins.[21,23] The fatty acid forms a noncovalent bond with albumin presenting in the circulation with slow dissociation rates, consequently increasing the residence time of the therapeutic agent *in vivo*.[24] Several therapeutic formulations exploiting this strategy have been approved by the FDA, such as Levemir (Insulin detemir), Tresiba (Insulin degludec), Victoza (Liraglutide), and Ozempic (Semaglutide).[25] These drugs have been developed through chemical conjugation of fatty acids to lysine residues of peptide and exhibit similar activity to the unmodified parent drugs. However, chemical conjugation to lysine residues is not suitable for IL-2, since the presence of multiple lysine residues is expected to complicate the homogeneous conjugation onto the protein, necessitating cumbersome separations and characterization.[23,26] In addition, multiple lysine residues lead to several modifications of fatty acids, causing much hydrophobic and low activity of bioconjugate.[23,26,27]

SortaseA (SrtA), a membrane protein expressed in *Staphylococcus aureus*, is widely used for protein conjugation and modification.[28−33] Compared with chemical conjugation, the use of srtA has the advantage of a single modification site, homogeneous composition, mild reaction conditions, and environmental protection.[34] Some studies believed that cytokines like IL-2 with a four-helix bundle are suitable for modification by srtA, because the receptor contacts the sides of the helical bundles and moves the N-terminus and C-terminus of the protein away from the receptor.[26]

In this study, we used srtA for fatty acid modification to extend the half-life of IL-2. Since natural IL-2 has limited solubility at neutral pH, the fatty acid modification will further enhance hydrophobicity, resulting in low bioavailability and poor druggability.[14] Here, we designed an IL-2 analogue modified via srtA at the C-terminus, by adding a peptide linker between the C-terminus of IL-2 and srtA recognition sequence (LPETG) to reduce hydrophobicity while retaining maximal biological activity. Additionally, several fatty acid moieties with the same fatty acid and different hydrophilic spacers were synthesized, containing the −GGG motif at the N termini recognized by srtA. The introduction of a hydrophilic spacer can improve the hydrophobicity of the fatty acid moiety which is also conducive to the reaction of srtA at neutral pH. We validated the utility of sortase-mediated ligation through oriented conjugation of fatty acid moieties to the IL-2 analogue to generate a series of IL-2 bioconjugates and obtained a bioconjugate with better hydrophilic, similar biological functions as IL-2 *in vitro* and significantly extended half-lives *in vivo*.

RESULTS AND DISCUSSION

Design, Expression, and Purification of IL-2 Analogues. Three IL-2 analogues (A1: IL-2-$(GGGGS)_3$-LPETGG-His_6, A2: IL-2-$(PA)_6$-LPETGG-His_6, and A3: IL-2-$(ED)_6$-LPETGG-His_6) containing the srtA recognition sequence (LPETG) and different peptide linker were designed. $(GGGGS)_3$, the most commonly used flexible linker (FL), is generally rich in small or polar amino acids such as glycine and serine to enhance flexibility and solubility.[35] The flexible linker employed when the connected domains require a certain degree of movement or interaction. $(PA)_6$ is a representative rigid linker (RL) that enlarges spatial separation between domains.[35] $(ED)_6$ is a fusion protein linker developed by Bristol-Myers Squibb Company (NY, US) mainly used to prevent protein aggregation and increase solubility.[36] The success of expression of three IL-2 analogues was confirmed via mass spectrometry (Figures S1−S3, Supporting Information), with a close agreement between the theoretical and measured molecular weights (Table 1). The sequence of the IL-2 part used in three analogues is consistent with aldesleukin (125-L-serine-2-133-interleukin 2; Figure 1A). Correct formation of the only one disulfide bond between the cysteines at positions 58 and 105, which is essential for IL-2 analogue activity, was assessed via SDS-PAGE (Figure 1B).[37] The disulfide bonds in the purified proteins can be reduced by dithiothreitol (DTT), showing a slight migration of protein bands to higher mass. The purity and hydrophobicity of analogues were further examined using reversed-phase high-performance liquid chromatography (RP-HPLC; Figure 1C). The purity of the three analogues was ≥90%, and the relative retention time (RRT) with IL-2 of three analogues was <1. Under the same elution conditions of RP-HPLC, the more hydrophilic the protein was, the lower the value of RRT with IL-2. All three analogues had better hydrophilicity than IL-2, and the diverse linker had different effects on hydrophilicity, of which A3 with $(ED)_6$ was shown to be the best.

***In Vitro* Bioactivity of Purified IL-2 Analogues.** Biolayer interferometry (BLI) is used to analyze interactions between IL-2 analogues and IL-2R.[38] Interactions between biotinylated human IL-2Rα captured on streptavidin-coated biosensors and IL-2 analogues at six different concentrations were monitored at 30 °C using BLItz. The overlay plot of individual sensorgrams (Figure S18, Supporting Information) showed that all IL-2 analogues bind human IL-2Rα in a concentration-dependent manner. The binding rate (K_a), dissociation rate (K_d), and equilibrium dissociation constant (K_D, K_d:K_a ratio) were automatically calculated with the aid of BLItz software using a Langmuir 1:1 binding model with global fit analysis (Table 2). Table 2 also revealed that all three IL-2 analogues exhibited similar kinetic binding capabilities as IL-2 and their K_D values were in the same order of magnitude as the theoretical value ($K_D \sim 10^{-8}$ mol/L).[39]

To determine the biological activities of the purified proteins, the cell viability assay was performed on CTLL-2 and NK-92 cells. CTLL-2 is a mouse T-lymphocyte cell line expressing high levels of IL-2Rα that requires IL-2 for growth.[39] NK-92 is an IL-2-dependent human NK cell line with the functional and phenotypic characteristics of activated NK cells.[40] After culturing, cells were washed, starved, and

Table 1. MS Data on IL-2 Analogues[a]

analogue	linker	style	molecular mass (MS) calculated	MS observed	RRT with IL-2
A1	$(GGGGS)_3$	FL	17652.15	17651.65	0.82
A2	$(PA)_6$	RL	17715.47	17714.97	0.77
A3	$(ED)_6$	N/A	18171.52	18170.99	0.76

[a]Abbreviations: FL, flexible linker; RL, rigid linker; RRT, relative retention time.

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Bioconjugate Chem. 2021, 32, 615−625

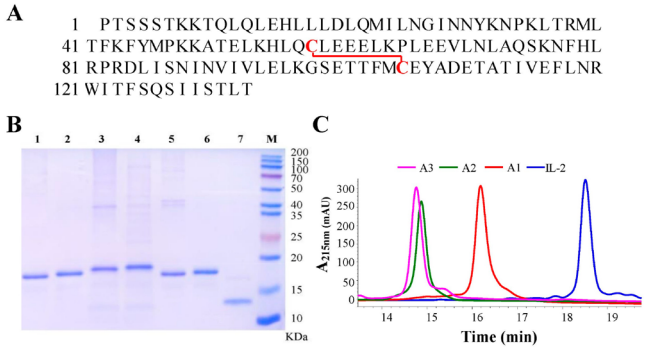

Figure 1. (A) Sequence of IL-2 part used in three analogues. Cysteine 58 and cysteine 105 can form only one disulfide bond (red). (B) IL-2 analogues purified and denatured IL-2 analogues via a final concentration of 100 mM dithiothreitol (DTT) were subjected to 15% SDS-PAGE and were assessed by Coomassie blue staining. 1, A1; 2, A1+DTT; 3, A2; 4, A2+DTT; 5, A3; 6, A3+DTT; 7, IL-2; M, marker. (C) Hydrophobicity of three analogues and IL-2 monitored by RP-HPLC ultraviolet absorbance chromatograms at 215 nm.

then treated with different concentrations of purified IL-2 analogues to examine stimulation of proliferation. The relative viability of cells exposed to IL-2 analogues compared to IL-2 is shown in Figure 2A,B. For both CTLL-2 and NK-92 cell lines, A1 induced weaker proliferation ability than IL-2 while the activities of A2 and A3 were stronger. Specifically, A3 was significantly better than A2 in CTLL-2 cell proliferation while no significant differences in NK-92 cell proliferation were observed. Different linkers between the C-terminus of IL-2 and srtA recognition sequence (LPETG) exert distinct stimulatory effects on CTLL-2 and NK-92 cells *in vitro*. A3 with $(ED)_6$ linker showed superior activity may be due to the capability of this linker preventing protein aggregation.[36]

The ability of IL-2 analogues to stimulate signal transduction was determined in CTLL-2 using a STAT A/B pY694/699 ELISA kit measuring activation of STAT5A and STAT5B via phosphorylation (pSTAT5). As shown in Figure 2C, the EC_{50} value of IL-2 was 4.921 pM, which was 23.7 times smaller than A1 (EC_{50} 116.5 pM), 7.0 times smaller than A2 (EC_{50} 34.5 pM), and 2.5 times smaller than A3 (EC_{50} 12.3 pM). The distinct rates of receptor-mediated internalization and degradation of our IL-2 analogues and unmodified IL-2 can lead to the differences between pSTAT5 activation and in CTLL-2 proliferation. The C-terminal modification of IL-2 had an impact on IL-2Rγ (CD132) binding attributable to the distinct linkers of the three analogues and resulted in reduced signal phosphorylation levels. A3 with the $(ED)_6$ linker displayed the closest biological activity to IL-2 and was therefore selected for further modification by srtA.

Design, Synthesis, and Preparation of Fatty Acid Moieties. We designed fatty acid moieties containing the −GGG motif at the N-terminus. The −GGG motif needs to be recognized for site-specific modification by srtA. The fatty acid moieties designed introducing 8-amino-3,6-dioxaoctanoic acid (AEEA) and glutamic acid as hydrophilic spacers to improve the hydrophobicity and octadecanedioic acid to increase affinity for human serum albumin. Fatty acid moieties were synthesized by application of the lysine orthogonal protection scheme in a solid-phase synthesizer (Figure 3) and subsequently purified and identified via preparative RP-HPLC and mass spectrometry (Figures S5−S10, Supporting Information). As shown in Table 3, molecular weights of all the fatty acid moieties were consistent with the theory.

Preparation and Purification of IL-2 Bioconjugates. Conjugation with fatty acids is an important modification of proteins and peptides commonly applied to increase the half-life in the circulation through the formation of a noncovalent bond with albumin, thus avoiding rapid clearance in the spleen, liver, and kidney.[25,41] Because A3 has the best hydrophilicity and the closest biological activity to IL-2, here we selected it for fatty acid modification by srtA. Through the srtA-mediated transpeptidation reaction, The −GGG motif of the fatty acid moiety at the N-terminus was coupled with A3 containing −LPETG at the C-terminus to form an IL-2 bioconjugate. Cysteine 184 in srtA serves as a nucleophile that attacks the carbonyl carbon of the threonine residue (T-G), which ejects the C-terminal fragment and simultaneously develops an acyl-enzyme intermediate. Subsequently, the transient acyl-enzyme intermediate and fatty acid moiety comprising N-terminal glycine reacts and forms a new peptide bond between the threonine residue and N-terminus of the fatty acid moiety (Figure 4A). The reaction was monitored using RP-HPLC. In our initial research, we use 80× excess of fatty acid moieties and reaction in 37 °C for 2 h, and approximately 20% transient acyl-enzyme intermediates were found. To reduce the potential formation of transient acyl-enzyme intermediates and the impact on the analogues, we carried out the reaction at 4 °C and increased the reaction time and fatty acid moieties to shift the equilibrium and achieve a higher yield. As a result, after an overnight reaction under excess fatty acid moieties (160-fold) at 4 °C, no transient acyl-enzyme intermediate was detected (data not shown).

All IL-2 bioconjugates prepared by RP-HPLC were confirmed via mass spectrometry (Table 3; Figures S12−S17, Supporting Information), their hydrophobicity was assessed by RP-HPLC (Figures 4B). By modification of fatty acid moieties, all bioconjugates were more hydrophobic than A3. When it contains more than one AEEA, the bioconjugate had stronger hydrophilicity than IL-2. Although all the bioconjugates were eluted since the RP-HPLC gradient starts at 40% acetonitrile, which seemed quite hydrophobic, they showed better solubility at neutral pH than IL-2 except for B1. This is mainly due to the introduction of the $(ED)_6$ linker and the increase in the hydrophilic interval to assist in the neutral dissolution of the coupling. The purity of the IL-2 bioconjugates was further evaluated using SDS-PAGE (Figure 4C). All bioconjugates exhibited a single electrophoretic band. Besides, we analyzed

Table 2. BLI Analysis of IL-2 Analogues to Human IL-2Rα

compound	K_D (M)	K_a (1/Ms)	K_d (1/s)	R^2
IL-2	$(1.952 \pm 0.130) \times 10^{-8}$	$(3.317 \pm 0.093) \times 10^{5}$	$(6.147 \pm 0.563) \times 10^{-3}$	0.9901
A1	$(2.501 \pm 0.007) \times 10^{-8}$	$(2.226 \pm 0.034) \times 10^{5}$	$(5.601 \pm 0.407) \times 10^{-3}$	0.9899
A2	$(4.380 \pm 0.186) \times 10^{-8}$	$(8.028 \pm 0.016) \times 10^{4}$	$(3.516 \pm 0.157) \times 10^{-3}$	0.9959
A3	$(0.995 \pm 0.019) \times 10^{-8}$	$(3.949 \pm 0.010) \times 10^{5}$	$(3.930 \pm 0.067) \times 10^{-3}$	0.9861

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Bioconjugate Chem. 2021, 32, 615−625

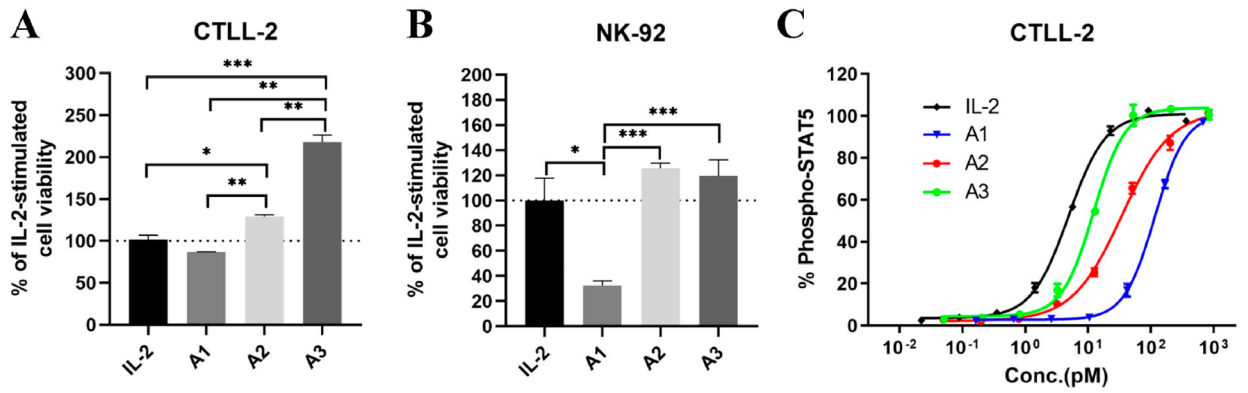

Figure 2. (A) Comparison of activities of IL-2 analogues relative to IL-2 in stimulating CTLL-2 cells, measured as percentage relative cell viability. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (B) Comparison of the activities of IL-2 analogues relative to IL-2 in stimulating NK-92 cells, measured as percentage relative cell viability. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C) Dose−response curves of IL-2 and IL-2 analogues activities in stimulating signal transduction based on STAT5 phosphorylation in CTLL-2 cells.

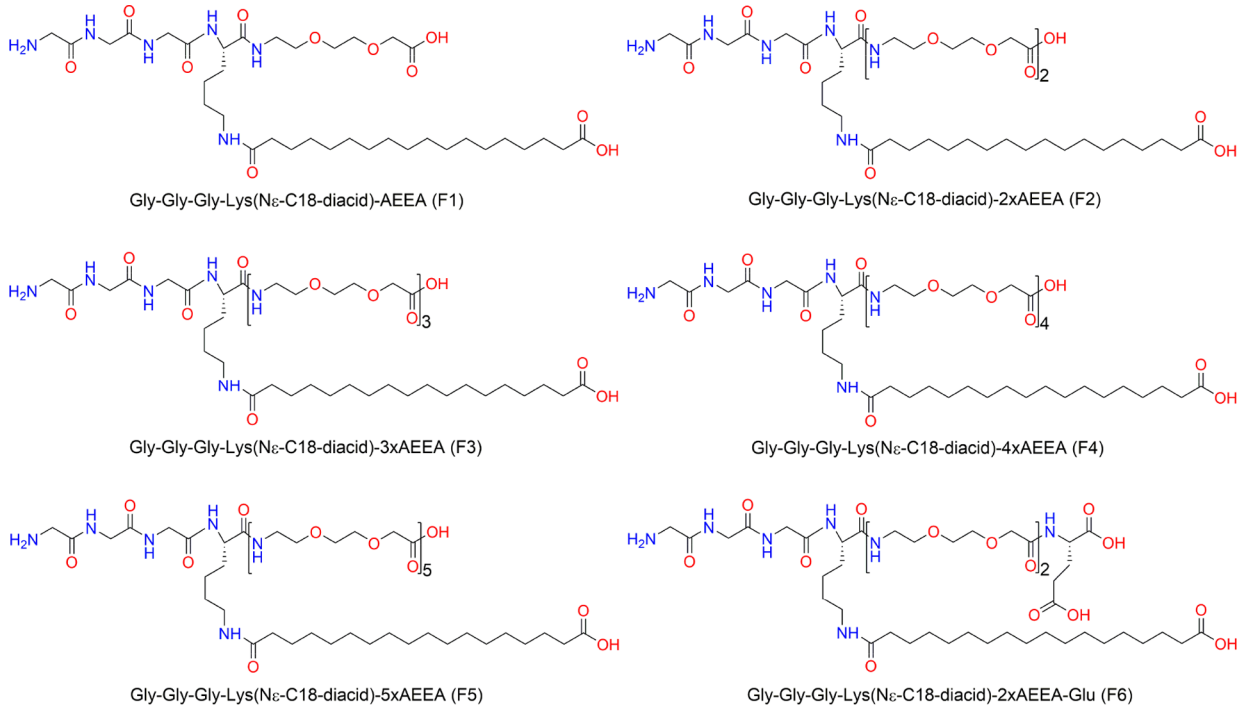

Figure 3. Structures of fatty acid moieties F1−F6 used for preparation of bioconjugates B1−B6.

Table 3. MS Data on Fatty Acid Moieties and IL-2 Bioconjugates

IL-2 analogue	fatty acid moiety	MS calculated	MS observed $[M + H]^+$	IL-2 bioconjugate	MS calculated	MS observed
A3	F1	758.48	759.68	B1	17975.04	17975.07
	F2	903.55	904.65	B2	18120.11	18120.26
	F3	1048.63	1049.81	B3	18265.19	18265.14
	F4	1193.70	1194.97	B4	18410.26	18410.24
	F5	1338.77	670.82[a]	B5	18555.33	18555.48
	F6	1032.60	1033.78	B6	18249.16	18249.34

[a] $[M + 2H]^{2+}$.

the structure of the analogue A3 (before the sortagging reaction) and representative bioconjugate (after the sortagging reaction) by circular dichroism (CD) using IL-2 as a positive control. The CD experiment showed that the sorting reaction and RP-HPLC purification will not affect the fold of structure and verified that the lipidation itself can increase helicity (Figure S19, Supporting Information).[42,43]

***In Vitro* Bioactivity of Purified IL-2 Bioconjugates.** The binding ability of the IL-2 bioconjugates to IL-2Rα at the molecular level was determined using BLI at 30 °C. Similar to IL-2 analogues, all bioconjugates showed concentration-dependent binding to human IL-2Rα (Figure S18, Supporting Information). Upon 1:1 global fitting analysis of the model through BLItz software, except for B1, all other bioconjugates

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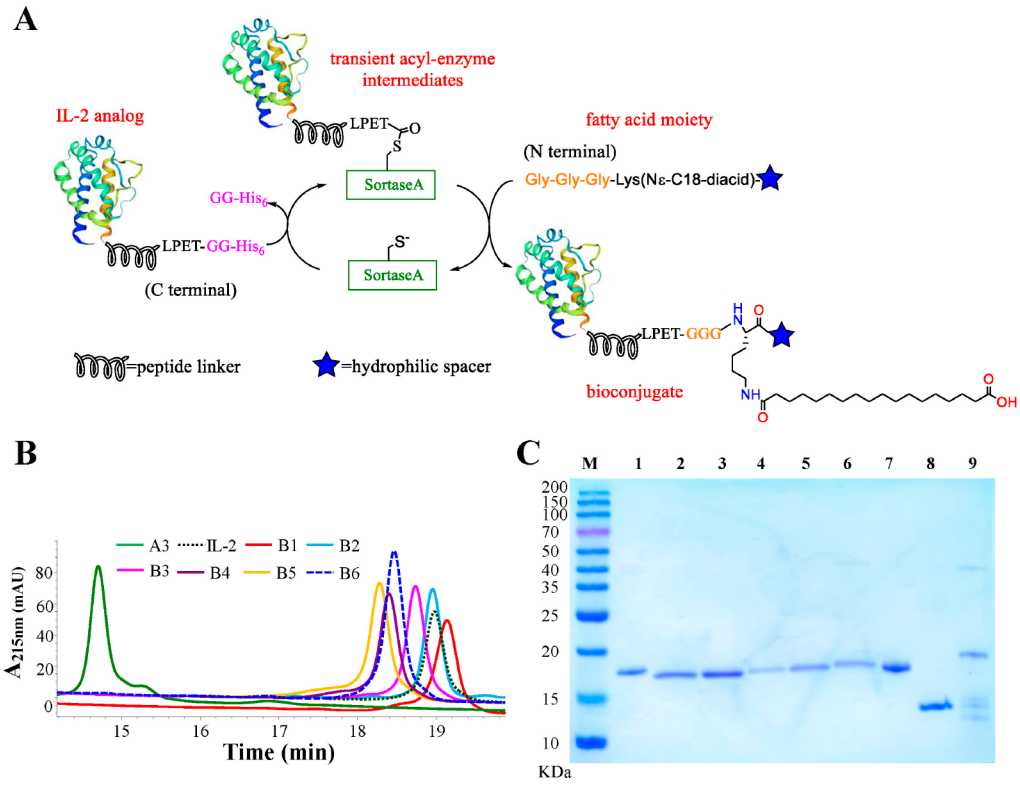

Figure 4. (A) Schematic diagram of the SrtA reaction in which transpeptidase attacks the carbonyl carbon of the threonine residue (T-G) and appends a fatty acids moiety. (B) Hydrophobicity of A3, IL-2, and six bioconjugates by RP-HPLC ultraviolet absorbance chromatograms at 215 nm. (C) Purified IL-2 bioconjugates were subjected to 15% SDS-PAGE and compared with IL-2, A3, and srtA in the reaction. M, marker; 1, A3; 2, B1; 3, B2; 4, B3; 5, B4; 6, B5; 7,B6; 8, IL-2; 9, srtA used for the reaction.

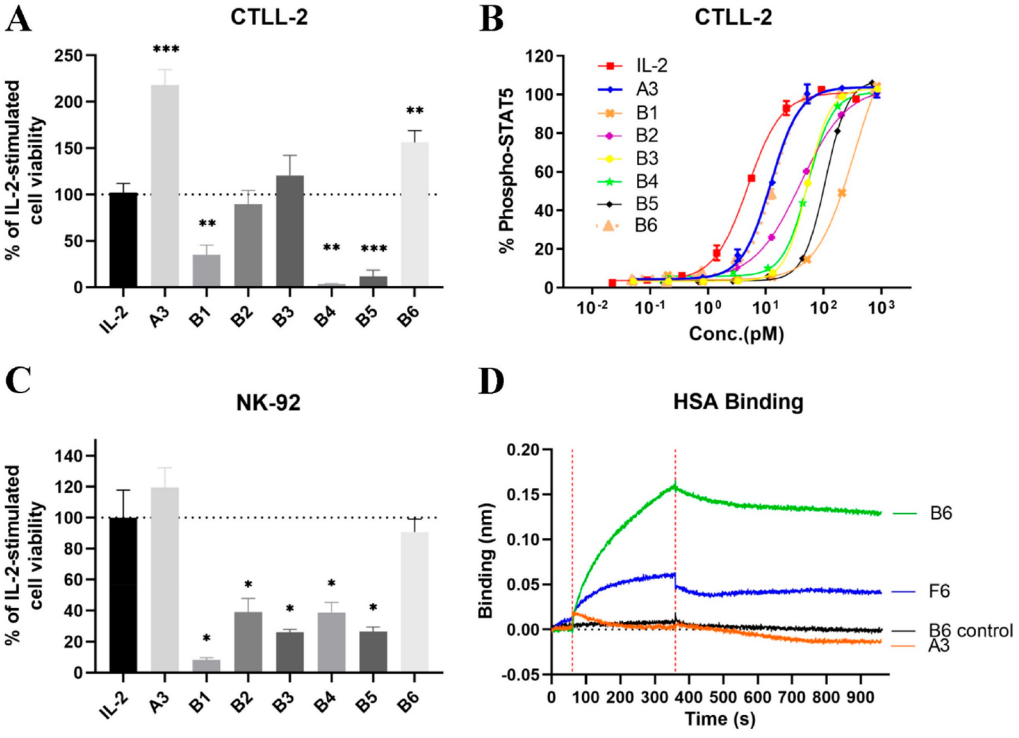

Figure 5. (A) Percentage relative viability of CTLL-2 cells treated with IL-2 bioconjugates compared to parent IL-2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to IL-2. (B) Dose−response curve of IL-2 bioconjugates in stimulation of signal transduction measured as STAT5 phosphorylation in CTLL-2 cells. (C) Percentage relative viability of NK-92 cells treated with IL-2 bioconjugates compared to parent IL-2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to IL-2. (D) BLI analysis of HSA binding with A3, F6, B6, and B6 control.

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Bioconjugate Chem. 2021, 32, 615−625

exhibited no significant differences in K_D from unmodified control IL-2 (Table S1, Supporting Information). The results suggested that more than one AEEA in the hydrophilic spacers of fatty acid moieties modified based on $(ED)_6$ linker are not likely to affect IL-2Rα interactions.

The biological activities of bioconjugates were compared with a viability assay using CTLL-2 and NK-92 cells. We additionally investigated the stimulatory effects of different fatty acid moieties conjugated to IL-2 analogues on signal transduction in CTLL-2. Our previous data suggested that different linkers between the C-terminus of IL-2 and the srtA recognition sequence (LPETG) had a significant impact on the biological activities of individual analogues and A3 with the $(ED)_6$ linker was the optimal IL-2 analogue. Accordingly, we further assessed whether the corresponding modified A3 bioconjugates were as effective as or better than A3 or IL-2. As shown in Figure 5A,B, B6 conjugation of F6 using srtA had the best proliferation activity and pSTAT5 activation of CTLL-2 cells among all bioconjugates and showed optimal potency to IL-2. With the extension of the hydrophilic spacers of fatty acid moieties, the proliferative activity and pSTAT5 activation of CTLL-2 cells showed a trend from low to high and back to low. Considering that all six bioconjugates had the same fatty acid structure (octadecanoic diacid), we speculated that B6 was the best bioconjugate, which may be due to the length of the hydrophilic spacer "2×AEEA-Glu" in its structure. In addition, we prepared a B6 control (Figure S21, Supporting Information) without octadecanoic diacid but with the same peptide linker and hydrophilic spacer of B6 and found that the fatty acid structure of fatty acid moiety also affected the activity of the bioconjugate (Table S2, Supporting Information).

Another investigation we have done showed that the bioconjugate with the same fatty acid moieties and the different peptide linkers had lower bioactivity compared to their parent analogues (Figure S22; Table S2, Supporting Information). Compared with IL-2, all peptide linker, hydrophilic spacer, and fatty acid in the C-terminus of the bioconjugate affect biological activity. Although the C-terminus of IL-2 was exposed outside the structure and did not directly participate in the binding of the receptor, we guess that the combined modification may affect some key amino acids not distant from the C-terminus of IL-2 such as $Gln\text{-}126^{IL\text{-}2}$ involved in IL-2Rγ (CD132) binding and signaling.[44,45] On the other hand, the previous binding experiment also verified that the modification in the C-terminus other than B1 can maintain the binding to the IL-2Rα, which is necessary for the proliferation of CTLL-2 cells with the high expression levels of IL-2Rα.[39] Both cell proliferation and signal transduction experiments were based on a comprehensive evaluation of CTLL-2 activity at the cell level. Compared with the studies on other long-acting derivatives in the C-terminus of IL-2 such as IL-2-HSA and IL-2-Fc, B6 had a better proliferation level in CTLL-2 cells and was 1.6 times as high as IL-2.[11,19] Besides, we also observed B6 showed optimal activity in the NK-92 cell proliferation experiment ($p < 0.05$; Figure 5C).

Because of its optimal *in vitro* activity among all the IL-2 bioconjugates generated, B6 is considered suitable for HSA binding research. We examined the hypothesis that our bioconjugates could form a noncovalent bond with albumin through modified fatty acid to extend the half-life *in vivo*. BLI has been previously used to study the binding of proteins to HSA.[46] However, due to the multiple fatty acid binding sites on HSA, only qualitative analysis of binding was performed in our study. Biotinylated HSA was captured on streptavidin-coated biosensors. As shown in Figure 5D, there are signal responses shown in the curves of B6 and F6, which means they could bind to HSA. Meanwhile, A3 and B6 control showed no signal to HSA binding, which indicates the peptide linker and the hydrophilic spacer are not involved in the binding of HSA. Therefore, B6 bound to HSA via octadecanedioic acid from modified F6, which is consistent with our assumption.

Pharmacokinetics. To further determine whether the bioconjugate B6 has a greater half-life than IL-2 through noncovalent interactions between fatty acid and albumin, we conducted pharmacokinetic (PK) experiments *in vivo*. Although B6 control showed no contribution to HSA binding, the effect of peptide linker and hydrophilic spacers on the pharmacokinetics is unknown. So, we also conducted pharmacokinetic studies on B6 control. IL-2, B6, and B6 control were examined in a BALB/c mouse model after single-dose intravenous (i.v.) administration (0.6 mg/kg). The plasma concentration of the drug was measured using sandwich ELISA. Plasma concentration−time profiles are presented in Figure 6. IL-2 and B6 control was rapidly eliminated *in vivo*, with a concentration of ≤1 ng/mL at 8 h, while the bioconjugate B6 with fatty acid modification could be detected at 24 h (≥1 ng/mL). Moreover, proliferating CTLL-2 was not detected in plasma collected at 6 h of IL-2 treatment but still detectable in the B6 group at 20 h (data not shown).

The PK parameters are shown in Table 4. The pharmacokinetics of all the compounds conformed to the two-compartment model. The plasma clearance rate of B6 was significantly reduced 15-fold slower than IL-2. After i.v. administration, both rapid initial phase half-life ($t_{1/2\alpha}$) and a second slower component half-life ($t_{1/2\beta}$) of B6 were extended significantly (15-fold longer $t_{1/2\alpha}$ and 13-fold longer $t_{1/2\beta}$), compared to IL-2. The total exposure to the circulating drug or area under the curve (AUC) of B6 was approximately 15-fold higher than that of IL-2. Additionally, B6 control showed similar pharmacokinetic parameters to IL-2, which indicated that the addition of peptide linker or hydrophilic spacers in B6 control will not improve the half-life. Although B6 control has a PEG-like structure (AEEA−AEEA) in the modified hydrophilic spacer, the molecular weight of our structure (~300 Da) is far from the theoretical molecular weight (~20 000 Da) that can reduce renal clearance and significantly improve pharmacokinetics.[47] Thus, the fatty acid contributed to a

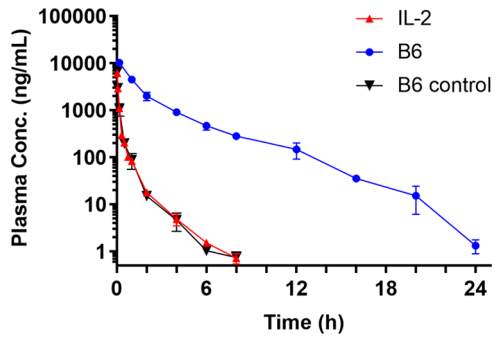

Figure 6. Comparison of the pharmacokinetic profiles of IL-2, B6, and B6 control after single i.v. administration (0.6 mg/kg) in BALB/c mice. Data are presented as means ± SD (n = 4 per group).

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Bioconjugate Chem. 2021, 32, 615−625

Table 4. Pharmacokinetic Parameters of IL-2, B6, and B6 Control Following I.V. Administration (0.6 mg/kg) in BALB/c Mice[a]

compound	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	V_d (L/kg)	CL (L/h/kg)	$AUC_{(0-\infty)}$ (μg/L h)
IL-2	0.041 ± 0.006	0.273 ± 0.040	0.059 ± 0.009	0.454 ± 0.036	1329.586 ± 108.969
B6	0.613 ± 0.063	3.595 ± 0.518	0.053 ± 0.006	0.031 ± 0.004	19638.819 ± 2360.316
B6 control	0.035 ± 0.014	0.196 ± 0.114	0.050 ± 0.012	0.453 ± 0.026	1329.375 ± 74.369

[a]Abbreviations: V_d, volume of distribution; CL, clearance; AUC, area under the curve; $N = 4$ per compound. Average values and SD are indicated.

large extent to the observed half-life by binding with albumin. These studies indicate that following i.v. administration, B6 displays a more favorable pharmacokinetic profile than IL-2 and circulating levels of the bioconjugate are sustained for approximately 1 day.

CONCLUSIONS

In this study, we have developed a fatty acid conjugated IL-2 bioconjugate, B6, modified by srtA that has similar biological functions to IL-2 *in vitro* and significantly extended half-life *in vivo*. Initially, three IL-2 analogues with different peptide linkers between the C-terminus of IL-2 and srtA recognition sequence (LPETG) were designed, and among them, A3 containing an $(ED)_6$ linker was determined as optimal for further modification. Six fatty acid moieties with the same fatty acid and different hydrophilic spacers were conjugated to A3 through srtA. The different hydrophilic spacers between six modified fatty acid moieties have distinct impacts on the biological activity of six bioconjugates, with B6 showing optimal potency to that of IL-2 *in vitro*. Compared with IL-2, all peptide linkers, hydrophilic spacers, and fatty acids modified in the C-terminus of the bioconjugate have an effect on biological activity. B6 exhibited 160% to IL-2 in the proliferation activity of CTLL-2 cells and was better than other derivatives in the C-terminus of IL-2. B6 binds to albumin through a conjugated fatty acid, leading to a significant improvement in its pharmacokinetic properties in mice. The addition of peptide linker and hydrophilic spacer can improve the hydrophobicity of the bioconjugate, but are unrelated to extending the half-life. Compared with unmodified IL-2, B6 showed a remarkable prolonged plasma exposure, with a 15-fold longer $t_{1/2\alpha}$ of 0.613 ± 0.063 h, 13-fold longer $t_{1/2\beta}$ of 3.595 ± 0.518 h, and an increase of $AUC_{(0-\infty)}$ by a factor of 15. Taken together, the present study provides an effective approach for the development of long-acting fatty acid-modified IL-2 bioconjugates and will be of significant interest to others working in the area of long-acting therapeutic proteins.

EXPERIMENTAL PROCEDURE

Cell Lines and Reagents. The IL-2-dependent CTLL-2 mouse T-lymphocyte cell line was purchased from the National Institutes for Food and Drug Control (Beijing, China). The IL-2-dependent NK-92 cell line was provided by Chia Tai Tianqing Pharmaceutical Group Co. Ltd. (Nanjing, China). CTLL-2 cells were cultured at 37 °C and 5% CO_2 in RPMI 1640 (GlutaMAX, Gibco, Grand Island, NY, US) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM HEPES (Gibco, Grand Island, NY, US), and 100−200 IU/mL IL-2. NK-92 cells were cultured at 37 °C and 5% CO_2 in Alpha Minimum Essential medium (without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate, Gibco, Grand Island, NY, US) containing 12.5% horse serum, 12.5% FBS, 0.1 mM 2-mercaptoethanol (Gibco, Grand Island, NY, US), 0.2 mM inositol, 0.02 mM folic acid (Sigma-Aldrich, St. Louis, MO, US) and 100−200 U/mL IL-2.

Recombinant human IL-2 was purchased from the National Institutes for Food and Drug Control (Beijing, China). This human IL-2 was also the unmodified IL-2 as a control in this study.

The srtA used was $m^9SrtA_{\Delta N59}$ from *Staphylococcus aureus*, which was expressed and purified in a previous study.[48]

Reagents used for the synthesis of fatty acid moieties are described in Supporting Information.

Expression and Purification of IL-2 Analogues. Three recombinant IL-2 analogues, IL-2-$(GGGGS)_3$-LPETGG-His_6, IL-2-$(PA)_6$-LPETGG-His_6, and IL-2-$(ED)_6$-LPETGG-His_6, were engineered via gene fusion. The amino acid sequence and structure of parts of IL-2 were consistent with aldesleukin (125-L-serine-2-133-interleukin 2). All three analogues were expressed in *E. coli* and purified via reversed-phase C4 chromatography. The detailed methodology is provided in Supporting Information.

Synthesis and Preparation of Fatty Acid Moieties F1−F6. Fatty acid moieties were synthesized with an application of the lysine orthogonal protection scheme in a solid-phase synthesizer. The detailed methodology is provided in Supporting Information.

Preparation and Purification of IL-2 Bioconjugates B1−B6. Conjugation of the N-terminus of the fatty acid moiety to the C-terminus of the srtA recognition sequence (LPET-) in IL-2-$(ED)_6$-LPETGG-His_6 was performed as follows: 10 μM protein was mixed with 0.25 μM SrtA, 160-fold excess fatty acid moiety (1600 μM), 50 mM NaCl, and 10 mM $CaCl_2$ in Tris-HCl buffer (pH 7.0) and overnight at 4 °C.

The reaction mixture was analyzed via reversed-phase HPLC (Agilent, Santa Clara, CA, US) and terminated by adjusting the pH to 3.0 with AcOH. The IL-2 bioconjugate was purified using a reversed-phase C4 preparative chromatography column (YMC park pro C4 30 μm, 10 × 250 mm, 300 Å; Kyoto, Japan). The mobile phase comprised gradient elution with 0.1% TFA (v/v) water−acetonitrile (60:40, v/v, solution A) and 0.1% TFA (v/v) acetonitrile (solution B). The target bioconjugate was subjected to gradient elution in 15−45% of solution B buffer. Pooled fractions were evaporated on a rotary evaporator and pH adjusted to 7.4 with 1 M NaOH. The solution was replaced three times with PBS buffer in 3 kDa ultrafiltration centrifuge tubes (Millipore, Billerica, MA, US) and analyzed via SDS-PAGE under reducing conditions (Coomassie brilliant blue (CBB) staining), followed by reversed-phase HPLC (Agilent, Santa Clara, CA, US) and ESI-MS (Waters Xevo G2-XS QTof; Milford, Massachusetts, US). The bioconjugate concentration was determined using a bicinchoninic acid (BCA) assay kit (Sangon Biotech, Shanghai, China) with BSA as a standard.

Binding Studies. Binding studies were performed with biolayer interferometry (BLI). Binding data were collected in

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Bioconjugate Chem. 2021, 32, 615−625

BLItz (ForteBio, San Francisco, CA, US) and processed using integrated software of the instrument using a 1:1 binding model.[38] Biotinylated target receptors, either human serum albumin (HSA; Sigma-Aldrich, St. Louis, MO, US) or human IL-2Rα (Genscript Biotech, Nanjing, China), were functionalized to streptavidin-coated biosensors (SA; ForteBio, San Francisco, CA, US) at a concentration of 100 nM in binding buffer (PBS, pH 7.4, 0.02% Tween-20) for 600 s. Analytes were diluted from concentrated stock solutions into the binding buffer. After baseline measurement in binding buffer alone for 60 s, binding kinetics were monitored by dipping biosensors in wells containing the target analyte at the indicated concentration (association step) for 300 s and then back into baseline/buffer (dissociation) for 600 s. All interacting biosensors were regenerated by exposure in 10 mM sodium acetate, 1 M $MgCl_2$, pH 5.4 for 5 s, and subsequently, binding buffer (PBS, pH 7.4, 0.02% Tween-20) for 20 s for 5 cycles.

Assessment of CTLL-2 Proliferation and Activity. For details of the methodology to determine IL-2 biological activity, please refer to Chinese Pharmacopoeia. The proliferation of CTLL-2 was assessed using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, St. Louis, MO, US).[18] Briefly, after culturing for 48−60 h, cells were collected via centrifugation at 180 × *g* for 7 min, washed three times with IL-2-free medium, and resuspended in IL-2-free medium. An aliquot of cell suspension (50 μL) containing 30 000 cells was seeded in a 96-well culture plate (Corning, NY, US). Serial dilutions of analyte stocks were generated and added to cells in a total volume of 50 μL at a final concentration range of 0−1000 pM, followed by incubation at 37 °C and 5% CO_2 for 20 h. MTT solution (20 μL of 5 mg/mL) was added to each well and cells incubated for 5 h, followed by the addition of 150 μL of 15% SDS solution and incubation for 20 h. Finally, cell proliferation was quantified using dual-wavelength (570 nm detection, 630 nm reference) spectrophotometry on a microplate reader (SpectraMaxi3x, Molecular Devices, San Jose, CA, US).

Signaling in CTLL-2 cells was assessed by activation of pSTAT5 after analyte stimulation.[2] A STAT A/B pY694/699 enzyme-linked immunosorbent assay kit (ELISA; Abcam, Cambridge, MA, USA) was used to quantify phosphorylation of STAT5A and STAT5B in cellular lysates. On the day of the assay, cells were preincubated in IL-2-free medium for ≥4 h before treatment and 20 μL cell suspensions containing 10 000 cells plated on a precoated 96-well microplate. Analytes were prepared as 4× serial dilutions and added to cells in 20 μL aliquots. Treated cells were stimulated for 15 min at 37 °C and 5% CO_2. In keeping with the manufacturer's instructions on cell lysis and assay protocols, signals (OD_{450}) were read on a microplate reader (SpectraMaxi3x, Molecular Devices, San Jose, CA, US).

NK-92 Cell Viability Assay. After culturing for 48−60 h, NK-92 cells were collected via centrifugation at 130 × *g* for 10 min, washed three times with IL-2-free medium three times, and subsequently starved in IL-2-free medium for 2 h at 37 °C and 5% CO_2. Cells were resuspended in an IL-2-free medium and a 12.5 μL suspension containing 10 000 cells seeded on a 384-well culture plate (black, clear-bottom, Thermo Fisher, Waltham, MA, US). Analytes were prepared as 10× serial dilutions before assay and added to cells (12.5 μL), followed by incubation of plates for 48 h at 37 °C and 5% CO_2. Using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, US), the ATP content was measured according to the manufacturer's instructions to determine cell viability.[49] Finally, cell proliferation activity was measured in a microplate reader (SpectraMaxi3x, Molecular Devices, San Jose, CA, US).

Pharmacokinetic Studies. *In vivo* pharmacokinetic (PK) experiments were reviewed and approved by the Ethical Committee of Experimental Animals of the Chia Tai Tianqing Pharmaceutical Group. Female Balb/c mice aged 6−8 weeks were housed under standard conditions with *ad libitum* access to food and water.

Mice (approximately 20 g) were divided into two groups and intravenously administered a single dose of IL-2 bioconjugate B6 (i.v., 0.6 mg/kg). Serum samples were collected at 10 min and 2, 6, 12, 20, and 36 h for group I and 1, 4, 8, 16, 24, and 48 h for group II by retro-orbital bleeding (n = 4 animals per time-point). Mice from another group (∼20 g) were divided into two groups and intravenously administered a single dose of IL-2 (i.v., 0.6 mg/kg). Serum samples were collected at 0, 2, 5, 20, 30, and 45 min for group I and 10 min and 1, 2, 4, 6, and 8 h for group II by retro-orbital bleeding (n = 4 animals per time-point). The third group of mice (∼20 g) were divided into two groups and intravenously administered a single dose of B6 control (i.v., 0.6 mg/kg). Serum samples were collected at 0, 5, and 30 min and 2, 6, and 12 h for group I and 2, 10, and 60 min and 4, 8, and 24 h for group II by retro-orbital bleeding (n = 4 animals per time-point). Blood was collected in an EDTA-coated test tube and stored on ice until centrifugation at 1200 × *g* for 10 min at 4 °C. Plasma was transferred to a micronic tube and stored at −80 °C until analysis.

Human IL-2 was assayed with a sandwich-type ELISA kit (Cat. No. 555190; BD Biosciences, San Jose, CA, US). B6 and B6 control were generated using the same antibody from the ELISA kit for capture and a biotinylated polyclonal antibody (BAF202, R&D systems, San Jose, CA, US) for detection. Blocking and dilution were performed with 10% FBS in PBS, and individual standard curves were constructed to determine the plasma levels of each protein. Each sample was used to calculate concentrations in plasma (ng/mL) using parameters derived from the standard curve (log−log regression analysis).

Pharmacokinetic parameters were calculated using a two-compartment model with Drug And Statistics software (DAS, ver. 2.0; Mathematical Pharmacology Professional Committee of China, Shanghai, China).

Statistical Analysis. Prism software ver. 8.01 (GraphPad, La Jolla, CA, US) was used for nonlinear regression and statistical analyses. The half-maximal effect concentration (EC_{50}) was determined by fitting a sigmoidal dose−response model "log (agonist) vs. response-variable slope (four parameters)". Graphs depict the average ± SD of at least three independent experiments. All normally distributed data were compared using Student's t test or one-way ANOVA with post-hoc analysis (Games-Howell's procedure) as appropriate. P-values of <0.05 were considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

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Bioconjugate Chem. 2021, 32, 615−625

Reagents and general experimental details, including preparation and characterization of the compounds (PDF)

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Author Contributions

Mengxin Qian designed and conducted the experiments. Qingbin Zhang provided srtA and performed the binding studies. Jianguang Lu designed and carried out purification of IL-2 analogs. Jinhua Zhang contributed experiments involving the synthesis of fatty acid moieties. Yapeng Wang helped with HPLC and SDS-PAGE analyses. Wenwen Shangguan revised the manuscript. Meiqing Feng and Jun Feng supervised the experiments. Mengxin Qian wrote the manuscript, which was edited by all the authors.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

IL-2, interleukin 2; srtA, sortase A; NK, natural killer; JAK, Janus kinase; STAT5, signal transducer and activator of transcription 5; i.v., intravenous; PEG, polyethylene glycol; HSA, human serum albumin; Fc, fragment crystallizable; FL, flexible linker; RL, rigid linker; RP-HPLC, reversed-phase high-performance liquid chromatography; RRT, relative retention time; MS, molecular mass; DTT, dithiothreitol; BLI, Bio-Layer Interferometry; EC_{50}, median effect concentration; pSTAT5, STAT5A and STAT5B by phosphorylation; AEEA, 8-amino-3,6-dioxaoctanoic acid; PK, pharmacokinetic; SD, standard deviation; V_d, volume of distribution; CL, clearance; AUC, area under curve; IL-2R, interleukin 2 receptor; ELISA, enzyme-linked immunosorbent assay; ATP, adenosine-triphosphate; EDTA, ethylenediaminetetraacetic acid

REFERENCES

(1) Morgan, D. A., Ruscetti, F. W., and Gallo, R. C. (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science 193*, 1007−1008.

(2) Charych, D. H., Hoch, U., Langowski, J. L., Lee, S. R., Addepalli, M. K., Kirk, P. B., Sheng, D., Liu, X., Sims, P. W., and VanderVeen, L. A. (2016) NKTR-214, an engineered cytokine with biased IL2 receptor binding, increased tumor exposure, and marked efficacy in mouse tumor models. *Clin. Cancer Res. 22*, 680−690.

(3) Spolski, R., Li, P., and Leonard, W. J. (2018) Biology and regulation of IL-2: from molecular mechanisms to human therapy. *Nat. Rev. Immunol. 18*, 648−659.

(4) Mizui, M. (2019) Natural and modified IL-2 for the treatment of cancer and autoimmune diseases. *Clin. Immunol. 206*, 63−70.

(5) BOYMAN, O, PURTON, J, SURH, C, and SPRENT, J (2007) Cytokines and T-cell homeostasis. *Curr. Opin. Immunol. 19*, 320−326.

(6) Klapper, J. A., Downey, S. G., Smith, F. O., Yang, J. C., Hughes, M. S., Kammula, U. S., Sherry, R. M., Royal, R. E., Steinberg, S. M., and Rosenberg, S. J. C. (2008) High-dose interleukin-2 for the treatment of metastatic renal cell carcinoma: a retrospective analysis of response and survival in patients treated in the surgery branch at the National Cancer Institute between 1986 and 2006. *Cancer 113*, 293−301.

(7) He, J., Zhang, X., Wei, Y., Sun, X., Chen, Y., Deng, J., Jin, Y., Gan, Y., Hu, X., and Jia, R. (2016) Low-dose interleukin-2 treatment selectively modulates CD4+ T cell subsets in patients with systemic lupus erythematosus. *Nat. Med. 22*, 991−993.

(8) Rosenzwajg, M., Lorenzon, R., Cacoub, P., Pham, H. P., Pitoiset, F., Soufi, K. E., Ribet, C., Bernard, C., Aractingi, S., and Banneville, B. (2019) Immunological and clinical effects of low-dose interleukin-2 across 11 autoimmune diseases in a single, open clinical trial. *Ann. Rheum. Dis. 78*, 209−217.

(9) Tahvildari, M., and Dana, R. (2019) Low-Dose IL-2 Therapy in Transplantation, Autoimmunity, and Inflammatory Diseases. *J. Immunol. 203*, 2749−2755.
(10) He, J., Zhang, R., Shao, M., Zhao, X., Miao, M., Chen, J., Liu, J., Zhang, X., Zhang, X., and Jin, Y. (2020) Efficacy and safety of low-dose IL-2 in the treatment of systemic lupus erythematosus: a randomised, double-blind, placebo-controlled trial. *Ann. Rheum. Dis. 79*, 141−149.
(11) Melder, R. J., Osborn, B. L., Riccobene, T., Kanakaraj, P., Wei, P., Chen, G., Stolow, D., Halpern, W. G., Migone, T.-S., and Wang, Q. (2005) Pharmacokinetics and in vitro and in vivo anti-tumor response of an interleukin-2-human serum albumin fusion protein in mice. *Cancer Immunol. Immunother. 54*, 535−547.
(12) Adabi, E., Saebi, F., Hasan-Abad, A. M., Teimoori-Toolabi, L., and Kardar, G. A. (2017) Evaluation of an albumin-binding domain protein fused to recombinant human il-2 and its effects on the bioactivity and serum half-life of the cytokine. *Iran. Biomed. J. 21*, 77.
(13) Rosalia, R. A., Arenas-Ramirez, N., Bouchaud, G., Raeber, M. E., and Boyman, O. (2014) Use of enhanced interleukin-2 formulations for improved immunotherapy against cancer. *Curr. Opin. Chem. Biol. 23*, 39−46.
(14) Katre, N. V., Knauf, M. J., and Laird, W. J. (1987) Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model. *Proc. Natl. Acad. Sci. U. S. A. 84*, 1487−1491.
(15) Leonardo, M. J. (1991) Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature 353*, 6347.
(16) Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Parkinson, D. R., Marincola, F. M., Weber, J. S., Seipp, C. A., White, D. E., and Rosenberg, S. A. J. C. (1995) The use of polyethylene glycol-modified interleukin-2 (PEG-IL-2) in the treatment of patients with metastatic renal cell carcinoma and melanoma. *Cancer 76*, 687−694.
(17) Verhoef, J. J., and Anchordoquy, T. J. (2013) Questioning the use of PEGylation for drug delivery. *Drug Delivery Transl. Res. 3*, 499−503.
(18) Lei, J., Guan, B., Li, B., Duan, Z., Chen, Y., Li, H., and Jin, J. (2012) Expression, purification and characterization of recombinant human interleukin-2-serum albumin (rhIL-2-HSA) fusion protein in Pichia pastoris. *Protein Expression Purif. 84*, 154−160.
(19) Vazquez-Lombardi, R., Loetsch, C., Zinkl, D., Jackson, J., Schofield, P., Deenick, E. K., King, C., Phan, T. G., Webster, K. E., and Sprent, J. (2017) Potent antitumour activity of interleukin-2-Fc fusion proteins requires Fc-mediated depletion of regulatory T-cells. *Nat. Commun. 8*, 1−12.
(20) Peterson, L. B., Bell, C. J., Howlett, S. K., Pekalski, M. L., Brady, K., Hinton, H., Sauter, D., Todd, J. A., Umana, P., and Ast, O. (2018) A long-lived IL-2 mutein that selectively activates and expands regulatory T cells as a therapy for autoimmune disease. *J. Autoimmun. 95*, 1−14.
(21) Cho, J., Park, J., Kim, S., Kim, J. C., Tae, G., Jin, M. S., and Kwon, I. (2020) Intramolecular distance in the conjugate of urate oxidase and fatty acid governs FcRn binding and serum half-life in vivo. *J. Controlled Release 321*, 49−58.
(22) van Witteloostuijn, S. B., Pedersen, S. L., and Jensen, K. (2016) Half-life extension of biopharmaceuticals using chemical methods: alternatives to PEGylation. *ChemMedChem 11*, 2474−2495.
(23) Cho, J., Lim, S. I., Yang, B. S., Hahn, Y. S., and Kwon, I. (2017) Generation of therapeutic protein variants with the human serum albumin binding capacity via site-specific fatty acid conjugation. *Sci. Rep. 7*, 1−12.
(24) Zaman, R., Islam, R. A., Ibnat, N., Othman, I., Zaini, A., Lee, C. Y., and Chowdhury, E. H. (2019) Current strategies in extending half-lives of therapeutic proteins. *J. Controlled Release 301*, 176−189.
(25) Erak, M., Bellmann-Sickert, K., Els-Heindl, S., and Beck-Sickinger, A. G. (2018) Peptide chemistry toolbox-Transforming natural peptides into peptide therapeutics. *Bioorg. Med. Chem. 26*, 2759−2765.
(26) Popp, M. W., Dougan, S. K., Chuang, T.-Y., Spooner, E., and Ploegh, H. L. (2011) Sortase-catalyzed transformations that improve the properties of cytokines. *Proc. Natl. Acad. Sci. U. S. A. 108*, 3169−3174.
(27) Muppidi, A., Lee, S. J., Hsu, C.-H., Zou, H., Lee, C., Pflimlin, E., Mahankali, M., Yang, P., Chao, E., Ahmad, I., et al. (2019) Design and Synthesis of Potent, Long-Acting Lipidated Relaxin-2 Analogs. *Bioconjugate Chem. 30*, 83−89.
(28) Pan, L., Zhao, W., Lai, J., Ding, D., Zhang, Q., Yang, X., Huang, M., Jin, S., Xu, Y., Zeng, S., et al. (2017) Sortase A-Generated Highly Potent Anti-CD20-MMAE Conjugates for Efficient Elimination of B-Lineage Lymphomas. *Small 13*, 1602267.
(29) Greineder, C. F., Villa, C. H., Walsh, L. R., Kiseleva, R. Y., Hood, E. D., Khoshnejad, M., Warden-Rothman, R., Tsourkas, A., and Muzykantov, V. R. (2018) Site-specific modification of single-chain antibody fragments for bioconjugation and vascular immunotargeting. *Bioconjugate Chem. 29*, 56−66.
(30) Silvius, J. R., and Leventis, R. (2017) A Novel "prebinding" strategy dramatically enhances sortase-mediated coupling of proteins to liposomes. *Bioconjugate Chem. 28*, 1271−1282.
(31) Gu, H., Ghosh, S., Staples, R. J., and Bane, S. L. (2019) *β*-Hydroxy-Stabilized Boron-Nitrogen Heterocycles Enable Rapid and Efficient C-Terminal Protein Modification. *Bioconjugate Chem. 30*, 2604−2613.
(32) Agwa, A. J., Blomster, L. V., Craik, D. J., King, G. F., and Schroeder, C. I. (2018) Efficient enzymatic ligation of inhibitor cystine knot spider venom peptides: using sortase A to form double-knottins that probe voltage-gated sodium channel NaV1. 7. *Bioconjugate Chem. 29*, 3309−3319.
(33) Lieser, R. M., Yur, D., Sullivan, M. O., and Chen, W. (2020) Site-specific bioconjugation approaches for enhanced delivery of protein therapeutics and protein drug carriers. *Bioconjugate Chem. 31*, 2272−2282.
(34) Markus, R. (2014) Sortagging: a robust and efficient chemoenzymatic ligation strategy. *Chem.-Eur. J.*, 20.
(35) Chen, X., Zaro, J. L., and Shen, W.-C. (2013) Fusion protein linkers: property, design and functionality. *Adv. Drug Delivery Rev. 65*, 1357−1369.
(36) Gosselin, M. L., Fabrizio, D., Swain, J. F., Mitchell, T., Camphausen, R., Cload, S. T., Furfine, E., Morin, P. E., Mukherjee, R., and Taylor, S. I. (2019). *Serum albumin binding molecules US*; Bristol-Myers Squibb Company, Princeton, NJ.
(37) Ju, G., Collins, L., Kaffka, K. L., Tsien, W.-H., Chizzonite, R., Robert, C., Ram, B., and Patricia, K. L. (1987) Structure-function analysis of human interleukin-2. Identification of amino acid residues required for biological activity. *J. Biol. Chem. 262*, 5723−5731.
(38) Silva, D.-A., Yu, S., Ulge, U. Y., Spangler, J. B., Jude, K. M., Labão-Almeida, C., Ali, L. R., Quijano-Rubio, A., Ruterbusch, M., and Leung, I. (2019) De novo design of potent and selective mimics of IL-2 and IL-15. *Nature 565*, 186−191.
(39) Doucette, C. D., Greenshields, A. L., Liwski, R. S., and Hoskin, D. W. (2015) Piperine blocks interleukin-2-driven cell cycle progression in CTLL-2 T lymphocytes by inhibiting multiple signal transduction pathways. *Toxicol. Lett. 234*, 1−12.
(40) Maki, G., Klingemann, H.-G., Martinson, J. A., and Tam, Y. K. (2001) Factors regulating the cytotoxic activity of the human natural killer cell line, NK-92. *J. Hematother. Stem Cell Res. 10*, 369−383.
(41) Sleep, D., Cameron, J., and Evans, L. R. (2013) Albumin as a versatile platform for drug half-life extension. *Biochim. Biophys. Acta, Gen. Subj. 1830*, 5526−5534.
(42) Yang, P.-Y., Zou, H., Chao, E., Sherwood, L., Nunez, V., Keeney, M., Ghartey-Tagoe, E., Ding, Z., Quirino, H., and Luo, X. (2016) Engineering a long-acting, potent GLP-1 analog for micro-structure-based transdermal delivery. *Proc. Natl. Acad. Sci. U. S. A. 113*, 4140−4145.
(43) Yang, P.-Y., Zou, H., Amso, Z., Lee, C., Huang, D., Woods, A. K., Nguyen-Tran, V. T., Schultz, P. G., and Shen, W. (2020) New generation oxyntomodulin peptides with improved pharmacokinetic profiles exhibit weight reducing and anti-steatotic properties in mice. *Bioconjugate Chem. 31*, 1167−1176.

https://dx.doi.org/10.1021/acs.bioconjchem.1c00062
Bioconjugate Chem. 2021, 32, 615−625

(44) Wang, X., Rickert, M., and Garcia, K. C. (2005) Structure of the Quaternary Complex of Interleukin-2 with Its α, ß, and γc Receptors. *Science 310*, 1159−1163.
(45) Stauber, D. J., Debler, E. W., Horton, P. A., Smith, K. A., and Wilson, I. A. (2006) Crystal structure of the IL-2 signaling complex: paradigm for a heterotrimeric cytokine receptor. *Proc. Natl. Acad. Sci. U. S. A. 103*, 2788−2793.
(46) Sankar, S., Yamaguchi, M., Kawabata, S., and Ponnuraj, K. (2020) Streptococcus pneumoniae Surface Adhesin PfbA Exhibits Host Specificity by Binding to Human Serum Albumin but Not Bovine, Rabbit and Porcine Serum Albumins. *Protein J. 39*, 1−9.
(47) Yamaoka, T., Tabata, Y., and Ikada, Y. (1994) Distribution and tissue uptake of poly (ethylene glycol) with different molecular weights after intravenous administration to mice. *J. Pharm. Sci. 83*, 601−606.
(48) Zhang, Q., Huang, Z., Lu, J., Zhao, W., and Feng, J. (2020) Prokaryotic Expression, Purification and Activity Determination of Sortase A Enzyme Mutant. *Chin. J. Pharm. 51*, 37−47.
(49) Ast, O., Bruenker, P., Freimoser-Grundschober, A., Herter, S., Hofer, T. U., Hosse, R., Klein, C., Moessner, E., Moessner, V. G., and Umana, P. (2019). *Mutant interleukin-2 polypeptides*. US, Roche Glycart AG, Schlieren (CH).

https://dx.doi.org/10.1021/acs.bioconjchem.1c00062
Bioconjugate Chem. 2021, 32, 615−625