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The Rational Design, Synthesis, Characterization, and Biological Evaluation of Cancer-Targeting Immunostimulatory Peptide-Protein Conjugates and Tripeptides

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THE RATIONAL DESIGN, SYNTHESIS, CHARACTERIZATION, AND BIOLOGICAL EVALUATION OF CANCER-TARGETING IMMUNOSTIMULATORY PEPTIDE-PROTEIN CONJUGATES AND TRIPEPTIDES

A Thesis Submitted by

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We certify that we read this thesis and in our opinion, it is sufficient in scientific scope and quality as a dissertation for the degree of Master's of Science in Biology.

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Dedication

This thesis is dedicated to my family for their unwavering support throughout my studies.

Abstract

With the advent of cancer immunotherapy and the rise in applications of synthetic biologics, there has been a steady decline in the incidence of cancer. Despite this trend, there is an anticipated 1.7 million new cancer cases with an estimated 610,000 deaths expected by the end of 2018.[2] Therefore, the call for continued efforts in creating more effective treatment options are still in high demand. In this thesis, the rational design of a semi-synthetic cancer-targeting immunostimulatory peptide-protein bioconjugate—using *N*-succinimidyl carbamate chemistry is described. This bio-orthogonal chemistry approach was used to conjugate the synthetic Pep42, cancer-targeting peptide (CTP) and the immunostimulatory recombinant B7H6 tumor associated antigen (TAA). Also reported within this thesis is the design, synthesis and biological evaluation of bifunctional tripeptides composed of the CTP and TAA targeting and effector ligands, respectively. The purported CTP was anticipated to bind to cell surface GRP78—a phenotype found exclusively in several cancers, while the TAA, was expected to bind and activate NKp30, an activating natural cytotoxicity receptor (NCR) found on the surface of NK cells. In this manner, the cancer-targeting immunostimulatory peptide-protein conjugates and tripeptides were hypothesized to behave as bifunctional antibody mimics, targeting and activating NK cells towards selective tumor cytolysis. Using Fmoc-SPPS, we have generated a library of tripeptides that were isolated and characterized by RP-LC/MS and UV/Vis spectroscopy. Using flow cytometry, the preliminary data confirmed tripeptide-GRP78 binding of the HepG2 cells and tripeptide-NKp30 binding of NK92-MI cells. We anticipate the specific binding of the tripeptides to their intended targets will provide the best candidates for translating our cancer immunotherapy approach *in-vivo*.

Keywords: Bioconjugation, CTP, TAA, GRP78, NKp30, cancer immunotherapy

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List of Abbreviations

ACN	Acetonitrile
AHX	Amino hexanoic acid
CTIP	Cancer-targeting immunostimulatory peptide
CTP	Cancer-targeting peptide
DMF	N,N-Dimethylformamide
DSC	Disuccinimidyl carbonate
ER	Endoplasmic Reticulum
ESI	Electrospray ionization
FA	Formic acid
FACS	Flow-assisted cell sorting
FITC	Fluorescein isothiocyanate
Gly	Glycine
GRP78	Glucose Regulated Protein 78
HCTU	O-(1H-6-Chlorobenzotriazole-1-yl)tetramethyluronium hexafluorophosphate
HPLC	High-performance liquid chromatography
IFN-γ	Interferon gamma
LC	Liquid chromatography
LCMS	Liquid chromatography - mass spectrometry
Lys	Lysine
mAB	Monoclonal antibody

Introduction

Cancer is a devastating disease characterized by a variety of biological hallmarks including: a reduced dependence of mitogenic growth factors, growth-inhibitory signal resistance, immortalization via indefinite proliferation, reduced influence by apoptosis, angiogenesis, metastatic ability, genomic instability, and ability to evade the immune system.[1] According the American Cancer Society, there have been over 2 million cancer deaths in the past 5 years and a predicted 1.7 million new cases with an estimated 610,000 additional deaths expected by the end of 2018. Despite the modern advances in cancer treatment and detection methods, the decline of cancer incidences have decreased by only about 2% over this same period of time.[2] This steady, yet small, decline in cancer incidence is in large part due to the advent of cancer immunotherapy and synthetic biologics which have led to more effective treatment options. In spite of their utility, synthetic biologics such as the monoclonal antibodies are plagued by poor pharmacological and pharmacodynamics properties in addition to immunotoxicity which limits their clinical utility and raises the need for new and improved therapeutics in the fight against cancer.[3]

Cancer-targeting peptides (CTPs) are a novel class of biologicals that have the ability to target tumor cells with high specificity, binding affinity, and modularity.[4] In this regard, CTPs are advantageous over conventional, nonselective forms of cancer therapies such as chemotherapy or radiation due to their ability to target and treat tumors while mitigating off-target side effects. One such example, the cyclic CTP Pep42 (CTVALPGGYVRVC-$CONH_2$), that has been identified through phage display as an excellent binding candidate towards the glucose regulated protein 78 (GRP78).[5] GRP78 is classified as a 78 kDa chaperone protein found in the lumen of the endoplasmic reticulum (ER) that assists in protein folding events as part of the unfolded protein response (UPR).[6] As such, GRP78 is ubiquitously expressed in all cells where, under normal

physiological conditions, it assists with protein folding in the ER, modulates Ca^{2+} flux, interacts with pro-apoptotic executors in the mitochondria and regulates nuclear gene expression.[7] However, under pathological conditions (i.e. cancer) GRP78 is translocated to the plasma membrane where it regulates proliferative and metabolic cell signaling pathways as a surface receptor.[7] In transformed cells, GRP78 has been found to be oncogenic in nature.[8] It is overexpressed and localized on the cell membrane where it is involved in the signaling pathways that lead to cancer proliferation, metastasis and chemo-resistance.[8] Moreover, although GRP78 is constitutively expressed in all healthy cells, it is exclusively expressed on the cell surface of many advanced cancers making it an excellent biomarker in the selective detection and treatment of resilient tumors.[9] Towards this goal, a wide range of small molecule[10], peptidic[11] and antibody ligands to GRP78[12] have been developed as tumor homing agents, in order to direct the payload at the targeted tumor site for selective detection and treatment.

B7H6, a member of the B7 protein family, is a transmembrane glycoprotein that has a molecular weight around 51 kDa, depending on its variable glycosylation pattern which contains immunoglobulin-like features on its ectodomain region.[13] B7H6 is expressed exclusively on the surface of tumor cells and has been identified as a tumor-associated antigen (TAA) for the NKp30 receptor found on NK cells.[14] Moreover, B7H6 has been shown to function, in conjunction with the absence of MHC class I, to activate the NK cell degranulation and enable the release of inflammatory cytokines (e.g. TNFα and IFNγ) which trigger tumor cell death via cytolysis.[14] In spite of its immunostimulatory activity, B7H6 retains moderate NKp30 binding affinity, limited stability and bioavailability which hinders its therapeutic efficacy.[13] Furthermore, cancers have evolved the ability to evade B7H6:NKp30 dependent immunity by using metalloprotease activity to cleave the B7H6 ectodomains from their cell surfaces, preventing NK cell effector functions.[13]

Moreover, certain tumors express minimal if any B7H6 in order to escape NK-dependent immunosurveillance.[15] Thus, new NKp30 binding and activating ligands are needed to address the shortcomings of B7H6 as a cancer immunotherapy target. Towards this goal, the discovery of the B7H6:NKp30 binding interaction provided the capabilities for developing new peptidic ligands that bound to NK cells and triggered NKp30-dependent immunostimulatory activity[16] Particularly, it was found that the TVPLN peptide sequence exhibited binding to NK92-MI cells in an NKp30 dependent manner that resulted in the release of TNF-α, an important pro-inflammatory cytokine that can result in direct tumor cytolysis.[16] This finding has provided the opportunity to design and develop cancer targeting immunostimulatory peptides (CTIPs) that can incorporate the targeting and effector functions of potent cancer immunotherapeutics.

In this thesis, the design, synthesis and biological evaluation of peptidic ligands derived from the GRP78 and B7H6 binding and immunostimulatory domains are reported. More specifically, a semi-synthetic strategy was developed for the incorporation of the GRP78 binding ligand Pep42 with the NKp30 binding and activating tumor associated antigen, B7H6. The synthesis, characterization and biological activity of the FITC-labeled Pep42-B7H6 conjugate is reported in this thesis. Moreover, a new class cancer-targeting immunostimulatory peptides based on Pep42 and other known GRP78-targeting sequences have been incorporated with the TVPLN peptide for binding and activating NKp30 derived NK cells. (Figure 1).

Figure 1: The structure-function properties of a semi-synthetic peptide-protein bioconjugate (a) and their corresponding (b) tripeptides.

Results & Discussion

Fmoc Solid-Phase Peptide Synthesis of Pep42-derived sequences

The Fmoc-SPPS of Pep42[5, 11b] CTVALPGGYVRVC-$CONH_2$, has been examined by conventional Rink amide-linker Merrifield resin, consisting of polystyrene-divinylbenzene (PS-DVB) cross-linked core[17] and on a Rink amide-linker amphiphilic polystyrene-*graft*-poly(ethylene

glycol) (TentaGel)[18] and hydrophilic poly(ethylene glycol) (NovaPEG)[19] based solid supports.[11a] The synthesis efficiency on the more hydrophobic Merrifield resin produced peptide crude purities of only 28% according RP-HPLC, whereas to 67% crude purities were obtained on a Tentagel S RAM resin and 92% on the poly(ethylene glycol) Rink Amide NovaPEG resin. Therefore, the Rink amide PEG-based resin was selected for making a small library of Pep42 sequences which resulted in good crude purities (34-75%). Following purification by RP-HPLC, the Pep42 sequences were isolated in good yields (10-45%) and excellent purities (>95%) according to RP-HPLC and the sequence identities were confirmed by molecular weight analyses using ESI-LCMS.[11a] Based on these initial results, the Rink amide PEG-based resin was selected as the optimal choice for making the Pep42 derived sequences in Table 1.

In this study, Pep42 was functionalized with either the *N*-terminal FITC or acetyl group onto the Ahx linker and with a PEG or Gly linker at the *C*-terminus (Figure 2). At the *C*-terminus, the orthogonally protected Lys(ivDde) group was removed with nucleophilic (4% $NH_2NH_2{\cdot}H_2O$:DMF) conditions to liberate the reactive ε-amino group.[20] Complete deprotection was monitored by RP-HPLC/MS following successive (3 x 5 min) NH_2NH_2 treatment. *N*-succinimidyl carbamate (NSC) chemistry was adopted to functionalize the reactive ε-amino group with disuccinimidyl carbonate (DSC).[21] This activation reaction was also monitored by RP-HPLC/MS which confirmed complete activation following 1 h. The NSC activated peptides (**1**-**4**) were isolated in good yields (28-40%) and >90% purities following LC/MS analyses and purification.

Figure 2. Representative solid phase peptide synthesis and LCMS characterization for peptides **1** and **2**. (A) Fmoc-solid-phase peptide synthesis and FITC labeling for peptides **1** and **2**, respectively. (B) RP HPLC analyses for FITC-Ahx-CTVALPGGYVRVC-PEG_3-K-$CONH_2$, **2** and (C) ESI-MS analyses for FITC-Ahx-CTVALPGGYVRVC-PEG_3-K-$CONH_2$, **2**.

Pep42-B7H6 Bioconjugation

The NSC-activated peptide sequences (**1-4**) were then subjected to bioconjugation with a recombinant human B7H6 Fc chimera protein (R&D Biosystems) (Figure 3). Several reaction conditions were examined in an effort to optimize conjugation yields. The reaction was initially conducted in solution with changes in reaction times (1 h – 24 h) in order to examine their influence on reaction conversions and yields. LCMS analysis proved difficult under these reaction conditions resulting in multiple product peaks and masses which weakly correlated to the expected mass per charge. In an attempt to facilitate the conjugation reaction, support-bound Pep42 sequences (**3-4**) with all side chain protecting groups intact and the Lys ε-amino selectively deprotected using the

above mentioned nucleophilic (4% $NH_2NH_2{\cdot}H_2O$:DMF) conditions were subjected to a solid-phase bioconjugation reaction with B7H6. This reaction was conducted in a buffer (PBS, 10mM pH 8.0) to enhance B7H6 solubility and efficient swelling of the Pep42-bound resin. The reaction proceeded overnight at room temperature. The reaction mixture was filtered to remove any unbound impurities and the resin was treated with 95:2.5:2.5 TFA:TES:H_2O for cleavage and deprotection which isolated the crude reaction product as a solid white pellet. This crude sample was analyzed by RP-HPLC and MS in order to track reaction conversions (Figure 3). In this case, Pep42 labeling of B7H6 was not detected by MS and the crude RP-HPLC analyses made it difficult to isolate pure Pep42-B7H6 bioconjugate potentially due to the large mass and heavy glycosylation patterns of B7H6. Regardless, this crude sample was tested for biological activity.

Figure 3. Bioconjugation and corresponding crude HPLC analysis of Pep42 and rB7H6 using NSC chemistry.

Synthesis and Characterization of GRP78 and NKp30-targeting tripeptides

Due to the difficulties associated with the bioconjugation of the GRP78-targeting Pep42 sequence with the NKp30-targeting B7H6 recombinant protein an alternative synthesis strategy was developed. In this synthetic approach, a series of tripeptides (Table 1, sequences **5-13**) incorporating NKp30 binding and NK cell-activating peptides[16] as linear dimers were extended into branching peptides to incorporate the GRP78-targeting peptides[22] from the ε-amino group of an internal Lys residue (Figure 4). Fmoc-SPPS was used to synthesize the bifunctional tripeptides on resin, which were isolated following cleavage and deprotection and then analyzed by LCMS to confirm peptide purities and identities. In this case, the GRP78 and NKp30-targeting tripeptides (Table 1, **5-13**) were isolated in good yields (61-85%) and purities >95% according to RP-HPLC. Moreover, ESI-MS analyses confirmed sample identities according to mass per charge ratios (Supporting Information, Figure S5-S29). With pure tripeptides in hand, peptide biology was next examined in collaboration with Drs. Robert Korngold and Dante Descalzi at Hackensack UMC working alongside Rachel Montel, a graduate student in the labs of Drs. Sabatino and Bitsaktsis at SHU.

Figure 4. Representative solid phase peptide synthesis and LCMS characterization for tripeptides **5** and **6**. (A) Fmoc-solid-phase peptide synthesis and FITC labeling for peptides **5** and **6**, respectively. (B) RP HPLC and MS for Peptide 5 and (C) peptide **6**.

Table 1: *Characterization Data of Synthetic Peptides*

Peptide	Sequence	Crude Purity (%)[a]	Crude Yield (%)[b]	Observed Mass (g/mol)[d]	Expected Mass (g/mol)[e]	Z[f]	Retention Time (min)[g]
1	Ac-AHX-CTVALPGGYVRVC-(PEG3)4-K-CONH2	74.65	40.5	1216.7 811.7	1216.5 811.3	2 3	7.474
2	FITC-AHX-CTVALPGGYVRVC-(PEG3)4-K-CONH2	52.43	28.1	1390.5	1391.2	2	14.477
3	Ac-AHX-CTVALPGGYVRVC-G-K-CONH2	33.66	N/A	839.0 559.7	838.5 559.4	2 3	8.561
4	FITC-AHX-CTVALPGGYVRVC-G-K-CONH2	59.02	N/A	1012.6 675.5	1013.2 675.8	2 3	9.798
5	Ac-TVPLNGK(Ac-AHX-CTVALPGGYVRVC-)GTVPLN-CONH2	77.23	95	1411.9	1412.7	2	8.589
6	Ac-TVPLNGK(FITC-AHX-CTVALPGGYVRVC-)GTVPLN-CONH2	84.25	43.6	1586.7	1587.4	2	9.821
7	Ac-TVPLNGK(Ac-AHX-RLLDTNRPLLPY-)GTVPLN-CONH2	83.07	31.8	1479.8 986.8	1480.3 987.2	2 3	8.005
8	Ac-TVPLNGK(FITC-AHX-RLLDTNRPLLPY-)GTVPLN-CONH2	74.1	25	1653.8 1102.7 827.2	1654.9 1103.6 827.9	2 3 4	9.023
9	Ac-TVPLNGK(Ac-AHX-RLLDTNRPFLPY-)GTVPLN-CONH2	68.04	48.05	1496.8 998.6	1497.3 998.5	2 3	8.142
10	Ac-TVPLNGK(FITC-AHX-RLLDTNRPFLPY-)GTVPLN-CONH2	68.56	60.3	1114.1 835.8	1114.9 836.5	3 4	9.089
11	Ac-TVPLNGK(Ac-AHX-RLLDTNRPFLFY-)GTVPLN-CONH2	69.05	71.2	1521.8 1015.0	1522.3 1015.2	2 3	8.545
12	Ac-TVPLNGK(FITC-AHX-RLLDTNRPFLFY-)GTVPLN-CONH2	60.67	66.6	1695.7 1130.7 848	1696.9 1131.6 848.9	2 3 4	9.421
13	Ac-TVPLNGK(FITC-AHX-WIFPWIQL-)GTVPLN-CONH2	85	37	1470 980 735	1470 980.3 735.5	2 3 4	18.4

[a] Crude purity was determined from RP HPLC at 220nm using 2-80% ACN/H2O with 0.1% FA over 25 minutes. Peptides with crude purities<95% were subjected to an additional purification step using the aforementioned RP HPLC conditions. In these cases, purified peptides were isolated with purities>95%. [b] Based on resin loading. [c] Isolated yield based on resin loading. [d] Observed mass is based on the mass/charge using [M + H+]/Z detected using MS direct injection in positive mode. [e] Expected mass as determined using ChemDraw. [f] The peptide charge states using positive mode in MS direct injection. [g] Based on RP HPLC at 220nm using 2-80% ACN/H2O with 0.1% FA over 25 minutes.

Binding Studies of the Bioconjugate and Tripeptides

The ability for a selected tripeptide (**13**) and the FITC labeled Pep42-B7H6 bioconjugate to bind to HepG2 cells' GRP78 was initially evaluated by flow cytometry (Figure 5). The FITC-labeled tripeptide (**13**) displayed good binding (~91%, 0.039 μg/μL) on the HepG2 cells, which was found to be diminished in the presence of a GRP78 peptide blocker (~76%, 0.25 μg/μL) and the anti-GRP78 primary antibody (~68%, 0.25 μg/μL). These results suggests that tripeptide **13**

exhibited GRP78-dependent binding on the HepG2 cells. Alternatively, the FITC-labeled Pep42-B7H6 bioconjugate displayed little binding to the HepG2 cells (~6%, 0.5 μg/μL) which was not disrupted when the HepG2 cells were pretreated with either the GRP78 blocking peptide or the anti-GRP78 primary antibody. Thus, the Pep42-B7H6 bioconjugate demonstrated little HepG2 cell binding in a non-GRP78 dependent manner.

Figure 5. GRP78 Binding on the HepG2 cells. A) Incubation with tripeptide **13**, B) incubation with GRP78 blocking peptide followed by treatment with tripeptide **13**, C) incubation with unlabeled sc-1050 GRP78 primary antibody followed by treatment with tripeptide **13**, D) incubation with FITC-Pep42-B7H6 bioconjugate, E) incubation with GRP78 blocking peptide followed by treatment with FITC-Pep42-B7H6 bioconjugate, F) incubation with unlabeled sc-1050 GRP78 primary antibody followed by treatment with FITC-Pep42-B7H6. n=1

The binding capabilities of the FITC-labeled synthetic tripeptide, **13**, and the FITC-Pep42-B7H6 bioconjugate to the cell surface of the human NK cell line, NK92-MI, was compared to that of the APC-labeled anti-NKp30 mAb (Figure 6). Flow cytometry revealed NK cell binding occupancy (~57 and 94%, 0.25 μg/μL) with the anti-NKp30 mAb, and the FITC-labeled tripeptide,

13, whereas the Pep42-B7H6 bioconjugate displayed little (~0.5%, 0.5 μg/μL) binding to the NK cells. A competitive binding assay was also conducted in between the FITC-labeled tripeptide, **13**, the FITC-Pep42-B7H6 bioconjugate and the APC-labeled anti-NKp30 mAb to evaluate NKp30 binding specificities (Figure 6). In this assay, the NK92-MI cells were initially incubated with either the FITC-labeled tripeptide, **13**, or the Pep42-B7H6 bioconjugate followed by treatment with the APC-labeled anti-NKp30 mAb. Flow cytometry revealed complete displacement of the tripeptide **13**, resulting in little (~1.5%) bound peptide and replacement with bound anti-NKp30 mAb (~94%, 0.25 μg/μL). Comparatively, FITC-Pep42-B7H6 bioconjugate displayed negligible displacement upon APC-labeled anti-NKp30 mAb treatment, presumably due to the little binding occupancy observed (0.14-0.52%) for the bioconjugate which was found to be completely replaced by the anti-Nkp30 mAb (~92%, 0.25 μg/μL) (Figure 6).

Figure 6*. NKp30 binding and displacement studies on the NK92-MI cells. A) NK92-MI cells incubated with tripeptide* ***13****, NK92-MI cells incubated with tripeptide* ***13*** *followed by APC-*

labeled anti-NKp30 antibody treatment, B) FITC and C) APC detection, D) NK92-MI cells incubated with FITC-Pep42-B7H6, NK92-MI cells incubated with FITC-Pep42-B7H6 followed by APC-labeled anti-NKp30 antibody treatment, E) FITC and F) APC detection. n=1

Conclusions

In this thesis, the design, synthesis, and biological evaluation of a new class of bifunctional semi-synthetic peptide-protein bioconjugates and synthetic tripeptides have been described. These bifunctional ligands were designed to contain GRP78 binding capabilities on the surface of a selected liver cancer cell line (i.e. HepG2 cells) as well as NKp30 binding and effector functions of NK cells (i.e. NK92-MI cells). These biomolecular ligands were synthesized by a combination of Fmoc-based SPPS and NSC biorthogonal chemistry. Following characterization by LCMS, a selected tripeptide (**13**) displayed GRP78 specific binding on the HepG2 cells and concomitant NKp30-dependent binding on NK cells. The latter may prove to be an important lead in follow-up immunostimulatory studies aimed towards developing synthetic antibody mimics that may potentiate targeted tumor immunotherapy responses in cells and *in vivo*.

Future Work

Our future work is geared towards: 1) screening the library of bifunctional tripeptides reported in Table 1 in order to assess their GRP78 specific binding on HepG2 (and related) cancer cells as well as NKp30 binding and activation of NK cells, 2) detection of secreted inflammatory cytokines (e.g. TNF-α, IFN-γ) and GRP78-dependent tumor cytolytic activity in cell co-cultures and 3) within tumor bearing mice xenografts. The latter research objectives will validate the cancer immunotherapy potential of our constructs with the goal of translating this pre-clinical study into clinical applications.

Experimental Section

Materials and Methods

Amino acids for the synthesis of all peptides, Fmoc-Lys(Boc), Fmoc-Lys(ivDde), Fmoc-Lys(Dde), Fmoc-Leu, Fmoc-Pro, Fmoc-Thr(tBu), Fmoc-Val, Fmoc-Gly, Fmoc-Phe, Fmoc-Met, Fmoc-Ser(tBu), Fmoc-Tyr(tBu), Fmoc-Cys(Trt), Fmoc-Ala, Fmoc-Arg(Pbf), Fmoc-Asp(otBu), Fmoc-Asn(Trt), Fmoc-His(Trt), Fmoc-Trp(Boc), and Fmoc-Ahx, were purchased from Novabiochem (San Diego, CA, USA) and Advanced ChemTech (Louisville, KY, USA). Peptide syntheses were conducted on a Rink Amide ChemMatrix (0.54mmol/g) (Biotage Inc., Charlotte NC, USA). HCTU was purchased from Advanced ChemTech (Louisville, KY, USA). Recombinant human B7H6 Fc chimera protein was purchased from R&D Systems (Minneapolis, MN, USA) and reconstituted in 10mM phosphate saline buffer (PBS) at pH 8.0 prior to use. Fluorescein isothiocyanate, FITC, was purchased from Thermo Scientific (Rockford, IL, USA) as an isomeric mixture and used in the dark to fluorescently label all peptides. Trifluoroacetic acid (TFA), Bio-grade, was purchased from VWR (Radnor, PA, USA); *N*-*N*-dimethylformamide, DMF, acetonitrile (ACN), methanol (MeOH), and dichloromethane (DCM) were all purchased from MACRON in ACS grade (Center Valley, PA, USA). Piperidine was purchased from EMD Millipore (Billerica, MA, USA); formic acid (97%) (FA), triethylsilane (>98%) (TES), and pyridine (ACS, 99%) were purchased from Alfa Aesar (Ward Hill, MA, USA). *N*-methylmorpholine (99%) (NMM), was purchased from Acros Organics (Pittsburg, PA, USA). Diethyl ether (99%, ACS) (Et_2O), used to precipitate peptides, was purchased from Sigma Aldrich (St. Louis, MO, USA). All chemicals were used directly as received.

Peptide Synthesis

All peptides were synthesized by stepwise manual or semi-automated solid phase peptide synthesis on a PSI 200C Peptide Synthesizer (Glen Oaks, NY, USA) using Fmoc-SPPS chemistry.[23] Fmoc-amino acids (3 equivalents (eq), 0.1 mmol) were coupled on a Rink amide linker poly (ethylene glycol) solid support (0.54 mmol/g, 0.1 mmol) for 30 minutes using HCTU (3 eq., 0.1 mmol), NMM (6 eq., 0.1 mmol) in DMF (4 mL). A 20% piperidine in DMF solution (4 mL) was used for Fmoc deprotection, reaction time 20 min. Amino acid couplings and Fmoc deprotections were repeated until the desired sequences were completed. For tripeptide sequences, **5 – 13,** Fmoc-SPPS was initially accomplished as previously described to generate the linear NKp30-targeting peptides followed by acetylation (Ac_2O:pyr:DMF, 50mmol%:50mmol%:4mL) of the *N*-terminus. Branching from an internal Lys(ivDde) residue was accomplished by ivDde deprotection using 4% NH_2NH_2:DMF (3 x 5 min) followed by DMF washing and Fmoc-SPPS from the liberated ε-amino to generate the GRP78-targeting peptide sequences. The *N*-terminus was then coupled with an amino hexanoic acid (Ahx) linker followed by acetylation or FITC-coupling.

The solid support bound peptide was separated (200 mg, 0.05 mmol) for FITC-labeling or acetylation at the *N*-terminus. For FITC-labeling procedures, the resin was swollen in DMF for 1 h. A mixture of FITC (1.1 equiv., 0.05 mmol) in pyridine/DMF/DCM (12:7:5 v/v) was prepared and added to the reaction vessel for overnight (at least 18 h) reaction on an overhead shaker. For N-terminal acetylation, the peptide bound resin (200 mg, 0.05 mmol) was treated with a solution of Ac_2O (472 µL, 0.1 mmol), pyridine (403 µL, 0.1 mmol) in DMF (3 mL) and reacted for 30 min. After FITC-labeling or acetylation was completed, the resin was washed with DMF (3 x 3 mL), MeOH (3 x 3 mL), and DCM (3 x 3 mL). Peptide cleavage and deprotection from the solid support

was accomplished using a mixture of TFA:TES:H_2O, (95:2.5:2.5 v/v/v) for 4 h. Peptide samples were concentrated under either air or nitrogen (air if disulfide bridges were expected) to a viscous oil, precipitated with cold Et_2O, and centrifuged to a white pellet. The supernatant was decanted, and the peptide pellets were dissolved in ACN/H_2O for RP-HPLC and MS analyses.

Pep42-B7H6 Bioconjugation

The Pep42 sequences, **1-2**, were activated at the *C*-terminal ε-amino group of Lys with DSC (16.6 mg, 65 μmol), triethylamine (0.45 μL, 3.2 μmol) dissolved in dry DMF (85 μL) at rt under N_2. The mixture was shaken at rt for 1 h. The crude mixture was diluted with 20 mL of 1% TFA:H_2O and purified by RP-HPLC. Fractions were collected and lyophilized to produce the activated NSC peptides that were analyzed by LC/MS to confirm purity and identity. The Pep42 sequences, **3-4**, remained on solid support prior to NSC chemistry. After activation of C-terminal ε-amine group of lysine with the same conditions as above, a small aliquot of the peptide bound resin was transferred to minimize excessive stoichiometric amounts peptide relative to B7H6.

Recombinant human B7H6 Fc chimera protein (R&D Systems, cat. 7144-B7-050, 50 μg, 9.38 x 10^{-4} μM) was reconstituted in PBS buffer (500 μL) and added to the NSC peptides **3-4** (4.3 μmol) on solid support (Rink amide PEG based resin). The mixture was shaken at rt for 24 h. Following the reaction, the mixture was diluted in H2O:MeOH (1:1 v/v, 1 mL) and analyzed by RP-HPLC and MS.

LCMS Analyses and Purification

Sample analyses were performed on an Agilent 1100 series ESI-MS with single quadrupole mass analyzer in positive mode. Analytical RP-HPLC was performed using a Waters 2695 Symmetry® C18 column (3.9 x 150 mm, 5 μm particle size) using a linear binary gradient, 2-80%

ACN/H_2O, 0.1% FA, over 25 min at 25°C, with a 1 mL/min flow rate and detection at 220 nm. Samples collected after purification were lyophilized to a white solid and re-dissolved in 50:50 v/v H_2O:ACN to confirm purity by LC and identity by molecular weight using MS analyses.

UV-Vis Spectroscopy

The concentrations of the peptide solutions were determined by UV/Vis spectrophotometry at 214 nm (ε value of peptides was calculated as described by Kuipers *et. al*. $\varepsilon_{214} = (\varepsilon_{peptidebond})(n_{peptidebonds}) + \Sigma\,(\varepsilon_{aminoacid(i)})(n_{aminoacid(i)})$.[24] The analyses were conducted on an 8452A Diode Array Spectrophotometer from Hewlett Packard, and concentrations were standardized accordingly for each peptide.

Flow Cytometry

For detection of cell surface expression levels of GRP78 or NKp30, the HepG2 cells or NK92-MI cells, respectively, were washed with FACS buffer (1X PBS, 1% BSA), suspended in APC-labeled human NKp30 or GRP78 mAb (0.5 μg/μL in FACS, SC1050 from R&D Systems), and incubated at room temperature for 15 minutes in the dark. Cells were then washed and analyzed by flow cytometry on a MACSQuant® analyzer (MiltenyilBiotec).

To determine direct binding of the bioconjugate and the synthetic tripeptide, **13**, HepG2 or NK92-MI cells were washed with FACS buffer and suspended with either bioconjugate or tripeptide (0.5 and 0.039 μg/μL in 1X PBS respectively) for 15 min (R.T., dark), washed and analyzed by flow cytometry.

For competitive blocking studies, HepG2 cells were initially treated with the unlabeled human anti-GRP78 mAb (0.5 μg/μL in FACS) for 15 min (R.T., dark). HepG2 cells were washed with FACS buffer, centrifuged and suspended with either the FITC-labeled tripeptide, **13,** or

bioconjugate (0.5 μg/μL in 1X PBS) for an additional 15 min (R.T., dark). Cells were finally washed with FACS buffer and analyzed by flow cytometry.

For competitive displacement studies, NK92-MI cells were initially suspended with either the FITC-labeled tripeptide, **13,** or bioconjugate (0.5 μg/μL in PBS) for 15 min (R.T., dark). NK cells were washed with FACS buffer, centrifuged and suspended with the APC-labeled human NKp30 mAb (0.25 μg/μL in FACS) for another 15 minutes (R.T., dark). Cells were then washed with FACS buffer and analyzed by flow cytometry.

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Supporting Information

Figure S1. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **1** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (center) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **1** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S2. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **2** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **2** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S3. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **3** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **3** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S4. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **4** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **4** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S5. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **5** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **5** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S6. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **5** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **5** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S7. Quantitation of tripeptide using UV-vis spectroscopy. Purified peptide **5** was lyophilized into a powder and solubilized in 60:40 ACN:H_2O for analysis. A wavelength scan from 190 to 700 nm was performed, determining the concentration of the peptide based on the absorbance of the peptide bond at 214 nm.

Figure S8. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **6** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **6** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S9. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **6** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **6** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S10. Quantitation of tripeptide using UV-vis spectroscopy. Purified peptide **6** was lyophilized into a powder and solubilized in 60:40 ACN:H_2O for analysis. A wavelength scan from 190 to 700 nm was performed, determining the concentration of the peptide based on the absorbance of the peptide bond at 214 nm.

Figure S11. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **7** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **7** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S12. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **7** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **7** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S13. Quantitation of tripeptide using UV-vis spectroscopy. Purified peptide **7** was lyophilized into a powder and solubilized in 60:40 ACN:H2O for analysis. A wavelength scan from 190 to 700 nm was performed, determining the concentration of the peptide based on the absorbance of the peptide bond at 214 nm.

Figure S14. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **8** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **8** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S15. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **8** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **8** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S16. Quantitation of tripeptide using UV-vis Spectroscopy. Purified peptide **8** was lyophilized into a powder and solubilized in 60:40 ACN:H_2O for analysis. A wavelength scan from 190 to 700 nm was performed, determining the concentration of the peptide based on the absorbance of the peptide bond at 214 nm.

Figure S17. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **9** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **9** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S18. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **9** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **9** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S19. Quantitation of tripeptide using UV-vis spectroscopy. Purified peptide **9** was lyophilized into a powder and solubilized in 60:40 ACN:H2O for analysis. A wavelength scan from 190 to 700 nm was performed, determining the concentration of the peptide based on the absorbance of the peptide bond at 214 nm.

Figure S20. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **10** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **10** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S21. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **10** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **10** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S22. Quantitation of tripeptide using UV-vis spectroscopy. Purified peptide **10** was lyophilized into a powder and solubilized in 60:40 ACN:H_2O for analysis. A wavelength scan from 190 to 700 nm was performed, determining the concentration of the peptide based on the absorbance of the peptide bond at 214 nm.

Figure S23. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **11** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **11** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S24. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **11** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **11** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S25. Quantitation of tripeptide using UV-vis Spectroscopy. Purified peptide **11** was lyophilized into a powder and solubilized in 60:40 ACN:H_2O for analysis. A wavelength scan from 190 to 700 nm was performed, determining the concentration of the peptide based on the absorbance of the peptide bond at 214 nm.

Figure S28. Crude peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of crude peptide **12** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **12** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S27. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **12** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **12** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide

Figure S28. Quantitation of tripeptide using UV-vis spectroscopy. Purified peptide **12** was lyophilized into a powder and solubilized in 60:40 ACN:H_2O for analysis. A wavelength scan from 190 to 700 nm was performed, determining the concentration of the peptide based on the absorbance of the peptide bond at 214 nm.

Figure S29. Purified peptide analysis by RP-HPLC and MS (A) Direct-injection mass spectra of pure peptide **13** in positive mode. The arrows indicate confirmed m/z of the observed mass. The structure depicted (top right) was created using ChemDraw software. (B) RP-HPLC chromatograph of peptide **13** using 2-80% ACN/H2O with 0.1% FA over 25 minutes. The arrow indicates the peak that was confirmed for the peptide