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Mix to Validate: A Facile, Reversible PEGylation for Fast Screening of Potential Therapeutic Proteins, TRAIL, *In Vivo*[**]

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Abstract

Mix to Validate: To advance the rate of novel protein therapies entering the clinic, we provide researchers a facile tool for protein drug efficacy testing in animal models in a high throughput manner. Here, we utilize the concept of PEGylating proteins through complementary interactions between His-tag and Ni^{2+} complex of NTA, a well-established practice in protein research, to improve blood half-life of therapeutic protein candidates after systemic administration *in vivo*.

Keywords

Histidine tag; *in vivo* drug screening; pegylation; protein delivery; protein modification

A number of novel proteins with high therapeutic potential are being discovered every year. Once a potent protein is verified, the next step towards clinical studies includes validation in an appropriate animal model. Unfortunately, most candidate protein drugs are inadequate for direct testing in a high-throughput fashion *in vivo* because of inherently short biological half-lives from non-specific proteolysis and renal clearance.[1] It is well-known that proteins with short half-lives do not exhibit similar potency *in vivo* as they do *in vitro*.[1b] For decades, PEGylation, the chemical attachment of poly(ethylene glycol) (PEG), has been considered the gold standard for enhancing stability, half-life, and aqueous solubility of protein drugs.[2] Especially, site-specific PEGylation improves protein stability *in vivo* while minimizing loss of activity associated with conventional random PEGylation.[3]

Here we introduce a facile technique that offers protein drug efficacy testing in animal models by extending the blood half-life of any selected protein candidate without compromising bioactivity. The platform delivers the benefits of site-specific PEGylation without time-consuming and costly chemical modification and purification processes, enabling high-throughput testing of protein drugs *in vivo*. The general concept is PEGylation of proteins via complementary interaction between the oligo-histidine tag (His-tag) and Ni^{2+} complex of nitrilotriacetic acid (NTA), which is now widely employed in protein research.[4] For example, protein immobilization techniques[5] and protein labelling with fluorophores[6] utilize the properties of His-tag to NTA.

In spite of numerous His-tag/NTA pair-based applications and a couple for protein therapeutic research,[7] no studies have successfully utilized this specific and strong interactive pair to improve the potency of therapeutic proteins *in vivo* after systemic administration. We hypothesize that PEG analogs with an NTA moiety could be selectively labelled at specific sites of His-tagged proteins by simple mixing and exhibit the benefits of site-specific PEGylated proteins. Then, *in vivo* efficacy of any protein candidate can be tested in a facile fashion. Here, with a rationally designed Ni-NTA-PEG analog and a biologically relevant His-tagged protein, we exemplify a practical technique for use *in vivo*.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) was chosen as a model protein drug. TRAIL selectively induces apoptosis in a variety of cancer cells by delivering apoptotic signals through binding to cancer cell death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), while providing negligible toxicity to non-malignant cells.[8] These unique features make TRAIL one of the most promising and versatile anticancer protein drugs. However, it suffer from inherent instability, requiring stabilizers at high concentrations (e.g. > 200 μg mL^{-1}), and a short half-life of approximately 3, 5 and 30 min

in mouse, rat and human, respectively.[9] Our previously reported covalently N-terminal PEGylated TRAIL analogs demonstrate superior pharmacokinetic (PK) and pharmacodynamic (PD) profiles.[10] Such analogs involve considerable time in synthesis and optimization to validate bioactivity *in vivo*. By applying the technique reported here, novel protein candidates such as TRAIL can be immediately applied to screen and/or validate *in vivo* therapeutic efficacy quickly. Selected proteins could be further optimized by established formulation techniques, e.g. PEGylation, for clinical translation.

The general design of the reactive Ni-NTA-PEG analogs, **1** and **4**, and its reaction process with a His-tag, hexahistidine (H_6), and fused protein are illustrated in Fig. 1. Binding affinities as the dissociation constant (K_D) between fluorophore-labelled mono-NTA or multivalent NTA analogs and H_6 have been reported.[6b] K_D of a monoNTA with H_6 remains weak at 10–18 μM, however bisNTA exhibits a significantly lower K_D at 0.27 μM towards an H_6 moiety. TrisNTA further lowered the K_D ten-fold compared to bis-NTA, but tetraNTA demonstrated no further improvement.[6b, 11] To avoid complicated synthetic processes and potential toxicity that may be induced by multiple Ni^{2+} ions, we designed PEG analogs having monoNTA **1** and bisNTA **4** and investigated their applications *in vitro* and *in vivo* compared with PEG without the NTA functionality. Compound **1** was synthesized by reacting methoxy PEG *N*-hydroxylsuccinimide ester (PEG-NHS) and NTA-Lys, N_α,N_α-bis(carboxymethyl)-L-lysine, followed by chelation with Ni^{2+} in $NiCl_2$ solution. Unlike other reported multivalent NTA analogs, **4** was synthesized using a combination of commercially available compounds including NTA-Lys, Traut's reagent (2-iminothiolane), bis-maleimide amine and PEG-NHS, without the need for harsh protection/deprotection schemes. Briefly, a sulfhydryl group was first introduced to NTA-Lys by incubating NTA-Lys with the amine-reactive Traut's reagent. Thiolated NTA-Lys, NTA-Lys-SH **2**, was then reacted with a bis-maleimide amine to result in bisNTA analog **3**. Compound **3** was finally conjugated with PEG-NHS and labelled with Ni^{2+} to produce **4**. A methoxy PEG with a molecular weight of 5 kDa was used as a backbone. Details of the synthesis and characterization of **1** and **4** are described in the Supporting Information.

The formation of interaction complexes between TRAIL and Ni-NTA-PEGs were confirmed by size exclusion chromatography (SEC). An active TRAIL including an N-terminal H_6 and trimer-forming zipper sequence, H_6-ILZ-hTRAIL (114 – 281) (m.w. 22 kDa), was purified and used as previously reported.[10a] As shown in Fig. 2A, TRAIL mixed with PEG without the NTA moiety, TRAIL/PEG, did not form any complexes. However, the addition of **1** and **4**, producing TRAIL/**1** and TRAIL/**4**, exhibited increasing hydrodynamic radii due to the interaction of TRAIL with the NTA appendage possessing high affinity towards H_6. TRAIL/**4** showed a similar SEC profile compared to that of covalently bound N-terminal PEGylated TRAIL-PEG_{5K} without free TRAIL at a feed molar ratio of TRAIL:**4** above 1:5. In contrast, TRAIL/**1** failed to reach complete complexation at any ratio. To explore a substantial difference in complexation profiles between TRAIL/**1** and TRAIL/**4**, the interaction kinetics of **1** and **4** with TRAIL was studied by measuring binding constants using BIAcore. **1** and **4** demonstrated K_D of 41.6 μM and 17.7 μM with TRAIL, respectively (Supporting Information Fig. S3). The results demonstrate that the incomplete complexation of TRAIL/1 is probably due to the lower K_D and induced steric hindrance of **1** in the buffer. After fixing the ratio at 1:5, the stability of each formula was investigated in 20 mM PBS, pH 7.4, at 37 °C, without any stabilizing agents such as Tween 20 and concentrated glycerol and sucrose.[1b] Native TRAIL and TRAIL/PEG at a concentration of 400 μg mL^{-1} (based on the protein concentration) showed rapid aggregation and precipitation, losing more than 70% of the protein in an hour (Fig. 2B) because of its low stability and solubility at physiological pH. In contrast, both **1** and **4** improved stability and reduced precipitation of TRAIL under the same conditions. More than 50% of TRAIL/**4** was found to be stable

twelve hours after incubation, however, it gradually lost stability. The bioactivity of each formula was examined based on tumor cell-specific cytotoxicity measured by MTT assays following incubation of TRAIL-based formulas (protein concentration from 10^{-1}–10^{4} ng mL^{-1}) in human colon cancer HCT116 cells (Fig. 2C and Table 1). TRAIL and TRAIL/PEG showed a marked apoptotic effect on HCT116 cells. Cytotoxicity of TRAIL associated with **1** and **4** was slightly decreased with increasing NTA affinities but retained 43.7±7.1 and 20.3±1.6% of bioactivity from native TRAIL. The observed IC_{50} value of TRAIL/**4** was similar to that of previously reported TRAIL-PEG_{5K}.[10a] To confirm TRAIL's tumor cell specificity, the same TRAIL formulas were treated in normal cells (fibroblast CCD-986sk) and showed no toxicity. In terms of cytotoxicity of Ni-NTA-PEGs, **1** and **4** were nontoxic both to normal fibroblasts CCD-986sk and HCT116 cells (Supporting Information, Fig. S4). Taken together, *in vitro* assays demonstrated that simple addition of **1** and **4** to TRAIL was able to provide extended stability in solution and reduced aggregation, while salvaging the bioactivity of TRAIL.

After validation of TRAIL/Ni-NTA-PEGs *in vitro*, we analysed the PK of TRAIL/**1**, /**4** and TRAIL-PEG_{5K}, TRAIL covalently conjugated with the same molecular weight PEG, in rats after intravenous (IV) injection. Total active TRAIL plasma levels were measured by enzyme-linked immunosorbent assay (ELISA). All PK parameters are summarized in Table 1 and illustrated in Fig. 3A and Fig. S5(Supporting Information). It has been reported that TRAIL has a short half-life of 5–10 min in rat, mainly through rapid renal clearance.[9a] In accordance with reported values, intravenously injected TRAIL and TRAIL/PEG were rapidly eliminated from rats within 1 h (Fig. 3A). In contrast, TRAIL/**4** showed a prolonged elimination half-life and maintained activity up to 6 hours post-injection. Furthermore, bioavailability of TRAIL/**4**, as determined by the area under the curve (AUC) analysis, was enhanced by 3.8 ± 0.6 and 2.1±0.3-fold compared to that of native TRAIL and TRAIL/**1**, respectively (Fig. 3B). Since **4** was able to provide significantly extended stability in solution and improved PK parameters of TRAIL both *in vitro* and *in vivo* compared to **1, 4** was chosen for further PD testing in HCT116-tumor bearing mice. To demonstrate the utility of **4** for *in vivo* applications, the antitumor effect was investigated in tumor models by continually monitoring tumor volumes while treating mice with TRAIL every 5 days. As shown in Fig. 3C, all formulas, TRAIL, TRAIL/PEG and TRAIL/**4**, suppressed tumor growth. However, mean tumor growth with tumor growth inhibition (TGI) values were only maintained by TRAIL/**4** throughout the study period (at day 20, for TRAIL, TRAIL/PEG and TRAIL/**4**; 15.1 ± 7.6, 19.2 ± 5.3 and 51.8 ± 7.1%, respectively), and tumor size rebound was observed in all other formulas. TGI value was calculated using the formula [1-(T C^{-1}) ×100%], where T and C are the tumor size of drug treated and control groups, respectively. At the end of the study tumor tissues were harvested and apoptotic cells in tumor sections were visualized by TdT-mediated dUTP nick end labelling (TUNEL) assays (Fig. 3D). As expected, TRAIL/**4** treated tissues demonstrated increased tumor cell apoptosis compared to those of native TRAIL and the other formulas. At any injected dose of native TRAIL (50 to 1,000 μg per mouse), a 50% of TGI was not achieved under our experimental conditions. Since the major human adverse event related to high PEG exposure is renal toxicity,[1a] acute renal toxicity of **4** was examined by histological investigation of renal tissues after PD studies. No sign of toxicity was observed for any formula (Fig. 3D). The experimental results consistently exemplify that an appropriate Ni-NTA-PEG molecule can significantly and positively affect the physicochemical properties of His-tagged proteins *in vitro* and *in vivo* while maintaining bioactivity. Collectively, TRAIL/**4** demonstrated 3- to 4-fold improved efficacy over native TRAIL in terms of solution stability, *in vivo* half-life and bioavailability. This is a noteworthy effect but still not as effective as our previously reported TRAIL-PEG_{5K}, which retained more than 80% stability in physiological buffer for 24 hours and showed a 10-fold increase in bioavailability compared to TRAIL alone after

intraperitoneal (IP).[10a] Surprisingly, however, based on the TGI value, TRAIL/**4** demonstrated similar antitumor efficacy to TRAIL-PEG_{5K} in the same tumor model. This is probably because of the different *in vivo* bioactivities between TRAIL-PEG_{5K} and TRAIL/**4**; TRAIL-PEG has reduced activity (50% vs. TRAIL) for all time points, whereas TRAIL/**4** can fully recover its bioactivity once Ni-NTA-PEG is released from TRAIL in the blood. Since the administration route (IP vs. IV) and dosing profiles are different, the results cannot be directly compared. Note that our platform offers all of the benefits that site-specific PEGylation can provide without any further chemical modification and purification processes. Once **4** is added to protein in solution, the protein can be highly concentrated and freeze-dried. Since an excess amount of **4** does not interfere with the bioactivity and PK of the protein, one can easily add our analogs just as commercial stabilizers. Moreover, the observed pseudo-PEGylation effect can be easily achieved by simple incubation with total preparation time totalling less than 30 min.

We initiated this project with the aim of introducing a facile and versatile technique that can increase *in vivo* stability of any protein while maintaining that protein's bioactivity. Using TRAIL as a model protein, we demonstrated that a unique Ni-NTA-PEG analog associated with His-tagged protein is able to provide outstanding physicochemical stability without compromising bioactivity. Importantly, the Ni-NTA-PEG analog maximized the pharmacological efficacy of the protein drug *in vivo*. Significant efforts are under way to develop novel biologics with improved efficacy and reduced dosing profiles and toxicity compared to protein drugs. This current platform can contribute to the early development of biologics by reducing the cost of drug screening and streamlining evaluation in animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. a) Krejsa C, Rogge M, Sadee W. Nat. Rev. Drug Discov. 2006; 5:507–521. [PubMed: 16763661] b) Frokjaer S, Otzen DE. Nat. Rev. Drug Discov. 2005; 4:298–306. [PubMed: 15803194]
2. a) Harris JM, Chess RB. Nat. Rev. Drug Discov. 2003; 2:214–221. [PubMed: 12612647] b) Obermeier B B, Wurm F, Mangold C, Frey H. Angew. Chem. 2011; 50:7988–7997. [PubMed: 21751305]
3. a) Kang JS, Deluca PP, Lee KC. Expert Opin. Emerg. Drugs. 2009; 14:363–380. [PubMed: 19453284] b) Knop K, Hoogenboom R, Fischer D, Schubert US. Angew. Chem. 2010; 49:6288–6308. [PubMed: 20648499] c) Pasut G, Veronese FM. J. Control Release. 2012; 161:461–472. [PubMed: 22094104] c) Cong Y, Pawlisz E, Bryant P, Balan S, Laurine E, Tommasi R, Singh R, Dubey S, Peciak K, Bird M, Sivasankar A, Swierkosz J, Muroni M, Heidelberger S, Farys M, Khayrzad F, Edwards J, Badescu G, Hodgson I, Heise C, Somavarapu S, Liddell J, Powell K, Zloh M, Choi JW, Godwin A, Brocchini S. Bioconjg. Chem. 2012; 23:248–226.
4. a) Crowe J, Dobeli H, Gentz R, Hochuli E, Stuber D, Henco K. Methods Mol. Biol. 1994; 31:371–387. [PubMed: 7921034] b) Terpe K. Appl. Microbiol. Biotechnol. 2003; 60:523–533. [PubMed: 12536251]
5. a) Zhen G, Falconnet D, Kuennemann E, Vörös J, Spencer ND, Textor M, Zürcher S. Adv. Funct. Mater. 2006; 16:243–251.b) Bruckman MA, Soto CM, McDowell H, Liu JL, Ratna BR, Korpany KV, Zahr OK, Blum AS. ACS Nano. 2011; 5:1606–1616. [PubMed: 21361370] c) Ludden MJ, Mulder A, Tampe R, Reinhoudt DN, Huskens J. Angew. Chem. 2007; 46:4104–4107. [PubMed: 17443757] d) Roullier V, Clarke S, You C, Pinaud F, Gouzer GG, Schaible D, Marchi-Artzner V, Piehler J, Dahan M. Nano Lett. 2009; 9:1228–1234. [PubMed: 19216518]
6. a) Guignet EG, Hovius R, Vogel H. Nat. Biotechnol. 2004; 22:440–444. [PubMed: 15034592] b) Lata S, Gavutis M, Tampe R, Piehler J. J. Am. Chem. Soc. 2006; 128:2365–2372. [PubMed:

16478192] c) Goldsmith CR, Jaworski J, Sheng M, Lippard SJ. J. Am. Chem. Soc. 2006; 128:418–419. [PubMed: 16402822]

7. Mero A, Ishino T, Chaiken I, Veronese FM, Pasut G. Pharm. Res. 2011; 28:2412–2421. [PubMed: 21611874]
8. a) Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. Nat. Med. 1999; 5:157–163. [PubMed: 9930862] b) Ashkenazi A. Nat. Rev. Cancer. 2002; 2:420–430. [PubMed: 12189384] c) Voelkel-Johnson C. Nat. Rev. Urol. 2011; 8:417–427. [PubMed: 21670755]
9. a) Kelley SK, Harris LA, Xie D, Deforge L, Totpal K, Bussiere J, Fox JA. J. Pharmacol. Exp. Ther. 2001; 299:31–38. [PubMed: 11561060] b) Ashkenazi A, Holland P, Eckhardt SG. J. Clin. Oncol. 2008; 26:3621–3630. [PubMed: 18640940]
10. a) Chae SY, Kim TH, Park K, Jin CH, Son S, Lee S, Youn YS, Kim K, Jo DG, Kwon IC, Chen X, Lee KC. Mol. Cancer Ther. 2010; 9:1719–1729. [PubMed: 20515949] b) Kim TH, Youn YS, Jiang HH, Lee S, Chen X, Lee KC. Bioconjug. Chem. 2011; 22:1631–1637. [PubMed: 21751817] c) Jiang HH, Kim TH, Lee S, Chen X, Youn YS, Lee KC. Biomaterials. 2011; 32:8529–8537. [PubMed: 21855130]
11. Lata S, Reichel A, Brock R, Tampe R, Piehler J. J. Am. Chem. Soc. 2005; 127:10205–10215. [PubMed: 16028931]

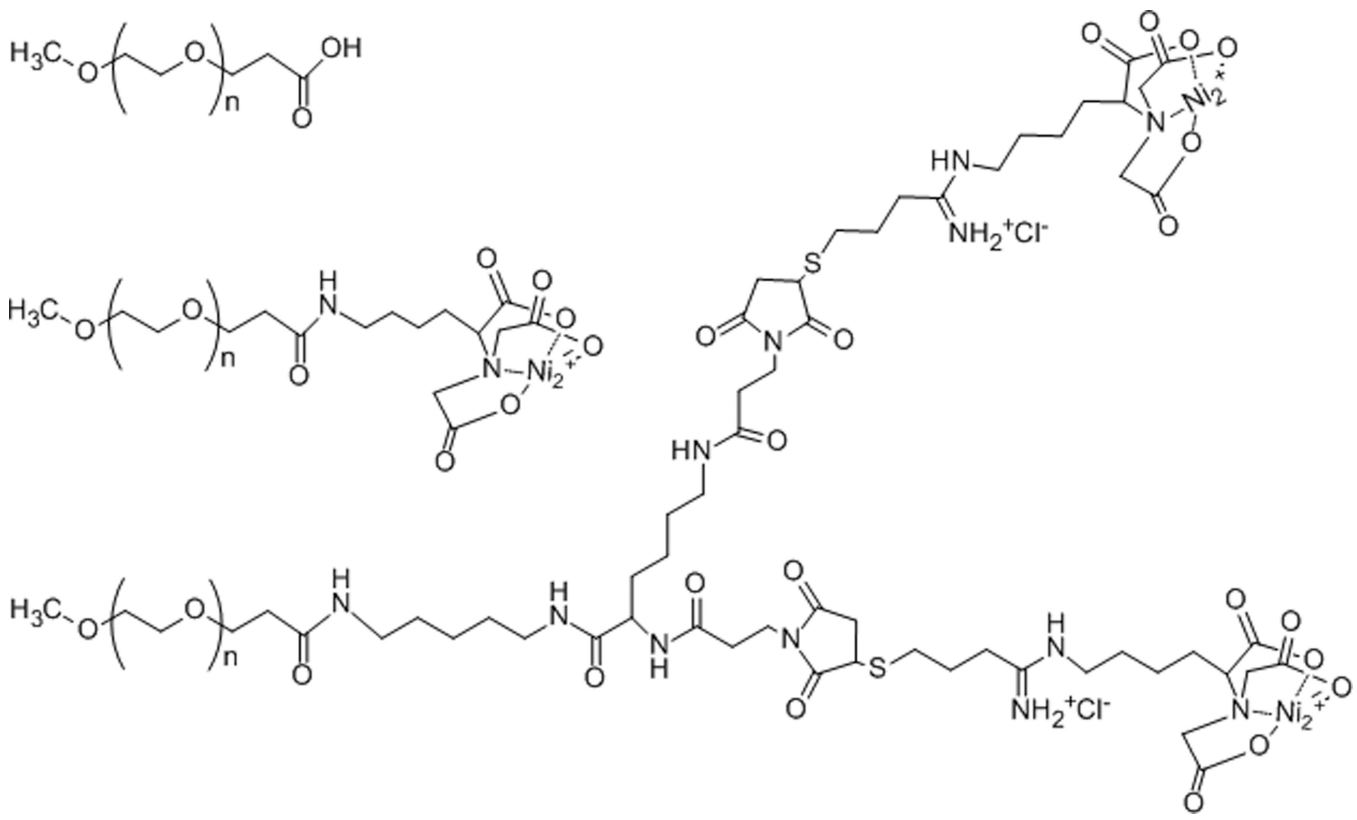

Figure 1.

Left: Site-specific pseudo-PEGylation of His-tag fused protein. Schematic illustration of PEGylating His-tagged protein with the reactive Ni-NTA-PEG such as **1** or **4**. Right: Chemical structures of methoxy PEG (PEG, m.w. 5 kDa) and Ni-NTA-PEG analogs, Ni-monoNTA-PEG **1** and Ni-bisNTA-PEG **4**. Synthetic details are described in the Supporting Information.

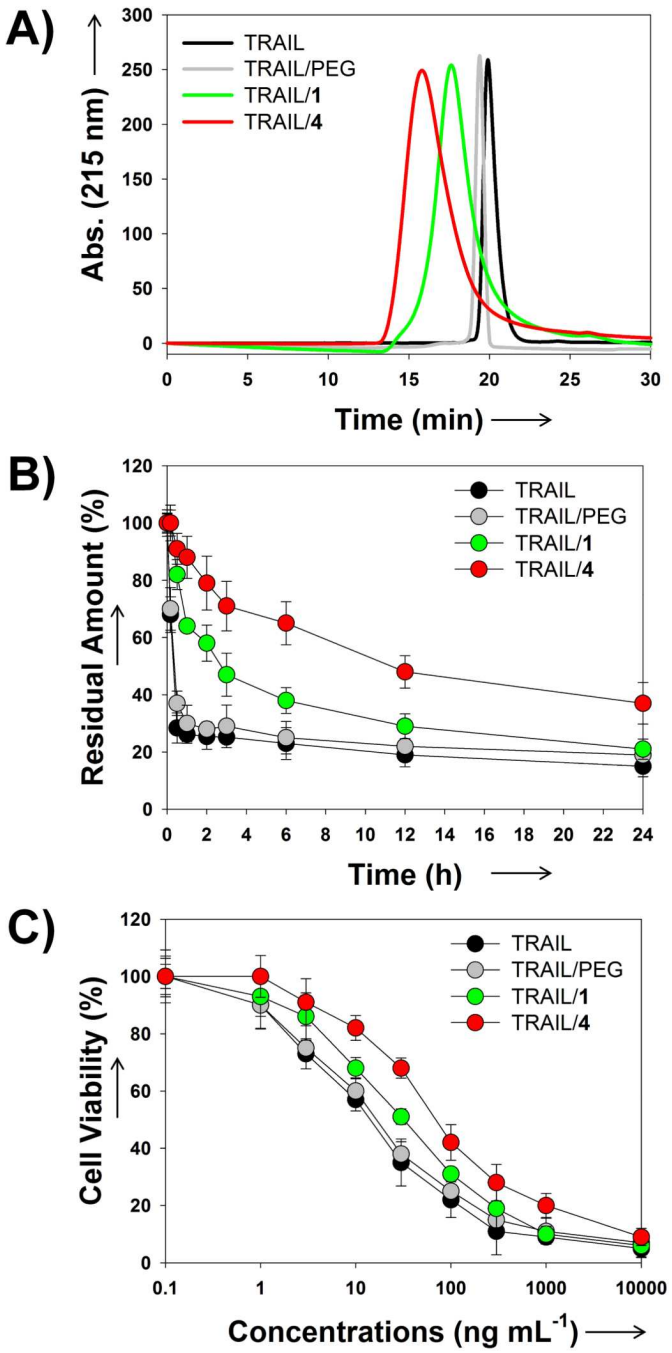

Figure 2.

The effects of Ni-NTA-PEGs on the stability and bioactivity of TRAIL. A) Size exclusion chromatography spectra of TRAIL (100 μg mL^{-1}) and TRAIL associated with PEG, **1** and **4** at a TRAIL:PEG molar ratio of 1:5 in 20 mM acetate buffer, pH 6.0. B) Time-dependent stability of TRAIL (400 μg mL^{-1}) and its mixtures relative to the stability at time 0 in 20 mM PBS, pH 7.4, at 37°C. C) *In vitro* biological activity of TRAIL (10^{-1}–10^{4} ng mL^{-1}) and its mixtures on HCT 116 cells. Cytotoxicities of formulas were determined by performing MTT assay after incubation for 24 hours. Graph represents mean ± s.d. (n=4).

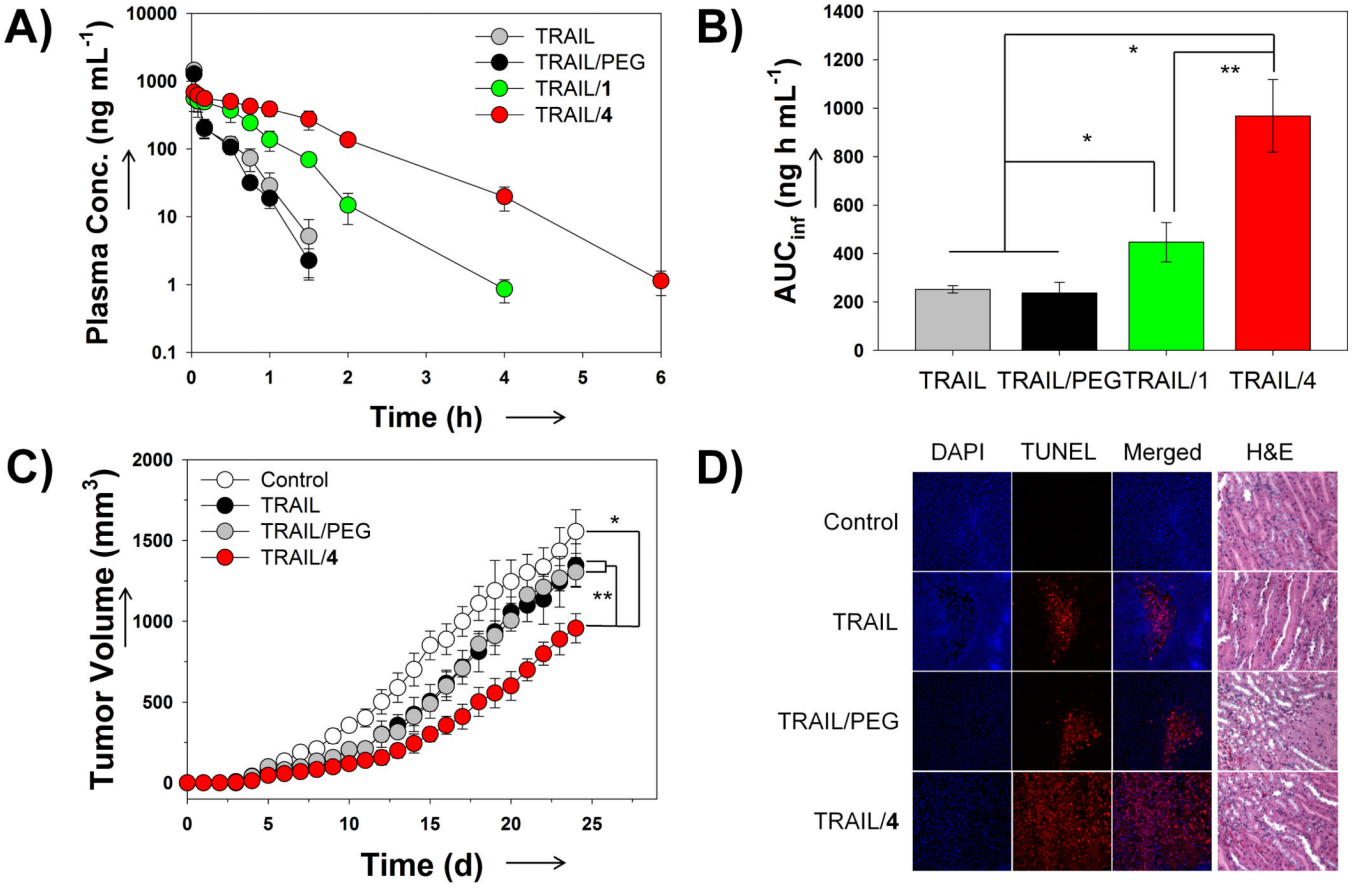

Figure 3.

The effects of Ni-NTA-PEGs on the pharmacological efficacy of TRAIL. A) PK profiles of TRAIL, TRAIL/PEG and TRAIL with Ni-NTA-PEG analogs. Cannulated Sprague-Dawley rats were adminstrered an IV injection of TRAILs (100 μg kg^{-1}, based on the TRAIL conc.) and plasma concentrations were monitored by ELISA assay (n=4). B) Area under the curve from zero to infinity (AUC) values derived from the PK analysis. *$p < 0.001$ v.s. TRAIL alone and TRAIL/PEG, **$p < 0.001$ v.s. TRAIL/1 C) Antitumor activity of TRAIL formulations in HCT116 human colon cancer-bearing mice. Tumor growth suppression was monitored while treating mice with TRAILs (150 μg per mouse, based on the TRAIL conc.) by IV injection every 2 days starting at 5 days after tumor inoculation (n=6). *$p < 0.001$ v.s. Control, **$p < 0.001$ v.s TRAIL alone and TRAIL/PEG. D) Left: TUNEL staining of apoptotic cell death in tumors from mice treated with TRAILs (nucleus stained with DAPI, blue; apoptotic cells, red) and right: histological images of kidney kindey stained using hematoxulin and eosin (H&E). Graphs represent mean ± s.d.

Table 1

Pharmacokinetic parameters

	TRAIL	TRAIL/PEG	TRAIL/1	TRAIL/4	TRAIL-PEG[d]
IC_{50}[a]	14.1±4.2	16.7±3.9	32.8±5.3*	69.8±5.6*	66.7±3.9
CL[b]	1.17±0.21	1.12±0.12	0.84±0.08*	0.61±0.05*	0.1±0.01
$t_{1/2}$[c]	11.4±3.1	11.5±2.2	24.1±1.3*	43.3±3.7*	337.3±16.5

[a] the half maximal inhibitory concentration, (ng mL^{-1})

[b] clearance, (mL min^{-1})

[c] elimination half-life, (min).

* $P < 0.001$ v.s. TRAIL alone and TRAIL/PEG.

[d] TRAIL covalently conjugated with PEG_{5K}.

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