molecular pharmaceutics

Brief Article

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Balancing the Pharmacokinetics and Pharmacodynamics of Interferon-α2b and Human Serum Albumin Fusion Protein by Proteolytic or Reductive Cleavage Increases Its *in Vivo* Therapeutic Efficacy

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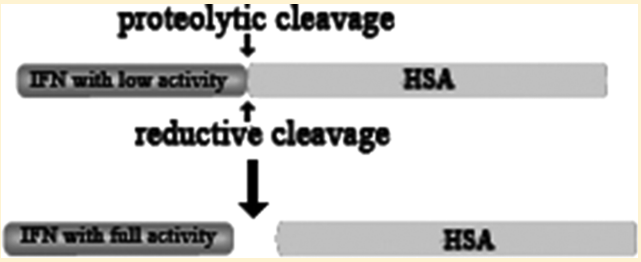
ABSTRACT: Human serum albumin (HSA) fusion (Albufusion) technology has evolved to be a general strategy to increase the *in vivo* half-lives of therapeutic proteins. However, because of the steric hindrance effect of HSA, conventional Albufusion technology improves the pharmacokinetics (PK) at the cost of pharmacodynamics (PD). To achieve balanced PK and PD of interferon-α2b (IFN-α2b) and HSA fusion protein, protease cleavage sites or disulfide linkage that enabled releasing of intact IFN-α2b with full activity was introduced between these two moieties. Nonreleasable and releasable fusion proteins showed similar cell surface receptor binding affinities; however, releasable fusion proteins exhibited release efficiency proportional increase of *in vitro* antiviral and antiproliferative activities. The release rate also had a profound impact on the *in vivo* pharmaceutical properties of fusion proteins. Releasable fusion proteins with intermediate release rate had the most balanced PK and PD, which translated into improved therapeutic efficacy in the HT29 human colon cancer xenograft model. Releasable Albufusion (rAlbufusion) allows tailored design of the PK/PD profile and greatly extends the utility of conventional Albufusion technology.



KEYWORDS: *human serum albumin fusion, long acting interferon, drug delivery*

INTRODUCTION

Interferons (IFNs) are a class of cytokines possessing antiviral, antiproliferative and immunomodulatory effects. Recombinant human interferon-α (IFN-α) has undergone extensive clinical investigations and has been widely used for both viral and oncological indications.[1] However, the short circulating half-life of unmodified IFN-α (4 to 8 h) necessitates frequent dosing (daily or three times weekly) over an extended period (6−12 months or more), which leads to increased frequency and severity of adverse events and decreased patient compliance.[2]

Pegylation, the covalent attachment of one or more polyethylene glycol (PEG) moieties to a biological molecule, is a well-established technology to improve the pharmacokinetics of therapeutic proteins.[3] Currently, two versions of pegylated IFN-α are commercially available. PEG Intron (Schering Plough), with a linear 12 kDa PEG molecule, has a half-life of 35 h. PEGASYS (Roche), with a branched 40 kDa PEG molecule, has a half-life of 77 h. The use of PEG-IFN-α allows the interval between doses to be extended to 1 week, a feature that resulted in improved adherence of patients.[4] However, while the attachment of PEG chains to proteins prolongs their *in vivo* half-lives, it often results in a dramatic reduction of *in vitro* biological activities. For example, depending on the molecular size of PEG chains, pegylated IFN-α retained 7% or 28% activity of native IFN-α.[5,6]

Albufusion, genetic fusion of therapeutic proteins to long-lived human serum albumin (HSA), is an alternative strategy to improve the pharmacokinetics of therapeutic proteins.[7] Clinical studies showed that IFN-α2b and HSA fusion protein had a much longer half-life (144 h) than its pegylated counterparts, which enabled treatment at 2 or 4 week intervals.[8] However, because of the bulky size of HSA, the decrease of *in vitro* activity was also more profound. IFN-α2b and HSA fusion protein possesses only about 1% of the *in vitro* bioactivity of wild type IFN-α.[9,10]

The longer half-life of IFN-α2b and HSA fusion protein originally raised high hopes that it would have improved efficacy.[11] However, two recently released large clinical trials showed that IFN-α2b and HSA fusion protein only had noninferior efficacy in patients with chronic hepatitis C virus when compared to pegylated IFN-α.[12,13] The inability of IFN-α2b and HSA fusion protein to show superior efficacy indicated that increased pharmacokinetics (PK) will not always compensate for decreased pharmacodynamics (PD). Thus, the clinical trial results questioned the validity of the rationale

Received: July 10, 2011
Revised: December 14, 2011
Accepted: January 7, 2012
Published: January 7, 2012

dx.doi.org/10.1021/mp200347q | *Mol. Pharmaceutics* 2012, 9, 664−670

Table 1. List of Primers Used for PCR

primer	sequence (5′-3′)
F	CGGGATCCAAACGATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCC
R	TTGAATTCTTACAAACCCAAAGCAGCTTGAGAAGC
RRF	TTGCAAGAGAGATTGAGAAGAAAGGAGGACGCTCACAAGTCTGAAGTTGCTCAC
RRR	GTGAGCAACTTCAGACTTGTGAGCGTCCTCCTTTCTTCTCAATCTCTCTTGCAA
RKRRF	TCCTTGTCCACCAACAGACAAAAGAGATTGAGAAGAAAG
RKRRR	CTTTCTTCTCAATCTCTTTTGTCTGTTGGTGGACAAGGA
SSF	TGTAGAAGAAGAAGAAGAAGAGAGGCTGAGGCTTGTGACGCTCACAAGTCTGAAGTTGCT
SSR	ACAAGCCTCAGCCTCTCTTCTTCTTCTTCTTCTACACTCCTTGGATCTCAAGGACTCTTG

for conventional long acting therapeutic protein development, in which the improvements of PK come at the expense of PD.

Recent research of Pegylation has explored a new approach to address the PK/PD dilemma. By designing a PEG and therapeutic protein conjugate capable of generating native protein at an appropriate rate under physiological conditions, balanced PK/PD profile that translated into improved efficacy can be achieved.[14−17] Releasable Pegylation (rPegylation) allows tailored design of critical pharmaceutical parameters and greatly extends the utility of conventional Pegylation.[18]

In order to address the PK/PD dilemma facing conventional Albufusion technology, we proposed a novel strategy termed as releasable Albufusion (rAlbufusion), in which native IFN-α was generated from fusion protein by either proteolytic or reductive cleavage. The pharmacokinetics, pharmacodynamics and *in vivo* therapeutic efficacy of IFN-α2b and HSA fusion proteins with different release mechanism and rate were compared in detail.

EXPERIMENTAL SECTION

Construction, Expression and Purification of Releasable IFN-α2b and HSA Fusion Proteins. The gene encoding nonreleasable IFN-α2b and HSA fusion protein (IFN-HSA) was constructed as previously described.[19] The genes encoding releasable IFN-α2b and HSA fusion proteins were amplified using a two-step PCR strategy with primers listed in Table 1.

IFN-α2b and HSA fusion proteins were secreted from *Pichia pastoris* and purified to homogeneity by a process involving a four-step (cation exchange chromatography, hydrophobic interaction chromatography, anion exchange chromatography and gel filtration chromatography) column purification procedure as described previously.[19]

Cell Surface Receptor Binding Assay. The binding affinities of fusion proteins toward interferon receptor highly expressing human amnion WISH cells were determined by cell ELISA. Briefly, fusion proteins were labeled with biotin using EZ-Link Sulfo-NHS-LC-Biotinylation kit (Thermo Scientific). WISH cells were plated in a 96-well plate at 10,000 cells/well. The plates were incubated overnight at 37 °C in 5% CO_2. Different concentrations of biotinylated proteins were incubated with cells at 4 °C for 4 h. The cells were then fixed and quenched using Pierce Colorimetric In-Cell ELISA kit (Thermo Scientific). Finally, the concentrations of biotinylated fusion proteins were determined by HRP−streptavidin conjugate (Invitrogen).

Antiviral and Antiproliferative Activity Assay. Antiviral activities of fusion proteins were determined by their capacity to protect human amnion WISH cells against vesicular stomatitis virus (VSV)-induced cytopathic effects. WISH cells (4.5×10^5 cells/well) were seeded in a 96-well plate (100 μL/well) and grown to 95% confluence. Serial dilutions of fusion proteins were added to the wells. After 24 h of incubation, optimal concentrations of the viruses were added. After an additional 24 h incubation, WISH cell viability was determined by MTT assay. Antiviral activity, expressed as EC_{50}, was calculated by Prism software (version 3.0; GraphPad Software Inc., San Diego, CA).

For antiproliferative activity assay, Daudi cells were plated at 2.0×10^4 cells/well in 96-well plates and incubated at 37 °C for 4 days in the presence of different concentrations of fusion proteins. Viable cell densities were determined by MTT assay. Dose−response curves were generated, and EC_{50} values were calculated using Prism software.

***In Vitro* Release of Fusion Proteins by Proteases or Reducing Agents.** Fusion proteins were incubated with factor Xa (New England Biolabs), thrombin (Merck), furin (New England Biolabs) or physiological reductants (cysteine, reduced glutathione) at 37 °C for different periods of time. The cleavage of fusion protein was detected by nonreduced SDS−PAGE.

Serum Stability of Fusion Proteins. An equal volume of different fusion proteins was mixed with mouse serum. After incubation at 37 °C for 12 h, the released IFN were detected by Western blotting using a mouse monoclonal antibody against recombinant human interferon α2 (Santa Cruz Biotechnology, Inc.).

Pharmacokinetics and Pharmacodynamics. Female Balb/c mice (18−20 g) from Experimental Animal Center of Beijing Medical Institute were used for pharmacokinetics and pharmacodynamics studies. Fusion proteins were administered as a single subcutaneous injection at 50 μg/mouse in equal volumes (100 μL), with 4 animals in each group. Mice were bled via the retro-orbital method at various time points (6, 12, 24, 36, 48, 72 h post injection). The blood was allowed to clot and centrifuged and the serum removed for storage at −70 °C until analysis. Concentrations of IFN-α2b in the serum samples were determined using a human interferon alpha ELISA kit (PBL Interferon Source) following the manufacturer's instructions. Since the activity of interferon is highly species specific and human interferon is largely inactive in rodents, for pharmacodynamics analysis, the IFN-α2b activity in serum was quantified by using an antiproliferation assay against Daudi cells.

***In Vivo* Antitumor Activity.** Female Balb/c nude mice (4−6 weeks old) from Experimental Animal Center of Beijing Medical Institute were used for *in vivo* antitumor activity evaluation. Mice were inoculated subcutaneously with 1.0×10^6 HT29 cells on day 0. Treatments were administered by subcutaneous injection of 100 μg of fusion proteins (in 200 μL) on days 1 and 5 (n = 5 for each group). Saline was used as a control treatment. Animals were monitored for tumor growth by caliper measurement of the shortest diameter and the longest perpendicular diameter. Tumor volume was calculated

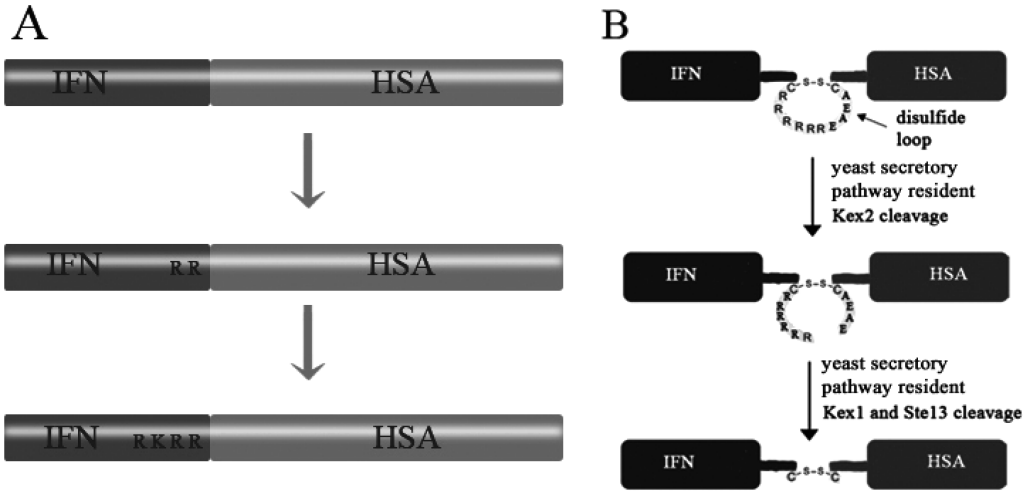


Figure 1. Design of releasable IFN-α2b and HSA fusion proteins. (A) Protease cleavable fusion proteins were constructed by introduction of basic amino acid residues at the C-terminus of IFN-α2b. (B) Reduction cleavable fusion protein was constructed by linking IFN-α2b and HSA by disulfide linker.

according to the formula (short diameter)^2 × (long diameter) × 0.4.

RESULTS

Design of Releasable IFN-α2b and HSA Fusion Proteins. Two strategies are available for release of IFN-α2b from fusion proteins: proteolytic or reductive cleavage.[20−23] Since IFN-α2b and HSA fusion protein mainly resides in the circulatory system, and many serum proteases (such as members of coagulation system) can cleavage behind basic amino acid residues (R or K), we chose to introduce extra basic amino acid residues at the C-terminus of IFN-α2b to render the fusion proteins susceptible to proteolytic cleavage.[24] To avoid the unwanted immunogenicity, we took advantage of the natural diversity of the human IFN-α family, and the basic amino acid residues were introduced at positions where such amino acids were occupied by some members of IFN-α family. For simplicity, IFN-α2b and HSA fusion protein with 2 extra basic amino acid residues (RR) at positions 160 and 163 of IFN-α2b was designated as IFN-RR-HSA; IFN-α2b and HSA fusion protein with 4 extra basic amino acid residues (RKRR) at positions 157, 159, 160, and 163 of IFN-α2b was designated as IFN-RKRR-HSA (Figure 1A).

Recently, an elegant strategy to produce disulfide-linked protein by a recombinant method has been published. In this strategy, different moieties of fusion protein were first linked by a thrombin-sensitive sequence containing disulfide loop and an intramolecular disulfide bond. After expression of the fusion protein, *in vitro* thrombin treatment generates fusion protein linked by disulfide bond.[25] We exploited the secretion signal processing proteases resident in the yeast secretory pathway, and by redesigning the amino acid sequence flanked by the cysteines, disulfide-linked IFN-α2b and HSA fusion protein was directly secreted from *Pichia pastoris*. IFN-α2b and HSA fusion protein linked by disulfide was designated as IFN-SS-HSA (Figure 1B).

Expression and Purification of IFN-α2b and HSA Fusion Proteins. Both the nonreleasable and releasable fusion proteins were efficiently secreted from *Pichia pastoris* (Figure 2A) and can be purified to >90% purity by the same purification process (Figure 2B). The final yields of IFN-

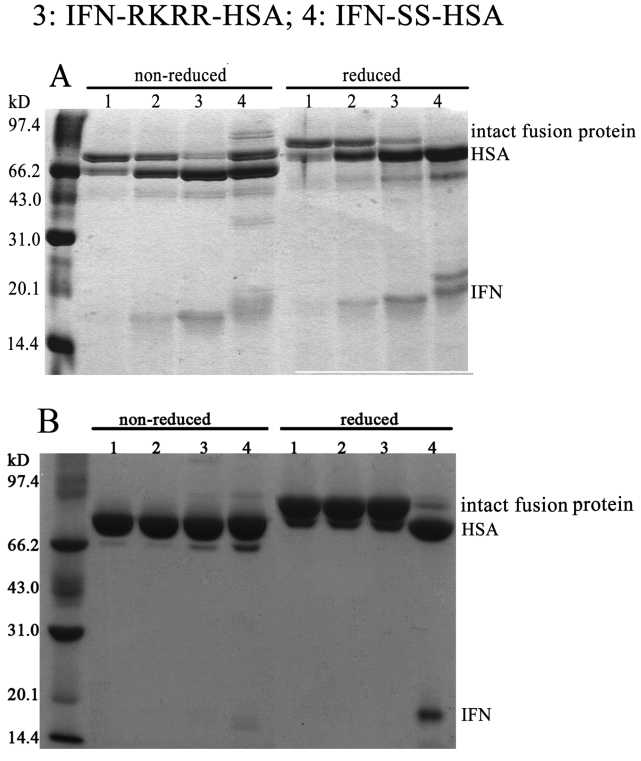


Figure 2. Expression and purification of IFN-α2b and HSA fusion proteins. (A) 10 μL samples of *Pichia pastoris* culture supernatants were analyzed by nonreduced and reduced SDS−PAGE. (B) 5 μg samples of purified fusion proteins were analyzed by nonreduced and reduced SDS−PAGE.

HSA, IFN-RR-HSA, IFN-RKRR-HSA and IFN-SS-HSA were about 200 mg/L, 150 mg/L, 80 mg/L and 120 mg/L, respectively. The susceptibility to proteolytic cleavage was in proportion to the number of introduced basic amino acid residues (Figure 2A, lanes 1, 2, 3), which indicated that the engineered cleavage sites were recognized by the proteases resident in the *Pichia pastoris* secretory pathway. The comparison of band pattern on reduced and nonreduced

dx.doi.org/10.1021/mp200347q | *Mol. Pharmaceutics* 2012, 9, 664−670

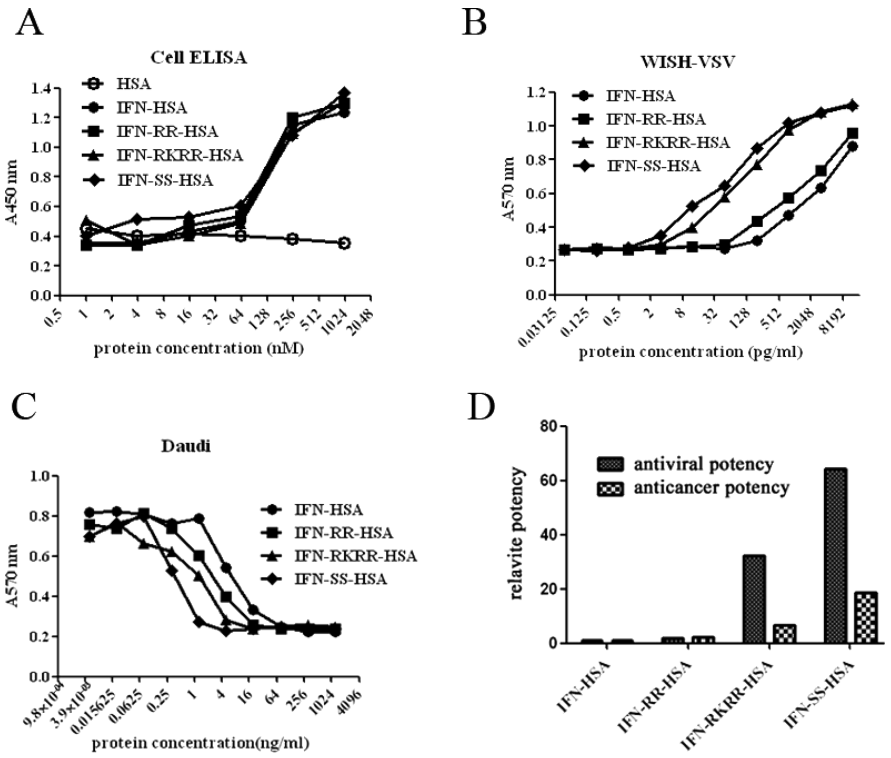


Figure 3. Cell surface receptor binding and biological activity assay of IFN-α2b and HSA fusion proteins. (A) The binding affinities of fusion proteins toward interferon receptor highly expressing human amnion WISH cells were determined by Cell ELISA. (B) Antiviral activities of fusion proteins were determined by their capacity to protect human amnion WISH cells against vesicular stomatitis virus (VSV)-induced cytopathic effects. (C) Anticaner activities of fusion proteins were determined by their capacity to inhibit the proliferation of Daudi cells. (D) Relative potency of fusion proteins. The antiviral and antiproliferative potency of nonreleasable IFN-HSA were arbitrarily defined as 1.

SDS−PAGE indicates that IFN-HSA, IFN-RR-HSA, and IFN-RKRR-HSA were single-chain molecular. On the contrary, IFN-SS-HSA was converted into two bands upon reduction. The low molecular weight band migrated at the position of IFN-α2b (about 20 kDa), indicating efficient cleavage at the disulfide loop during its passing through the secretory pathway (Figure 2B, lane 4).

Cell Surface Receptor Binding and Biological Activity Assay of IFN-α2b and HSA Fusion Proteins. As is shown in Figure 3A, all fusion proteins demonstrated very close affinity toward WISH cells (with a K_d of about 120 nM), indicating similar extent of structural perturbation by HSA. Depending on the activity assay systems, the releasable fusion proteins showed 1.84−64.4-fold increased antiviral and antiproliferative activities over nonreleasable fusion protein (Figure 3B−D). The discrepancy between the cell surface receptor binding and *in vitro* biological activity assay was assumed to be caused by different experimental conditions that disadvantaged and enabled release of IFN-α2b from fusion proteins, respectively. The different magnitude of increase of activity reflected the varied release efficiency and suggested a ranking following the order IFN-SS-HSA > IFN-RKRR-HSA > IFN-RR-HSA.

***In Vitro* Release of IFN-α2b and HSA Fusion Proteins.** *In vitro* protease cleavage assay showed that both IFN-RKRR-HSA and IFN-RR-HSA could be cleaved by factor Xa, with a half-life of several hours and days, respectively. On the contrary, thrombin, another member of the coagulation system, and furin, a ubiquitously expressed protease, act on dibasic amino acid sites could not cleave IFN-RKRR-HSA and IFN-RR-HSA (Figure 4A).

IFN-SS-HSA was relatively stable at physiological concentrations of reductants (10 μM), with a half-life of more than 50 h. One or even 2 order of magnitude higher concentrations were required for efficient reductive cleavage (Figure 4B). The stability of IFN-SS-HSA revealed by *in vitro* release assay was seemingly contradictory to the results of *in vitro* activity assay. The discrepancy might be explained by the presence of redox enzymes such as protein disulfide isomerase (PDI) in the serum or on the cell surface.[23]

Consistent with the protease and reducing agent cleavage assay, while nonreleasable fusion protein exhibited high stability in mouse serum, IFN-α2b was readily generated from the releasable fusion proteins after incubation in mouse serum (Figure 4C).

The release of IFN-α2b was associated with a marked increase of biological activity, indicating the generation of authentic IFN-α2b with full activity (Figure 4D).

Pharmacokinetics and Pharmacodynamics. The pharmcokinetical properties of releasable IFN-α2b and HSA fusion proteins, in terms of area under the curve (AUC), were generally lower than those of nonreleasable fusion protein (Figure 5A). The decrease of PK was in proportion to their release efficiency. IFN-RR-HSA, IFN-RKRR-HSA and IFN-SS-HSA retained 92%, 80% and 18% pharmacokinetics of the nonreleasable IFN-HSA, respectively (Figure 5C). The relationship between pharmacodynamics and release rate was not so straightforward (Figure 5B). Fusion protein with intermediate release rate (IFN-RKRR-HSA) exhibited the maximum AUC (4.5-fold of that of IFN-HSA), and fusion proteins with slow (IFN-RR-HSA) and fast release rate (IFN-

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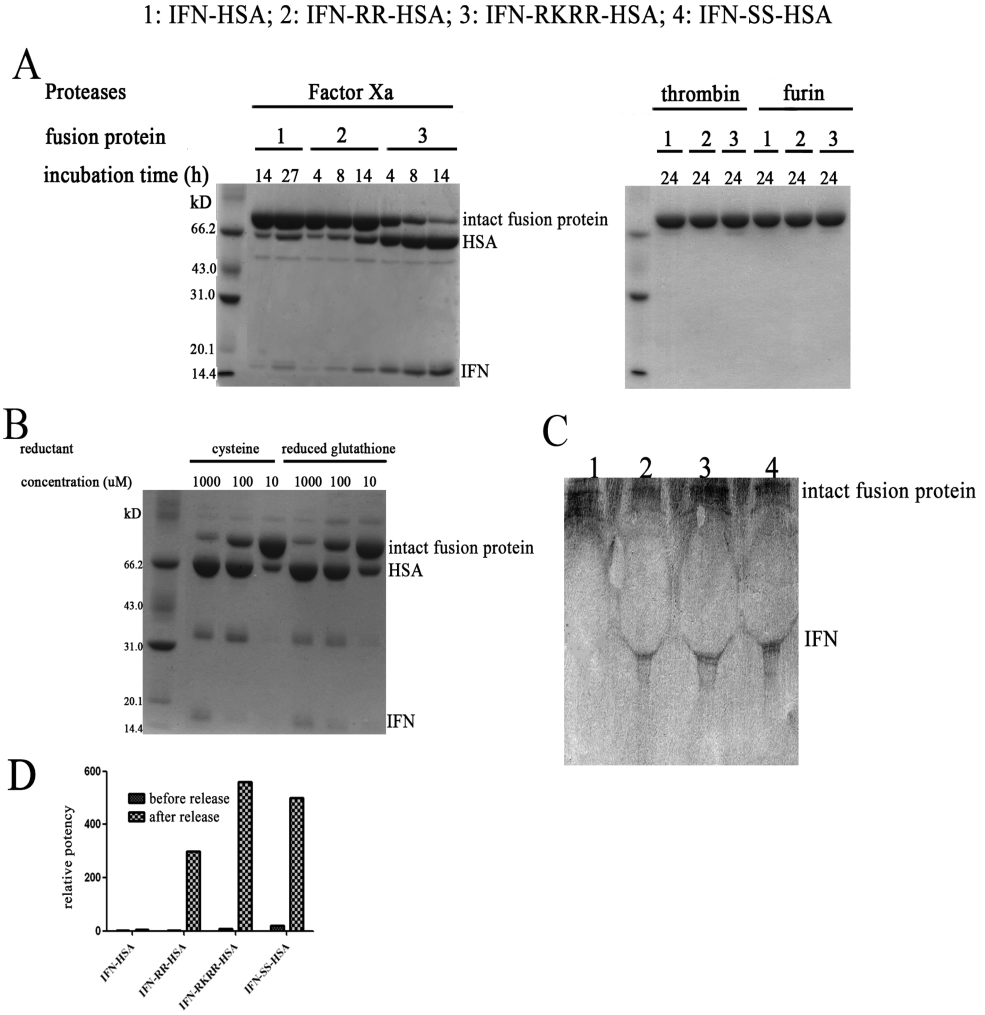

Figure 4. *In vitro* release of IFN-α2b and HSA fusion proteins. (A) Fusion proteins were incubated at 37 °C with factor Xa (1 U/mL), thrombin (1 U/mL) or furin (10 μg/mL) for indicated periods of time. The cleavage of fusion proteins were detected by nonreduced SDS−PAGE. (B) Fusion proteins were incubated at 37 °C with different concentrations of physiological reductants (cysteines or reduced glutathione) for 24 h. The cleavage of fusion proteins was detected by nonreduced SDS−PAGE. (C) An equal volume of fusion proteins was mixed with mouse serum and incubated at 37 °C for 12 h. Released IFN-α2b was detected by Western blotting. (D) The cleavage of fusion proteins led to increased antiproliferative potency. The potency of nonreleasable IFN-HSA was arbitrarily defined as 1.

SS-HSA) only showed a modest increase of AUC (25% and 47%, respectively) (Figure 5C).

***In Vivo* Antitumor Effects.** To investigate how the balanced PK/PD profile of releasable IFN-α2b and HSA fusion proteins translates into therapeutic efficacy, the *in vivo* anticancer effects were determined in HT29 human colon cancer xenograft models.

As shown in Figure 6, tumors of control mice treated with PBS grew rapidly, and at the end of the observation period, the average tumor size was 394.2 ± 111.7 mm^3. The treatment of IFN-HSA reduced the tumor volume to 307.2 ± 60.8 mm^3, which was statistically significant compared to the PBS group (P = 0.048). The tumor sizes of mice treated with fusion proteins with fast (IFN-SS-HSA) or slow (IFN-RR-HSA) release rate were 289.0 ± 41.0 mm^3 and 265 ± 42.6 mm^3, respectively. The IFN-SS-HSA and IFN-RR-HSA groups were statistically significant from the PBS group (P = 0.019 and 0.005, respectively), however, statistical significance toward the IFN-HSA group could not be achieved (P = 0.663 and 0.318, respectively). Fusion protein with intermediate release rate (IFN-RKRR-HSA) resulted in the most pronounced tumor growth inhibition effects. At the end of treatment, the average tumor size was 144.0 ± 39.2 mm^3, which was statistically significant not only from the PBS group but also from the IFN-HSA group (P = 0.000 and 0.001, respectively). Taken together, both pharmacokinetical and pharmacodynamical properties contributed to the *in vivo* anticancer effects, and by elaborately controlling the release rate, a balanced PK/PD that translated into improved efficacy can be achieved.

■ DISCUSSION

Albufusion technology has been widely applied to improve the pharmacokinetics of protein therapeutics, but none to our knowledge was releasable.[7] In conventional nonreleasable Albufusion technology, the improvement of pharmacokinetics (PK) comes at the expense of pharmacodynamics (PD). However, the results of recent phase III clinical trials of IFN-α2b and HSA fusion protein indicated that an increased PK may not always compensate for a decreased PD.[12,13] Studies on Pegylation have demonstrated that, by separating therapeutic protein from PEG moiety at an appropriate rate under physiological conditions, a balanced PK/PD profile that

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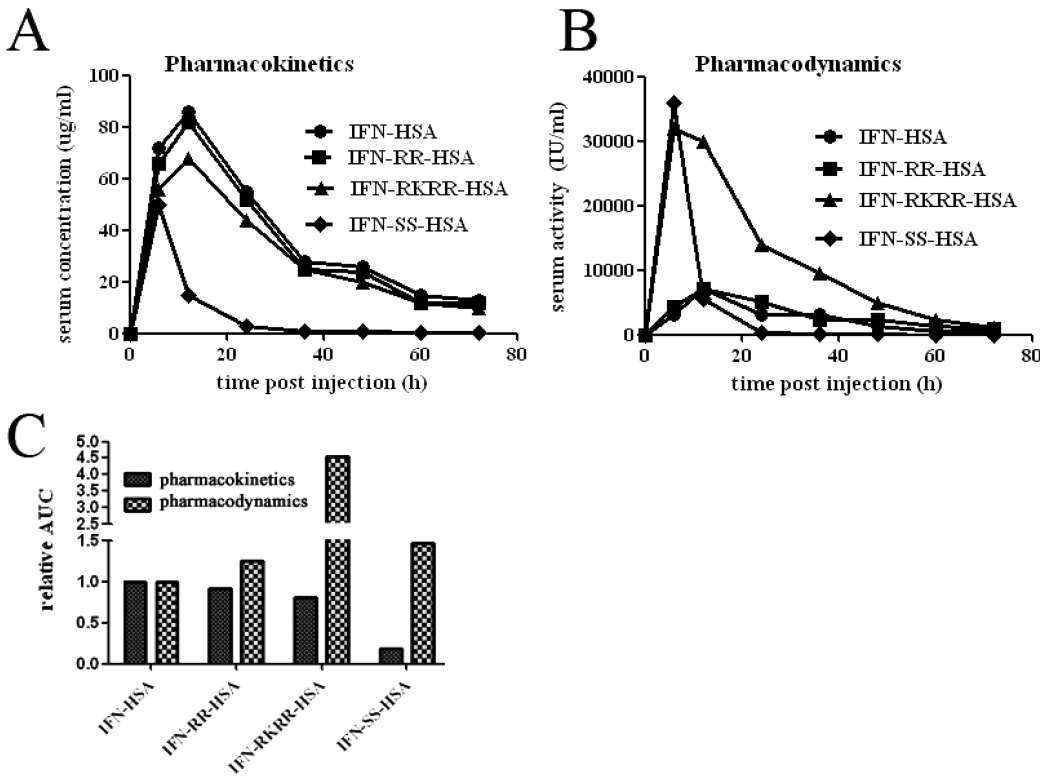

Figure 5. Pharmacokinetics and pharmacodynamics of IFN-α2b and HSA fusion proteins. (A) Serum concentrations of fusion proteins 6, 12, 24, 36, 48, 72 h post injection. (B) Serum antiproliferative activities of fusion proteins 6, 12, 24, 36, 48, 72 h post injection. (C) Relative pharmacokinetics and pharmacodynamics in terms of area under the curve (AUC). The pharmacokinetics and pharmacodynamics of nonreleasable IFN-HSA were arbitrarily defined as 1.

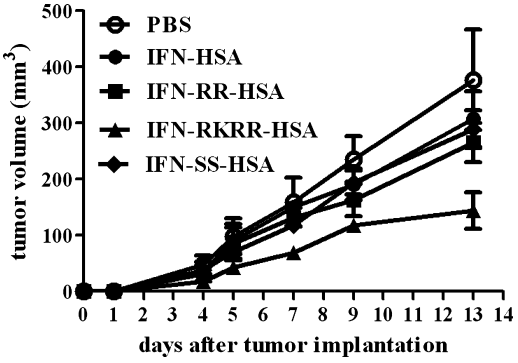

Figure 6. Antitumor effects of fusion proteins in nude mice bearing HT29 human colon cancer xenografts. Female balb/c nude mice (4–6 weeks old) were inoculated subcutaneously with 1.0×10^6 HT29 cells on day 0. Treatments were administered by subcutaneous injection of 100 μg of fusion proteins (in 200 μL) on day 1 and 5. PBS saline was used as a control treatment.

translated into increased efficacy can be achieved. The promising results of releasable Pegylation (rPegylation) provide important insights in addressing the PK/PD dilemma facing conventional Albufusion technology.[14−17]

rPegylation is achieved by linking PEG and a therapeutic protein with customized hydrolytically labile linkers.[18] Unfortunately, this strategy could not be transplanted directly to fusion proteins. In this study, two different strategies, proteolytic and reductive cleavage, were employed to separate IFN-α2b from HSA. The introduction of basic amino acid residues at the C-terminus of IFN-α2b conferred fusion proteins with factor Xa susceptibility, and disulfide-linked fusion protein can be reduced by cysteine or reduced glutathione in the presence of redox proteins. Both factor Xa and physiological reductants have been established as valid targets for controlled drug release, as they are tightly and stably controlled with low interindividual variations, which enabled reliable and predictable release of drugs.[21,23]

A prerequisite condition for releasable Albufusion technology is that the release takes place at an appropriate and homogeneous fashion *in vivo*. Protease or reduction cleavable IFN-α2b and HSA fusion proteins constructed in this study have release half-lives ranging from hours to days, which is within a therapeutically useful range. Moreover, by controlling the number of basic amino acid residues introduced, the release rate can be tailed to span an even larger range, which offers a new avenue to the application of releasable Albufusion technology to therapeutic proteins with different requirement for *in vivo* half-lives.

■ CONCLUSIONS

In this study, we proposed a novel strategy termed as releasable Albufusion (rAlbufusion), in which protein drugs were separated from HSA either by proteolytic or reductive cleavage. By controlling the release rate, optimal PK/PD profile that translated into increased *in vivo* therapeutic efficacy can be achieved. rAlbufusion allows tailored design of PK/PD profile, and greatly extends the utility of conventional Albufusion.

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■ ACKNOWLEDGMENTS

This research was supported by Natural Science Foundation of China (31000550, 81172968).

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