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Review Article

A new approach for prolonging the half-life of peptides, proteins and low-molecular-weight drugs *in vivo*

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CONTENTS

Introduction

Most peptide and protein drugs are short-lived species *in vivo*, having a circulatory half-life of several minutes. This is particularly valid for nonglycosylated proteins of a molecular mass less than 50,000 daltons. The short lifetime of proteins *in vivo* is attributed to several mechanisms, including glomerular infiltration in the kidney and proteolysis at several levels. Considering that peptide and protein drugs are not absorbed orally, prolonged maintenance of therapeutically active drugs in the circulation is a desirable feature of primary clinical importance. To achieve this goal, approaches such as pumps, liposomes, microspheres and protein pegylation have been studied and pursued. However, not all of these approaches can be applied to all drug categories.

In recent years, we have designed an approach for elongating the lifetime of insulin *in vivo* based on the fact that the major degradative pathway for this hormone is receptor-mediated endocytosis. The concept was later developed to include various peptides and proteins of therapeutic value, and was recently found to be applicable to the short-lived nonpeptidic low-molecular-weight drug category. In this regard, the progress and major findings made in this area are outlined here in a somewhat chronological manner.

Long-acting insulin

Insulin is a peptide hormone that regulates glucose and fatty acid metabolism. Diabetic patients, particularly those with type 1 diabetes, require several daily s.c. administrations of insulin in order to maintain normoglycemia (1, 2). Prolonged-acting insulin formulations for basal delivery are obtained by using insulin preparations made less soluble by complexing them with zinc and protamine. Upon dissolution, these suspended insulins are released gradually from the subcutis to the circulatory system (3, 4). Considerable efforts are presently being made to develop other approaches for long-acting insulins (5, 6). Our contribution in this area is described below.

General concept for developing long-acting insulins

Insulin is degraded primarily in the liver through a mechanism defined as receptor-mediated endocytosis (7, 8). Specifically, following the binding of insulin to its receptor sites and elicitation of rapid metabolic effects, hormone-receptor complexes are internalized by endocytosis ending up within endosomes and/or lysosomes where insulin is degraded. This pathway constitutes an efficient route aimed at terminating the action of insulin after the levels of glucose and other nutrients have been normalized. Consequently, the half-life of insulin after reaching the circulatory system is 5-6 min. Insulin variants with negligible receptor binding affinities are therefore longer-lived species within the circulation but are biologically ineffective (9, 11).

Our initial idea was to design an inactive insulin derivative with a reduced receptor binding capacity and

Table I: Half-maximal values for the hydrolysis of Fmoc and FMS conjugates under physiological conditions.

	Half-maximal hydrolysis (h)[1]
Fmoc-protein conjugates	24 ± 3
Fmoc-amino acids	170 ± 20
FMS-protein conjugates	6 ± 2
FMS-aminoglycosides	8 ± 2
FMS-amino acids	
FMS-L-serine	25 ± 3
FMS-L-phenylalanine	30 ± 4

[1]Rates of hydrolysis of Fmoc and FMS conjugates were determined either in normal human serum or in 0.1 M $NaHCO_3$ (pH 8.5) at 37 °C. Rates of hydrolysis in $NaHCO_3$ were found nearly identical to those obtained in normal human serum.

biological potency and, therefore, resistant toward receptor-mediated degradation. Such a derivative, however, should be capable of reverting spontaneously to the native hormone at physiological conditions with the desired rates and pharmacokinetic profiles.

Selecting Fmoc as a drug modifying moiety

Fmoc (9-fluorenylmethoxycarbonyl) is widely used in organic and peptide synthesis for the reversible protection of amino groups (12, 13). It is easily cleaved from the protein under basic conditions. For example, in organic solvents it is cleaved/hydrolyzed within several minutes after the addition of piperidine. We postulated that in aqueous buffers at neutral and slightly alkaline pH, Fmoc-proteins would be hydrolyzed at a slow rate in a homogeneous fashion. This was experimentally verified. Prior to modifying insulin, we linked Fmoc moieties to the amino side chains of several proteins. Under physiological conditions equivalent to those obtained in normal human serum at 37 °C, Fmoc moieties were hydrolyzed at a slow rate ($t_{1/2}$ = 24 ± 3 h) (Table I).

$Fmoc_2$-insulin

$Fmoc_2$-insulin is an HPLC-purified derivative of human insulin, in which two Fmoc moieties are covalently linked to the α-amino groups of phenylalanine B1 and the ε-amino group of lysine B29 (Fig. 1). This derivative has 1-2% of the biological potency and receptor binding capacity of the native hormone. $Fmoc_2$-insulin undergoes hydrolysis and reactivation to the fully active insulin upon incubation in normal human serum or in 0.1 M $NaHCO_3$ (pH 8.5, 37 °C) with a $t_{1/2}$ value of 32 ± 2 h. A single s.c. dose of $Fmoc_2$-insulin to streptozocin (STZ)-treated diabetic rats normalized their blood glucose levels and maintained the animals in an anabolic state for over 2-3 days (Fig. 2) (14). Hence, compared to regular insulin, $Fmoc_2$

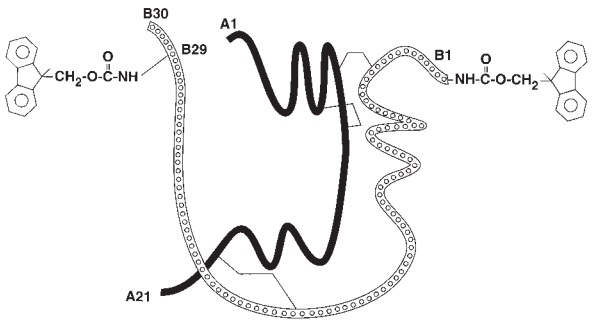

Fig. 1. Schematic model of $Fmoc_2$-insulin.

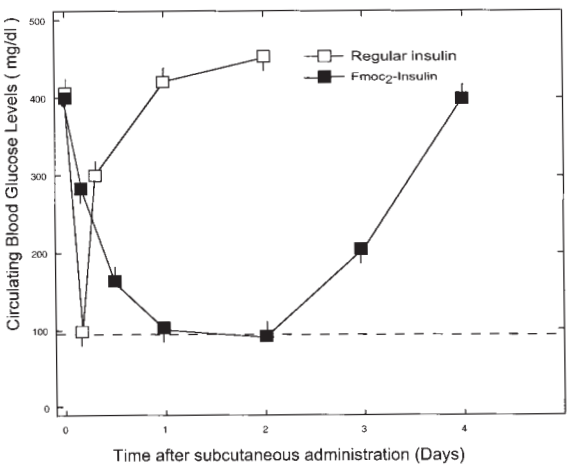

Fig. 2. Circulating glucose levels after a single s.c. dose (3 mg/rat) of $Fmoc_2$-insulin to STZ-treated diabetic rats. Blood glucose levels were determined at the indicated time points. The horizontal broken line indicates the arithmetic mean of blood glucose of control healthy rats. (Data from ref. 14.)

induces a 6- to 7-fold increase in the duration of antidiabetic actions in this diabetic rat model.

$Fmoc_2$-insulin cross-reacts weakly with antibodies to insulin. Hydrolysis (and reactivation) of this derivative closely parallels the elevation in immunoreactivity. Little circulating immunoreactive insulin is seen 1-2 h following s.c. administration of $Fmoc_2$-insulin. The level then rises, manifesting a broad peak of immunoreactive hormone which reaches a maximum 24 h postdosing; a subsequent gradual decrease occurs over the next 35 h (Fig. 3).

$Fmoc_2$-insulin is intrinsically less soluble than the native hormone in aqueous medium. Therefore, in theory, its prolonged action *in vivo* after s.c. administration can be attributed to a slower absorption rate and/or its ability to avoid receptor-mediated degradation in the circulation. To differentiate between these two mechanisms, the s.c. compartment was bypassed by administering $Fmoc_2$-insulin i.p. Following this type of administration, $Fmoc_2$-insulin still showed a prolonged glucose-lowering action

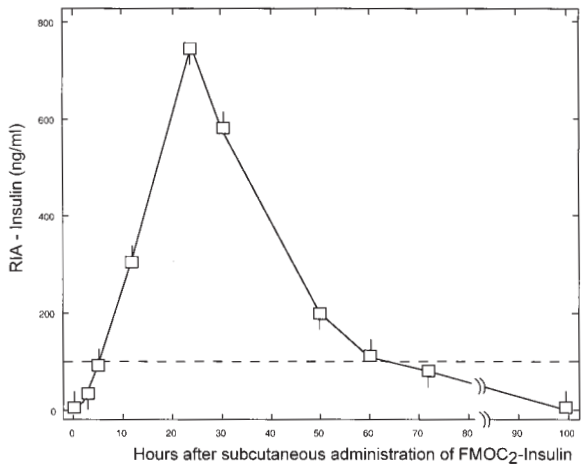

Fig. 3. Level of circulating immunoreactive insulin after a single s.c. dose (3 mg/rat) of $Fmoc_2$-insulin. Serum aliquots were withdrawn at the indicated time points to determine immunoreactive insulin. The horizontal broken line (95 ± 5 ng/ml) is the threshold level of immunoreactive insulin which is required to lower blood glucose level in this STZ-treated rat model. (Data from ref. 14.)

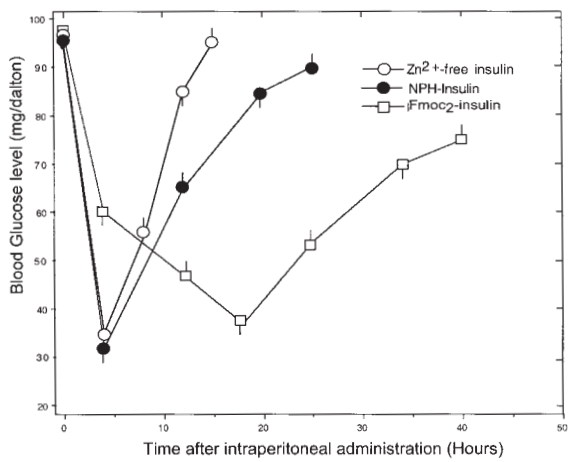

Fig. 4. Effect of a single i.p. dose (3 mg/rat) of $Fmoc_2$-insulin, rapid-acting (Zn^{2+}-free) insulin or long-acting (NPH) insulin on circulating glucose levels in normal healthy rats. Circulating glucose levels were then determined at the indicated time points. (Data from ref. 14.)

in rats ($t_{1/2}$ = 30 h). Regular insulin and NPH-insulin, a suspended preparation that facilitates a prolonged effect when administered s.c., had nearly the same glucose lowering effect in this experimental system ($t_{1/2}$ = 9 ± 1 h) (Fig. 4).

Thus, we tested a novel concept to prolong the action of insulin within the circulatory system *per se*. Protracted action is based on avoiding receptor-mediated clearance, prior to being reactivated *in vivo*, by hydrolysis.

Long-acting water-soluble insulin: an improved version of Fmoc

Although suspended long-acting insulins have been widely used for several decades, the current trend is aimed at designing long-acting, water-soluble preparations of insulin. Several approaches in this direction are in progress (5, 6). Regarding our technology, we searched for an Fmoc derivative that is *a priori* intrinsically soluble in aqueous media with aqueous solubility not appreciably decreasing following linkage to proteins. An acidic ionizable sulfonic acid moiety was introduced into the fluorene ring of Fmoc-OSu to obtain 2-sulfo-9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (FMS-OSu) (Fig. 5). Prior to testing insulin, we conjugated FMS moieties to the amino side chains of several proteins and tested their rates of hydrolysis *in vitro* under physiological conditions. FMS moieties undergo hydrolysis in a linear fashion with a $t_{1/2}$ value of 6 ± 2 h, indicating that hydrolysis is 4 times faster in comparison with Fmoc-protein conjugates. When FMS is conjugated to lower molecular-weight compounds such as amino acids and aminoglycosides, its hydrolysis generally proceeds at slower rates ($t_{1/2}$ = 8-30 h) (see Table I).

FMS_3-insulin

FMS_3-insulin is a derivative of human insulin that is comprised of three FMS moieties covalently linked to the three amino functional groups of the protein (Fig. 6). FMS_3-insulin is soluble in aqueous buffers at concentrations of 0.3-0.6 mM and has about 1% of both the biological potency and the receptor binding affinity of the native hormone. Upon incubation under physiological conditions, it undergoes hydrolysis with linear regeneration of insulin possessing full biological potency ($t_{1/2}$ = 17 ± 2 h in normal human serum at 37 °C). A single s.c. administration of FMS_3-insulin to STZ-treated rats lowered circulating glucose levels for a prolonged period (Fig. 7). As with $Fmoc_2$-insulin, the s.c. compartment was bypassed by administering this derivative i.p. Following administration, recovery from hypoglycemia proceeded with a $t_{1/2}$

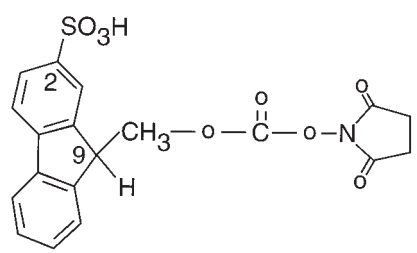

Fig. 5. Structure of (2-sulfo)-9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (FMS-OSu). The position of the sulfonyl group on the fluorene ring is indicated.

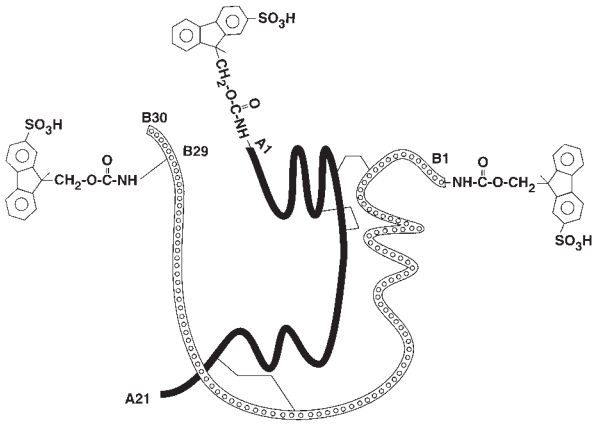

Fig. 6. Schematic model of FMS_3-insulin. The three FMS moieties of FMS_3-insulin were covalently linked to the α-amino groups of phenylalanine B1, glycine A1 and the ε-amino group of lysine B29.

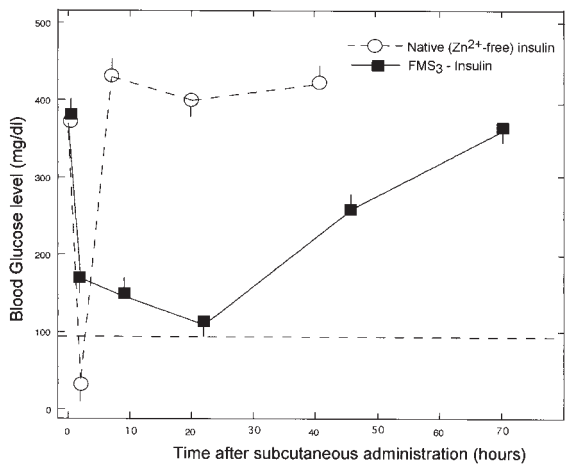

Fig. 7. Effect of a single s.c. dose of FMS_3-insulin or native insulin (3 mg/rat) on blood glucose levels of STZ-treated rats. Circulating glucose levels were determined at the time points indicated. The horizontal broken line represents the arithmetic mean of blood glucose in control healthy rats. (Data from ref. 16.)

value of 14 ± 1 h as compared with 9 ± 1 h obtained following administration of regular insulin and NPH-insulin under identical experimental conditions (16). Thus, a new version of a water-soluble, long-acting insulin was designed and introduced. Because of its low receptor binding affinity at the time of administration, it evades receptor-mediated degradation and maintains prolonged glucose-lowering action, undergoing a slow, spontaneous reactivation in the circulatory system *in vivo*.

Generality of the concept

The clearance of insulin through receptor-mediated endocytosis and degradation is quite unique and may not reflect the vast majority of other administered peptides and proteins. The short half-life of polypeptides *in vivo* is attributed to several other mechanisms, including degradation by serum proteases or proteases located at the surfaces of peripheral tissues and/or clearance by glomerular infiltration in the kidney (17). From a therapeutic point of view, a rapidly inactivated protein *in vivo* becomes therapeutically ineffective shortly after administration, even though its clearance by the kidney proceeds at significantly lower rates.

To analyze the generality of our concept, we administered various FMS-containing radiolabeled peptides and proteins and their non-FMS-modified counterparts s.c. to rats. The study included a variety of proteins and synthetic peptides of molecular weights varying from 0.6-17 kDa that contain one to three FMS moieties/mol. Pharmacological patterns were constructed by withdrawing blood aliquots at various time points after administration and determining the trichloroacetic acid (TCA)-soluble and TCA-precipitable counts for each sample. The major findings of this study are summarized in Table II. The overall conclusions are as follows: (a) Subcutaneously administered non-FMS-containing peptides and polypeptides (0.6-17 kDa) reach peak values at 1-2 h after administration and decline with $t_{1/2}$ values of 9-12 h. (b) All peptides and proteins containing two FMS/mol or more are long-lived species *in vivo* with no exceptions; the circulating lifetime is extended 2-4 times as compared to the non-FMS-containing counterparts. (c) In general, the introduction of a single FMS/peptide or protein is not sufficient to prolong the lifetime of the compound *in vivo*. (d) In many instances, the covalent introduction of two FMS/mol appears to be sufficient. Virtually all proteins containing two FMS/mol or more were found to be resistant to proteolysis *in vivo*. Proteolysis was decreased by 50-70% at all time points after administration (manuscript in preparation). (e) It seems that a distance of 4-6 amino acids between two FMS moieties within a random nondisulfide-bonded polypeptide may be optimal for maximal prolongation (currently under investigation). Also shown in Table II are the parameters for *N*-acetyl-^{125}I-histidine, as a representative of low-molecular-weight substances. Subcutaneously administered *N*-acetyl-histidine in rats peaked at 20 min and declined with a $t_{1/2}$ of 2.0 ± 0.2 h (Table II).

Long-acting interferon-α_2

Interferon-α_2 (IFNα_2) is an 18 kDa cytokine that possesses a variety of antiviral, immunomodulating and antiproliferative activities. It is FDA approved and currently used to treat chronic hepatitis C, chronic hepatitis B, Kaposi's sarcoma in HIV-infected patients and leukemia. IFNα_2 is a short-lived protein *in vivo*. It undergoes proteolytic inactivation in body fluids and is cleared by the kidneys (18-21).

We prepared FMS_7-IFNα_2 by covalently linking seven FMS moieties to the amino groups of human

Table II: Pharmacological parameters of s.c. administered radiolabeled peptides and proteins[1] in rats.

Designation	FMS/mol peptide or protein	Maximal peak height after administration (h)	$t_{1/2}$ of declining phase (h)
N-Acetyl-peptide 27[2]	0	2	12
FMS-peptide 27[2]	1	2	12
YGGFLK	0	1	9
FMS-YGGFLK	1	1	9
$Acetyl_2$-glucagon	0	2	10
FMS_2-glucagon	2	8	16
(FMS)-YGGFLK-(FMS)	2	12	34
$Succinyl_3$-insulin	0	2	9
FMS_3-insulin	3	7	24
$Acetyl_3$-peptide-23[3]	0	2	11
FMS_3-peptide-23[3]	3	7	21
N-Acetyl-histidine	0	0.3	2

[1]Peptides and proteins were radiolabeled with NaI^{125}. Male Wistar rats (170 ± 10 g) received s.c. 10 μg (2 ± 0.2 x 10^7 c.p.m.) of peptide or protein/rat. Blood samples were withdrawn from the tail vein at many time points after administration, weighed and counted for their radioactive content prior to and after precipitation with 10% cold trichloroacetic acid. [2]A nonlysine-containing synthetic peptide of 27 amino acids with one acetyl or FMS moieties linked to the α-amino function. [3]A synthetic peptide of 23 amino acids containing two moieties of lysine.

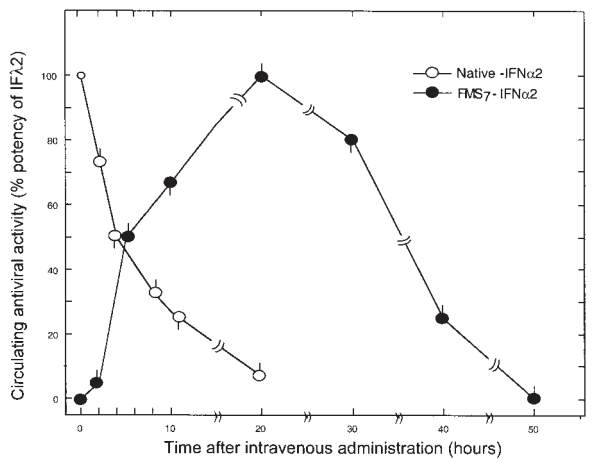

Fig. 8. Administration of FMS_7-IFNα_2 in mice facilitates prolonged circulating antiviral activity. Mice received either native IFNα_2 or FMS_7-IFN-α_2 i.v. (10 μg/mouse). Blood aliquots were withdrawn at the indicated time points. The half-maximal potency of each aliquot to protect human WISH cells from vesicular stomatitis virus (VSV)-induced cytopathic effects was determined. (Data from ref. 22.)

IFNα_2. FMS_7-IFNα_2 has approximately 4% the antiviral potency of the native cytokine, but undergoes linear, time-dependent reactivation upon incubation at physiological conditions, with a $t_{1/2}$ value of 5 ± 0.5 h (22). When FMS_7-IFNα_2 was administered i.v. to mice, there was a progressive elevation in circulating antiviral-active protein. The protein level began to increase 2 h after administration, peaked at 20 h and then declined with a $t_{1/2}$ value = 35 ± 4 h. The antiviral activity of native IFNα_2 administered to mice under identical experimental conditions, decayed with a $t_{1/2}$ value of 4 ± 0.4 h (Fig. 8). Similar antiviral profiles were obtained following incubation of either IFNα_2 or FMS_7-IFNα_2 in human serum albumin at 37 °C *in vitro*. The antiviral activity of the native cytokine decreased with a $t_{1/2}$ of 4 ± 0.4 h. Following a 2-h lag period, the antiviral potency of serum incubated with FMS_7-IFNα_2 increased steeply, was maintained at a high plateau over 10 h and declined with a $t_{1/2}$ of 32 ± 2 h (Fig. 9). Thus, the predominant reason for the short lifetime of antiviral-active IFNα_2 *in vivo* is inactivation by serum proteases.

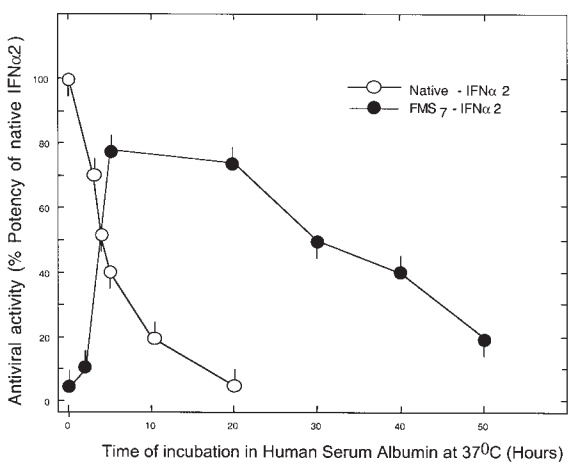

Fig. 9. Resistance of FMS_7-IFNα_2 to inactivation in human serum. FMS_7-IFN-α_2 and native IFN-α_2 (10 μg/ml each) were incubated in human serum at 37 °C. Aliquots were withdrawn at the indicated time points and tested for their half-maximal antiviral potencies to inhibit VSV-induced cytopathic effects in human WISH cells. (Data from ref. 22.)

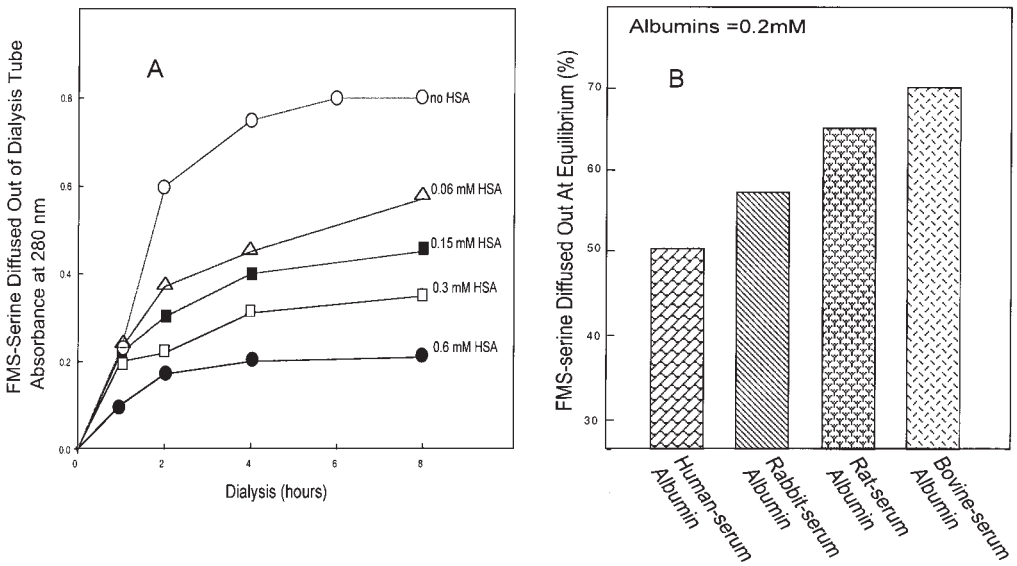

Fig. 10. FMS-L-serine associates with human serum albumin in an equilibrium-dialysis procedure. (A) Dialysis sacs containing 0.06-0.6 mM albumin and 0.4 mM FMS-serine were dialyzed against 9 volumes of PBS-buffer. At the indicated time points, the absorbance at 280 nm (corresponding to nonbound FMS-serine that diffused out of the dialysis sac) was monitored. (B) The same procedure was applied with 0.2 mM albumin for different species. The absorbance at 280 nm was monitored following 8 h dialysis at 25 °C.

These findings and others (22) led us to conclude that the prolonged antiviral activity of FMS_7-IFNα_2 *in vitro* and *in vivo* results from its resistance to inactivation in serum, coupled with the gradual recovery of its antiviral potency following hydrolysis of the FMS moieties. We thus concluded that our technology is applicable to protein drugs that are inactivated *in vivo* by serum proteases.

Prolonging the half-life of low-molecular-weight nonpeptidic drugs

More recently we tested whether our approach for prolonging the half-life was also applicable to low-molecular-weight drugs of nonpeptidic origin. This drug category differs from the polypeptide category in that it undergoes faster clearance *in vivo*, predominantly via glomerular filtration in the kidney. Elimination through degradative pathways in this case is of little or no significance.

Albumin is an abundant protein in the extracellular fluid, reaching a concentration of about 0.6 mM in serum and about 0.3 mM in s.c. interstitial fluid. Albumin binds a wide variety of ligands, such as long-chain fatty acids, metal ions and numerous drugs. Aliphatic compounds containing carboxyl groups and hydrophobic substances with hydrophilic carboxylates (*i.e.*, bilirubin) bind to albumin preferentially (23). Association of low-molecular-weight drugs with albumin often leads to lower clearance rates from the circulation. Attempts have been made to link such ligands (*i.e.*, long-chain fatty acids) to short-lived protein drugs to extend their action *in vivo* (6). For this purpose, conjugates must remain therapeutically active and maintain sufficient affinity toward albumin.

Using equilibrium dialysis, we recently observed that a single FMS moiety (*i.e.*, FMS-serine) associates with albumin (Fig. 10A). The relative affinities toward albumins from different species were not significantly different. Ratios of 1.0, 0.9, 0.8 and 0.7 were estimated for human, rabbit, rat and bovine albumin, respectively (Fig. 10B). A single moiety of FMS binds to human-serum albumin with a Ka value of approximately 0.5 x 10^5 M^{-1} (K_D = 20 μM). Since the half-life *in vivo* of monomodified FMS-short peptide conjugates was not elongated (see Table II), we concluded that this order of affinity toward albumin is insufficient for significantly prolonging the half-life *in vivo*.

Albumin, however, has multiple sites for diverse types of ligands. We therefore postulated that a small ligand containing two FMS moieties (or more) might form a more stable complex with albumin, provided that a bivalent ligand capable of associating simultaneously with two adjacent sites of albumin is formed (Fig. 11).

Indeed, several low-molecular-weight drugs containing two FMS moieties/mol compound were prepared. Affinity toward human serum albumin (HSA) was increased nearly 3-fold (K_D = 6-8 μM) as compared to monosubstituted low-molecular-weight compounds. Such bis-modified conjugates are long-lived species *in vivo*. An optimal distance of about eight atoms between two adjacent FMS moieties appears to be optimal for half-life elongation (manuscript in preparation). Inactive FMS-low-molecular-weight conjugates undergo FMS hydrolysis and reactivation in the circulatory system.

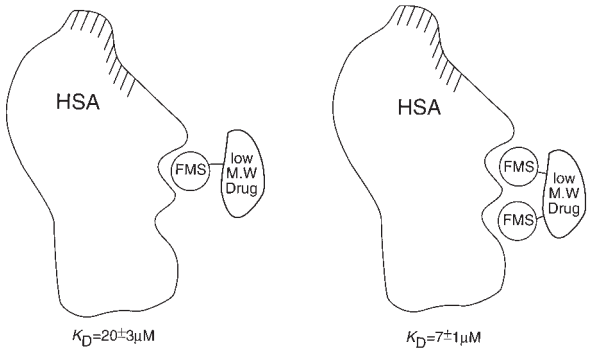

Fig. 11. Model for the higher binding affinities toward albumin of low-molecular-weight compounds containing two moieties of FMS. A bivalent ligand was formed capable of associating simultaneously with two adjacent sites of albumin, thus forming a more stable complex.

Lower toxicity indices

Although our original intention was to design and develop an approach solely for prolonging the life-time of drugs *in vivo*, another significant advantage of this technology has emerged in the course of our study. It is well documented that many types of drugs (*e.g.*, anticancer, cardiac glycosides and insulin) suffer from a narrow pharmacological window. Thus, circulating levels just 2-3 times higher than the desired therapeutical values may lead to adverse, undesirable toxic effects (1-3).

As expected, most drugs we studied underwent inactivation following derivatization with FMS. Clearly, when a drug such as an insulin derivative is inactive at the time of administration, the risks of toxicity, hypoglycemia or desensitization are largely minimized. Low circulatory levels of an active drug can then be maintained, depending on the rates of reactivation and clearance *in vivo*. Dosages to be administered can be calculated or experimentally determined based on these two *in vivo* parameters.

The most serious adverse reaction among diabetic patients receiving multiple injections of insulin is hypoglycemia. This may result from inappropriately large doses, missing a meal after administration or a variety of other nutritional, physiological and mental factors, each of which increases body consumption of glucose more than expected (1, 2).

We were interested in determining whether this risk factor was reduced when FMS_3-insulin replaces regular insulin. For this purpose, we injected hyperglycemic mice s.c. with either regular (Zn^{2+}-free) insulin or FMS_3-insulin. For each treatment, we determined the lowest effective dose that decreased circulating glucose levels after administration and the maximum tolerated dose defined as the highest dose which could be administered without severe hypoglycemic episodes at any time point after administration. These two values were 3 and 18 μg/mouse, respectively, for the native (Zn^{2+}-free) hormone, and 20 and 400 μg/mouse, respectively, for FMS_3-insulin (Table III). Ratios of 6 and 20 between these parameters were calculated for administered insulin and FMS_3-insulin, respectively. Thus, FMS_3-insulin can be administered over a broad range of glucose-lowering doses prior to the manifestation of hypoglycemia.

Conclusions

A conceptually novel approach for elongating drug lifetime *in vivo* has been presented. This concept, initially designed to extend the action of insulin, was later found to be applicable to many other peptides, proteins and, more recently, to low-molecular-weight drugs. The lifetime *in vivo* was elongated irrespective of whether the short-living character of the compound was attributed to receptor-mediated endocytosis, proteolysis at tissue surfaces, inactivation by serum proteases or clearance through glomerular filtration in the kidneys. Two or more FMS moieties should preferably be covalently linked for converting a short- to a long-lived drug in the circulation. The loss of therapeutic potency that often accompanies FMS derivatization provides a profound advantage in this technology as conjugates are hydrolyzed and reactivated. A desirable pharmacological profile consisting of a flat and broad peak of circulating levels of the agent was ordinarily obtained following a single administration.

Finally, FMS moieties are nontoxic (LD_{50} in rats > 3 g/kg). It also appears that the concern that FMS-protein conjugates are immunogenic is unfounded. FMS_3-insulin and FMS_7-IFNα_2 were only weakly or nonimmunogenic. Our attempts to prepare antibodies to FMS-KLH conjugates by repetitive immunization in rabbits failed. Further

Table III: Lowest levels of circulating glucose in STZ-treated diabetic mice[1] following s.c. administration of regular or FMS_3-insulin.

Dose of native (Zn^{2+}-free) insulin (μg/mouse)	Lowest blood glucose level after administration (mg/dl)
None	580 ± 30
3	504 ± 17
7	320 ± 10
11	270 ± 17
15	130 ± 7
18	90 ± 4[2]
FMS_3-insulin	
20	495 ± 20
50	180 ± 15
200	120 ± 15
400	90 ± 72

[1]Diabetes was induced in CD-1 mice by a single i.v. injection of a freshly prepared solution of streptozocin (STZ, 128 mg/kg body wt). [2]Although circulating glucose level of healthy nondiabetic mice was 140 ± 7 mg/dl, we arbitrarily applied a glucose level of 90 ± 5 mg/dl, below which the diabetic mouse was considered severely hypoglycemic.

aspects of this technology are currently under investigation, including an attempt to elevate gastrointestinal prodrug absorption and/or passage through the blood-brain barrier.

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