N-[(2-Sulfo)-9-fluorenylmethoxycarbonyl]$_3$-gentamicin C_1 Is a Long-Acting Prodrug Derivative

Yoram Shechter,*,† Haim Tsubery,†,‡ and Mati Fridkin*,‡

Departments of Biological Chemistry and Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Received January 28, 2002

Most low-molecular-weight drugs are short-lived species in the circulatory system, being rapidly eliminated by glomerular filtration in the kidney. However, binding to human serum albumin (HSA) can slow clearance and prolong lifetime profile in vivo. In this study, we have engineered a gentamicin derivative with affinity to albumin by linking three (2-sulfo)-9-fluorenylmethoxycarbonyl (FMS) to three amino groups of gentamicin C_1. FMS_3-gentamicin associates with HSA with a K_a value of $(1.31 \pm 0.2) \times 10^5$ M^{-1}. It has less than 1% the antibacterial potency of native gentamicin. Upon incubation at pH 8.5 and 37 °C, the FMS moieties from FMS_3-gentamicin undergo slow hydrolysis ($t_{1/2} = 8.0 \pm 0.2$ h), leading to a linear regeneration of the antibacterial potency with a $t_{1/2}$ value of 11 ± 0.7 h. FMS_3-gentamicin is a long-lived species in the rat circulatory system. Following a single subcutaneous or intravenous administration, it maintains a prolonged pharmacokinetic profile with a peak and a "through" concentration of immuno/antibacterial active gentamicin exceeding 4−5 times the duration obtained by administered native gentamicin. To sum up, an approach aimed at elongating the lifetime of low-molecular-weight drugs in vivo has been examined here with gentamicin. Two to three FMS per mole of compound are to be introduced to obtain an albumin associating affinity of $K_d = 7.6-9.2$ μM and, hence, to significantly extend the drug's lifetime in situ following administration. By use of this technology, the loss of pharmacological potency with derivatization is of no consequence, since FMS moieties are hydrolyzed and activity is generated at physiological conditions.

Introduction

Recently, we have introduced an approach for elongating the half-life of peptide and protein drugs in vivo[1] by covalently linking 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) moieties to their amino acid side chains. Such FMS derivatives containing two or more FMS moieties per protein show protracted action in vivo. This has been demonstrated for insulin,[2] interferon α2,[3] enkephalin, and other peptide and polypeptide drugs (in preparation). FMS moieties undergo slow and spontaneous hydrolysis under physiological conditions. Hydrolysis of FMS−protein conjugates in vivo proceeds with a $t_{1/2}$ value of 5−7 h.

FMS−peptide and −protein conjugates were found to be resistant to proteolysis both in vitro and in vivo. This was valid for peptides and proteins whose route of elimination in vivo takes place through receptor-mediated endocytosis, through inactivation by serum proteases in the circulatory system, or by proteolysis at the cell surfaces of tissues. With respect to FMS−peptide and −protein drugs, such resistance to proteolysis, prior to the release of the FMS moieties by hydrolysis, appears to be critical for maintaining prolonged circulating therapeutical levels.[2−4]

Our aim in this study was to investigate whether this conceptual approach can be extended to protract the actions, in situ, of low-molecular-weight drugs of *non-peptidic* origin. This drug category differs from the polypeptide category in undergoing a faster clearance in vivo, operating predominantly by glomerular filtration in the kidney; elimination through proteolysis in this case is none or negligible.[5] As a prototype for this drug category, we have selected gentamicin, a low-molecular-weight nonorally absorbed agent used for the treatment of many serious Gram-negative bacterial infections.[6] In theory, conjugation of FMS moieties to these types of drug is not expected to confer protracted action in vivo, unless the corresponding conjugates associate endogenously with albumin with a sufficient affinity to slowing their rate of clearance by glomerular filtration. Albumin, an abundant multifunctional transport protein, is known to bind reversibly a wide variety of endogenous substances and drugs.[7] Hence, low-molecular-weight drugs, when associated with albumin, are often cleared from the circulation at lower rates.[5] Our studies with gentamicin were largely based on this concept.

Experimental Section

Abbreviations. FMS, (2-sulfo)-9-fluorenylmethoxycarbonyl; FMS-OSu, FMS-*N*-hydroxysuccinimide; $(FMS)_3$-gentamicin, a gentamicin derivative having three FMS moieties covalently attached to the amino side chains of gentamicin; HSA, human serum albumin; IC_{50}, inhibitory concentration (half-maximal) needed to arrest *E. coli* replication; OD, optical density; HPLC, high-performance liquid chromatography; PNPA, *p*-nitrophenyl acetate; tBoc-Tyr-OSu, *N*-tBoc-L-tyrosine *N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; TNBS, trinitrobenzene sulfonic acid; FFA, free fatty acids.

Materials. Gentamicin sulfate, HSA, tBoc-Tyr-OSu, PNPA, and chloramine T were purchased from Sigma, and [^{125}I] iodine

* To whom correspondence should be addressed. For Y.S.: (phone) 972-8-9344530; (fax) 972-8-9344118; (e-mail) y.shechter@weizmann.ac.il. For M. F.: (phone) 972-8-9342505; (fax) 972-8-9344142; (e-mail) mati.fridkin@weizmann.ac.il.

† Department of Biological Chemistry.

‡ Department of Organic Chemistry.

10.1021/jm020042t CCC: $22.00
Published on Web 08/15/2002

(carrier-free) was from Amersham. 9-Fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-OSu) is a product of Novabiochem (Laüfelfingen, Switzerland). All other materials used in this study were of analytical grade.

Chemical Procedures. Ultraviolet spectra were obtained with a Beckman DU 7500 spectrophotometer in 1 cm path length UV cuvettes. Mass spectra were determined using MALDI-TOF and ESMS techniques (Bruker-Reflex-Reflectron model, Germany, and VG-platform-II electrospray single quadropole mass spectrometer, Micro Mass, U.K., respectively). Reverse-phase HPLC was performed with a Spectra-Physics SP8800 liquid chromatography system (Spectra-Physics, San Jose, CA) equipped with an applied Biosystem 757 variable-wavelength absorbance detector.

Preparation of (2-Sulfo)-9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (FMS-OSu). This compound was prepared by modifying the procedure of Merrifield and Bach.[8] Briefly, Fmoc-OSu (337.4 mg, 1 mmol) was dissolved in 4 mL of CH_2Cl_2 and cooled to 0 °C. A solution of $ClSO_3H$ (60 μL, 0.9 mmol) in 2 mL of CH_2Cl_2 was added with constant stirring and cooled over a period of 15 min. The solution, which turned yellow, was allowed to warm to room temperature. A white precipitate was formed within 1 h. At 2 h, cyclohexane (4 mL) was added to dissolve the unreacted Fmoc-OSu. The suspension was centrifuged and washed four times with 6 mL of 1:1 cyclohexane/CH_2Cl_2. The white solid thus formed was dried under P_2O_5 in vacuo for 24 h and had the following characteristics: yield 290 mg (86%); mp 140−146 °C; TLC (1 butanol/acetic acid/water, 8:1:1), $R_f = 0.31$; mass spectrum (ES^-), *m/z* 416 (100%, M − 1).

FMS moieties, either free or covalently bound to peptides and proteins, absorbed in the UV region with a molar extinction coefficient (ϵ_{280}) of 21.200 ± 100.

Synthesis of FMS_3-gentamicin. Gentamicin sulfate (10 mg, ~20 μmol) was dissolved in 1.0 mL of 0.5 M $NaHCO_3$ (pH 8.5) and cooled to 0 °C. Solid FMS-OSu was added gradually in several aliquots over a period of 2 h. Overall, 10 molar excesses (~200 μmol) of FMS-OSu were added over gentamicin. The reaction mixture was then dialyzed against H_2O at 7 °C for 3 days and finally lyophilized. A dialysis bag with a cutoff of 1200 ± 50 Da was used, which allowed for the diffusion of excess reagents and any residual amounts of native gentamicins or mono- and bis-modified FMS-gentamicins that might have been left following derivatization. The product was further purified by HPLC using a prepacked LichroCart RP-18 column (250 mm × 10 mm; 7 μm bead size), employing a binary gradient formed from 0.1% TFA in water (solution A) to 0.1% TFA in 75% acetonitrile in water (solution B), eluting at $t = 0$ min with B = 0% and at $t = 60$ min with B = 90%, at a flow rate of 12 mL/min. For purity evaluation of isolated fractions, analytical reversed-phase HPLC was performed using a prepacked Lichrospher-100 RP-18 column (250 mm × 4 mm, 5 μm bead size) and the following binary gradient: at $t = 0$ min, B = 0%; at $t = 30$ min, B = 100% at a flow rate of 0.8 mL/min. Separations were performed using a Spectra-Physics SP8800 liquid chromatography system equipped with an Applied Biosystems 757 variable-wavelength absorbance detector. The column effluents were monitored by UV absorbance at 220 nm. FMS_3-gentamicin was obtained with >95% purity and an overall yield of 56%.

Affinity of FMS-gentamicin Conjugates to HSA. Affinity was evaluated by a slight modification of the procedure of Koh and Means.[9] The analysis is based on the potency of several compounds that associate with HSA to arrest PNPA hydrolysis by HSA.[9] HSA, 11.4 μM in 0.4 mL of 0.05 M HEPES buffer, pH 6.7, was incubated for 10 min with and without increasing concentrations of FMS-gentamicin conjugates. PNPA (100 mM in DMSO) was then added to obtain a final concentration of 1 mM. The absorbance at 410 nm was determined 60 min after the addition of PNPA, and IC_{50} values were calculated. In preliminary studies, by use of an equilibrium dialysis procedure, FMS-serine was found to associate with HSA with a K_a value of $(0.5 \pm 0.02) \times 10^5$ M^{-1} ($K_d = 20 \pm 2$ μM) and to inhibit HSA-evoked PNPA hydrolysis with an IC_{50} value of 50 ± 3 μM (manuscript in preparation). A conversion factor of 2.5 was therefore used here for converting IC_{50} values in the PNPA assay to K_d value estimates.

Preparation of Gentamicin and FMS-gentamicin Conjugates That Can Be Radiolabeled with ^{125}I. tBoc-Tyr_1-gentamicin. Gentamicin (3 mg) was dissolved in 0.5 mL of 0.5 M $NaHCO_3$. Aliquots (2 μL) from a solution of tBoc-Tyr-OSu (180 mg/mL in DMSO) were added to the stirred gentamicin solution over a period of 3 h. Altogether, 1.5 mol of tBoc-Tyr-OSu/mol of gentamicin was added. The precipitate formed was collected by centrifugation, washed several times with H_2O, and resuspended in 0.1 M HCl, and excess tBoc-Tyr was extracted with ethyl acetate. The aqueous fraction was lyophilized and solubilized in 0.1 M $NaHCO_3$, pH 8.5. The product contained about 1 mol of tBoc-Tyr/mol of gentamicin, as judged by the absorbance at 278 nm and by the presence of tyrosine following acid hydrolysis. A fraction of this preparation was preserved for ^{125}I iodination.

$FMS_{2.0}$-tBoc-$Tyr_{1.0}$-gentamicin. FMS-OSu (11 mg, 26 μmol, 3.9 molar excess) was added to a solution of gentamicin (3.0 mg in 0.5 mM $NaHCO_3$, 6.7 μmol). The reaction was carried out for 1 h at 0 °C with constant stirring. tBoc-Tyr-OSu was then added in aliquots, as above, over a period of 1 h. FMS-OSu (10 mg) was then added, and after 30 min the reaction mixture was dialyzed for 3 days against H_2O, using a dialysis bag with a cutoff of 1200 ± 50 Da, and then was lyophilized.

Radiolabeling with Na ^{125}I. Gentamicin-containing tBoc-Tyr moieties were iodinated essentially by the procedure of Hunter and Greenwood[10] with slight modifications. Briefly, to 2 μL of PBS (concentrated ×20), tyrosyl-containing gentamicin derivative (2 μL, 1 nmol) and carrier-free Na ^{125}I (2 μL, 0.1 mCi) were added. This was followed by the sequential addition of 4 μL of chloramine T (from a fresh solution of 1 mg/mL, reaction period of 2 min), 4 μL of sodium metabisulfite (from a stock solution of 5 mg/mL, 2 min), and finally 2 μL of 10% BSA. Iodinated gentamicin derivatives were purified on a Sephadex G-10 column (0.4 cm × 7 cm), equilibrated and run with PBS/0.5% BSA (pH 7.4). Fractions corresponding to the radiolabeled derivative were pooled and frozen.

Amino acid composition was analyzed after acid hydrolysis in 6 N HCl at 110 °C for 22 h and precolumn reaction with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Waters 2690 separations module, Milford, MA).

Quantitation of Gentamicin. Following acid hydrolysis of native gentamicin and derivatization with 6-aminoquinolyl-*N*-hydroxysucciniimidyl carbamate (AQC), two peaks in a 1:1 ratio at the positions of proline (retention time RT = 24.94 min) and leucine (RT = 32.965 min) emerged on a standard amino acid analysis. An amount of 100 nmol of gentamicin yielded 179 nmol of either leucine-like or proline-like peaks. On the basis of that, gentamicin and derivatives were therefore quantitated by acid hydrolysis of an aliquot and determined by amino acid analysis, using peak areas of the leucine and the proline peaks. The obtained value was divided by 1.79.

Following Up the Circulating Levels of Subcutaneously Administered Gentamicin Derivatives in Rat. In this set of experiments, all derivatives were administered subcutaneously at 10 a.m. to male Wistar rats weighing 170 ± 10 g. Each rat received 0.3 mL of PBS/0.5% BSA containing 3 nmol of the unlabeled gentamicin derivative and a ^{125}I-labeled derivative to obtain a specific activity of 5000 ± 500 cpm/pmol derivative. Blood samples were withdrawn from the tail vein at various time points after administration and were absorbed onto a round (1 cm) Whatman filter paper (3 mm) that was weighed before and immediately after spotting. Each such aliquot contained about 25−35 mg of blood. Filters were counted for their radioactive content, and the obtained counts per minute were adjusted to obtain the amount of radioactivity corresponding to 1.0 mL of blood divided by 5000 to obtain the amount of the derivative in pmol/mL.

In an alternative procedure, native gentamicin and FMS_3-gentamicin were administered intravenously to rats. Blood aliquots were withdrawn at several time points, and gentami-

Table 1. Chemical and Biological Features of FMS_3-Gentamicin C_1

characteristic	FMS_3-gentamicin
solubility in aqueous buffer (pH 7.4), mg/mL	>30
molar extinction coefficient at 280 nm,[a] mol^{-1} cm^{-1}	63 600 ± 300
mol of FMS/mol of gentamicin[b]	3.0 ± 0.2
mass spectral data[c]	
calculated for FMS_3-gentamicin C_1, Da	1383.6
found $[M + H]^+$,[c] Da	1384.24
found $[M + Na]^+$,[c] Da	1405.74
retention time (analytical HPLC),[d] min	31.4
antibacterial potency[e]	<1% of the native potency
IC_{50}, μM	24 ± 3

[a] Native gentamicin does not absorb at 280 nm. [b] Based on the absorbance at 280 nm and derivative's concentration determined following acid hydrolysis and amino acid analysis (Experimental Section). [c] Mass spectra were determined using the MALDI-TOF technique (Bruker-Reflex-Reflectron model, Germany). Identical mass values were obtained by analyzing either dialyzed or HPLC-purified samples. [d] Column, Lichrosorb RP-18 (5 μM); linear gradient of 30−100% acetonitrile in 0.1% TFA; rate of 0.8 mL/min. [e] Determined by inhibiting replication of *E. coli*. Native gentamicin arrests replication half-maximally (IC_{50}) at a concentration of 0.22 ± 0.02 μM.

cin was quantitated by a fluorescence polarization immunoassay procedure.[11]

Results

Preparation and Features of FMS_3-gentamicin C_1. Initially, we have applied 10 molar excess of FMS-OSu over gentamicin. This was followed, at termination of reaction, by extensive dialysis in a dialysis bag having a cutoff of 1200 ± 50 Da (Experimental Section). Table 1 summarizes the characteristic features of the derivative thus obtained. It is a water-soluble, >95% pure compound (>30 mg/mL at pH 7.2) containing 3 mol of FMS/mol of gentamicin, as judged by its absorbance at 280 nm (ϵ_{280} = 63 600 ± 300). Mass spectrum analyses of native gentamicin (Sigma) used in our studies revealed primarily the presence of gentamicin C_1, ($[M + H^+]$ = 478.43 Da). Following derivatization, dialysis, and HPLC purification, gentamicin C_1 containing three FMS moieties was isolated (calculated MW = 1383.6 Da; found $[M + H^+]$ = 1384.24 Da and $[M + Na]$ = 1405.74 Da; Table 1). It should be noted that dialysis alone removes most of the conjugates containing less than 3 FMS/mol of gentamicin (Experimental Section) as well as residual, unreacted, gentamicin C_1. FMS_3-gentamicin C_1 migrates as a main peak on HPLC with an RT value of 31.4 min. FMS_3-gentamicin C_1 has less than 1% (IC_{50} = 24 ± 3 μM) of the native antibacterial potency of gentamicin (IC_{50} = 0.22 ± 0.02 μM; summarized in Table 1). The structure of gentamicin C_1 and the proposed structure of FMS_3-gentamicin C_1 are illustrated in Figure 1; FMS_3-gentamicin C_1 is TNBS-negative. TNBS does not react with secondary amines,[12] suggesting that in FMS_3-gentamicin C_1 prepared with up to 10 molar excess of FMS-OSu only the three primary amino side chains of this aminoglycoside have been derivatized. Reaction with a larger excess of FMS-OSu led to generation of tetra- and pentamodified gentamicin as revealed by mass spectrometry. For additional characterization of FMS_3-gentamicin C_1, ^1H NMR studies were performed. Thus, spectra in D_2O, DMSO-d_6, $CDCl_3/CD_3OD$, $CDCl_3$/TFA, and $CDCl_3/CD_3$-OD/TFA used as solvents were recorded at 25 °C on a Bruker Avance 400 spectrometer (400 MHz). However, very broad line NMR spectra in both aliphatic and aromatic regions indicated a possible liquid-crystal-like behavior, a fact that impairs the assignment of the compound's chemical shifts.

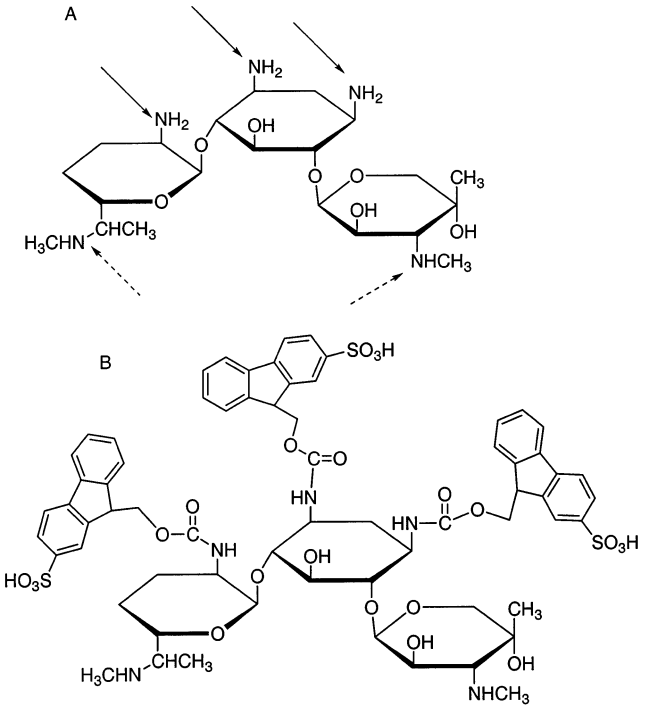

Figure 1. Structure of gentamicin C_1 (A) and the proposed structure of FMS_3-gentamicin C_1 (B). The arrows point to the five amino side chain moieties capable of reacting with FMS-OSu.

Association of FMS-gentamicin Conjugates with HSA. Figure 2 summarizes a set of experiments in which samples of gentamicin were modified with increasing concentrations of FMS-OSu to incorporate about 0.7, 1.3, 2.1, and 3.0 FMS/gentamicin. For each such treatment, the affinity toward HSA was determined by the PNPA assay (ExperimentalSection). Native gentamicin does not inhibit HSA-evoked PNPA hydrolysis, and gentamicin containing an average of 0.7, 1.3, 2.1, and 3.0 mol of FMS/mol of gentamicin inhibited HSA-evoked hydrolysis with IC_{50} values of 80 ± 3, 43 ± 4, 23 ± 2, and 19 ± 2 μM, respectively. These values correspond to K_d values of 32 ± 3, 17.2 ± 2, 9.2 ± 1, and 7.6 ± 0.7 μM, respectively. Thus, with the FMS modification, monomodified gentamicin was associated with HSA with a K_d value of 20−25 μM (see also FMS-serine), whereas with bismodified gentamicin, the affinity was increased about 2.5-fold (K_d = 9.2 ± 1.0 μM) and with the trismodified FMS derivative only slightly more (K_d = 7.6 ± 0.7 μM, Figure 2).

Rate of Hydrolysis of FMS_3-gentamicin. FMS_3-gentamicin was incubated in 0.1 M $NaHCO_3$, pH 8.5, at 37 °C. Aliquots were withdrawn at different time points and analyzed for the appearance of free amino groups with TNBS.[12] At pH 8.5, the rate of FMS hydrolysis from FMS−peptide and −protein conjugates is nearly the same as that found in vivo or in human serum in vitro.[2,3] Therefore, this pH was selected for further experiments. FMS moieties of FMS_3-gentamicin are hydrolyzed in a slow and homogeneous fashion with

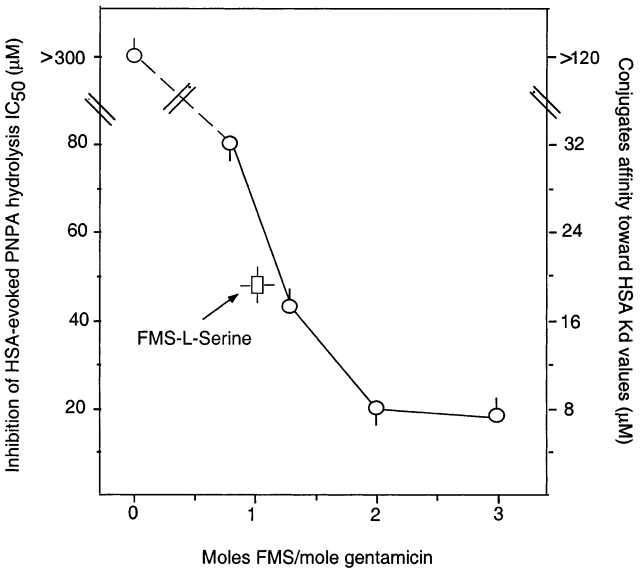

Figure 2. Increased affinity of FMS-gentamicin conjugates toward HSA as a function of FMS moieties incorporated into gentamicin. FMS-gentamicin conjugates containing 0.7−3 FMS/mol of gentamicin were assayed for their efficacy to inhibit HSA-evoked hydrolysis of *p*-nitrophenyl acetate (Experimental Section). Results are expressed as IC_{50} for each FMS-gentamicin conjugate (left *y* axis) and converted to K_d estimates (right *y* axis). The IC_{50} for FMS-L-serine (used here as an internal standard for monomodified, low-molecular-weight substance) is also included.

a $t_{1/2}$ value of 8.0 ± 0.2 h. Hydrolysis is complete after 20 h of incubation under these conditions (see Supporting Information).

Generation of the Antibacterial Potencies of FMS_1, FMS_2, and FMS_3-gentamicin. In Figure 3, gentamicin derivatives containing 1.0 ± 0.1 and 2.0 ± 0.3 FMS/mol of gentamicin, as well as FMS_3-gentamicin, were incubated in 0.1 M $NaHCO_3$ (pH 8.5) at 37 °C. Aliquots were withdrawn at different time points and were assessed for their antibacterial potencies. All three FMS-containing derivatives are nearly inactive prior to incubation (0.9−2% activity, time 0 in the figure). Upon incubation, the antibacterial potencies of FMS_1, FMS_2, and FMS_3-gentamicin are generated with $t_{1/2}$ values of 2 ± 0.3, 7 ± 0.6, and 11 ± 0.7 h, regaining the full (100%) potencies after 20 h (Figure 3A), 30 h (Figure 3B), and 40 h (Figure 3C) of incubation under these conditions.

FMS_3 -gentamicin Is a Long-Lived Species in Rats Following Subcutaneous Administration. In the set of experiments summarized in Figure 4, radiolabeled gentamicin-tBoc-Tyr, or $FMS_{2.0}$-$tBocTyr_{1.0}$-gentamicin, were administered subcutaneously to groups of rats (3 nmol of each, specific activity of 5000 ± 500 cpm/pmol, administered in 0.3 mL of PBS buffer/0.5% BSA, pH 7.4). The circulatory level of each derivative was determined at many time points after administration (Experimental Section). Administered ^{125}I-labeled tBocTyr-gentamicin peaked at 20 ± 3 min (15 ± 1 pmol/mL) and then declined with a $t_{1/2}$ value of 2 ± 0.2 h, reaching undetectable levels 20 h after administration. With administered ^{125}I-labeled $FMS_{2.0}$-$tBocTyr_{1.0}$-gentamicin, circulating levels increased more gradually, peaking at 2 h (17 pmol/mL), and then maintained at a high plateau for an additional 2 h. Subsequently, the levels declined with a $t_{1/2}$ value of 9 ± 0.3 h, reaching undetectable levels 35 h after administration (Figure 4). Thus, administered gentamicin containing 2.0 FMS/mol of gentamicin maintains prolonged circulatory levels over a period of 40 h, exceeding by 4.5 times the levels obtained after administration of non-FMS-containing gentamicin.

Correlation between Immunoreactivity and Antibacterial Potencies of FMS_3-gentamicin during FMS Hydrolysis. In the experiment summarized in Figure 5, FMS_3-gentamicin was incubated at pH 8.5, 37 °C, and aliquots were withdrawn at different time points and analyzed for both immunoreactivity and antibacterial potency (Experimental Section). FMS_3-gentamicin is immunoreactive-silent (time 0, Figure 5). Immunoreactivity, however, is elevated during incubation, fully overlapping the elevation in antibacterial potency (Figure 5). Thus, immunoreactivity correlates with the antibacterial potency of FMS_3-gentamicin during FMS hydrolysis.

FMS_3-gentamicin Is a Long-Lived Species in Rats Following Intravenous Administration. In Figure 6, rats have received intravenously native gentamicin, FMS_1, or FMS_3-gentamicin (1 μmol/rat, $n = 4$ in each group). Blood aliquots were taken at several time points, and levels of immunoreactive gentamicin were determined by the fluorescence polarization immunoassay procedure. Following native gentamicin administration, circulating levels peaked at 30 min (47 ± 4 nmol/mL blood), and then declined with $t_{1/2} = 0.9$ ± 0.1 h, reaching undetectable levels 2 h after administration (Figure 6A). With administered FMS_1-gentamicin, circulating immunoreactive gentamicin also peaked at 30 min (60 ± 7 nmol/mL blood) and declined with a $t_{1/2}$ value of 1.3 ± 0.2 h, reaching undetectable levels at 3.5 h after administration (Figure 6B). Following administration of FMS_3-gentamicin, immunoreactive gentamicin peaked at 1 h (27 ± 4 nmol/mL blood). This was followed by a flat wide peak over a prolonged period with a $t_{1/2}$ value of 3.8 ± 0.2 h, reaching undetectable levels 10 h after administration (Figure 6C), indicating the presence of circulating immunoreactive/antibacterial gentamicin over a prolonged period following intravenous administration of FMS_3-gentamicin.

Discussion

We found previously that a single moiety of FMS associates with HSA (and other albumins as well) with a K_a value of (0.5 ± 0.05) × 10^5 M^{-1}. The half-life of singly modified FMS−short peptide conjugates in vivo has not been appreciably prolonged (1.5- to 2-fold, manuscript in preparation). Albumins, however, have multiple binding sites for diverse types of ligands that are associated with this carrier protein.[13] We have therefore postulated that a small ligand containing two FMS moieties (or more) might form a more stable complex with albumin, provided a bivalent ligand is formed that is capable of associating simultaneously with two or more adjacent sites of albumin.[14]

In this study, we have applied a 10-fold excess of FMS-OSu over gentamicin and thus introduced 3 mol of FMS to 1 mol of gentamicin, with concomitant loss of the antibacterial potency (summarized in Table 1).

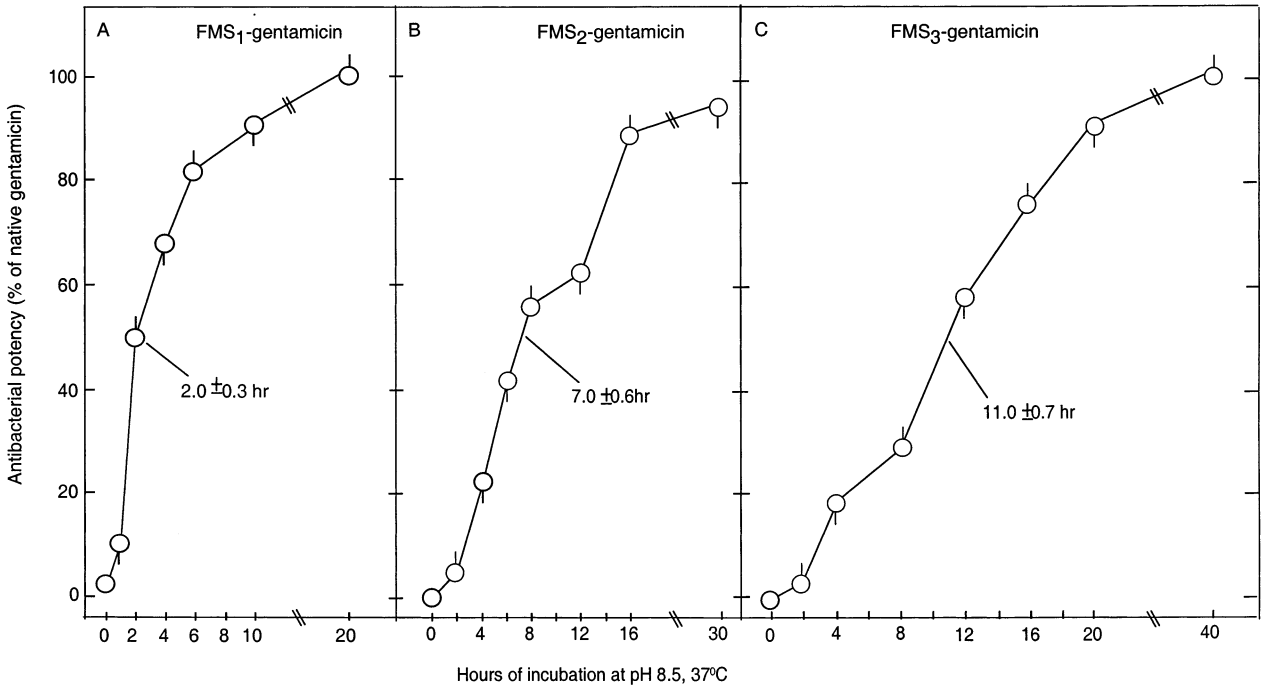

Figure 3. Time course of reactivation of FMS_1, FMS_2, and FMS_3-gentamicin. The three FMS-gentamicin derivatives (0.25 mM of each) were incubated at pH 8.5, 37 °C. Aliquots were drawn at the indicated time points and analyzed for their potency to arrest *E. coli* replication. The IC_{50} for each aliquot was determined. Native gentamicin inhibited *E. coli* replication with an IC_{50} value = 0.22 ± 0.02 μM. An FMS derivative with IC_{50} = 2.2 ± 0.2 μM in this assay was considered to have 10% the native antibacterial potency.

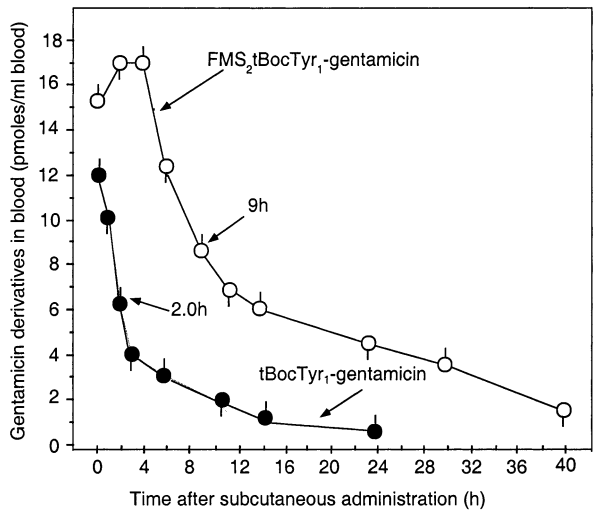

Figure 4. Pharmacokinetic profile of subcutaneously administered radiolabeled $FMS_{2.0}$-gentamicin in the rat. Comparison to non-FMS-containing gentamicin. Groups of male Wistar rats (170 ± 10 g, n = 4 in each group) received subcutaneously at 10 a.m. 0.3 mL of PBS (pH 7.4) 0.5% BSA, containing 3 nmol of either ^{125}I-labeled $tBocTyr_1$-gentamicin or ^{125}I-labeled $FMS_{2.0}$-$tBocTyr_{1.0}$-gentamicin (specific activity, 5000 ± 50 cpm/pmol). Blood aliquots from the tail vein were drawn at the indicated time points. The amount of each derivative in pmol/mL blood was estimated (Experimental Section). Each point is the arithmetic mean ± SE of four rats.

Upon incubation at pH 8.5, 37 °C, FMS moieties were hydrolyzed in a slow spontaneous fashion with a $t_{1/2}$ = 8.0 ± 0.2 h and full resumption of the antibacterial activity ($t_{1/2}$ = 11 ± 0.7 h, Supporting Information and Figure 3C).

As predicted, affinities of FMS-gentamicin conjugates toward HSA increased significantly when 2−3 mol of FMS were introduced into gentamicin. A K_d value of 7.6−9.2 μM has been obtained. Thus, bismodified gentamicin associates with HSA with a 2.5-fold higher affinity as opposed to monomodified gentamicin or to FMS-serine, suggesting that a bivalent ligand has been formed capable of associating with two adjacent sites of albumin.

We next studied whether this increase in the conjugate's affinity toward albumin is translated into a prolonged lifetime in vivo. Subcutaneously administered $FMS_{2.0}$-gentamicin compounds have maintained a prolonged elevated level of the derivative that exceeded 4.5 times that of subcutaneously administered non-FMS-containing gentamicin (Figure 4). The excellent correlation found between developed immunoreactivity and antibacterial potencies upon hydrolysis of FMS_3-gentamicin (Figure 5) further assisted us in determining pharmacokinetic profiles, which are more relevant to circulating bacterial killing potencies. Next, we have further established the short-lived character of immunoreactive gentamicin following intravenous administration of either native or FMS_1-gentamicin, and the prolonged pattern of circulating immunoreactive gentamicin following intravenous administration of FMS_3-gentamicin (Figure 6).

It is important to mention at this point that the desired pharmacokinetic pattern of circulating aminoglycosides following administration in humans is still a matter of debate among clinicians. Several types of dosing, adjustments, and monitoring were suggested for achieving maximal bacterial killing and minimal aminoglycosides-dependent kidney toxicity.[6] A pharmacokinetic profile, supplying an initial circulating peak level of >10 nmol/mL gentamicin, for inducing concentration-dependent bacterial killing, followed by a prolonged

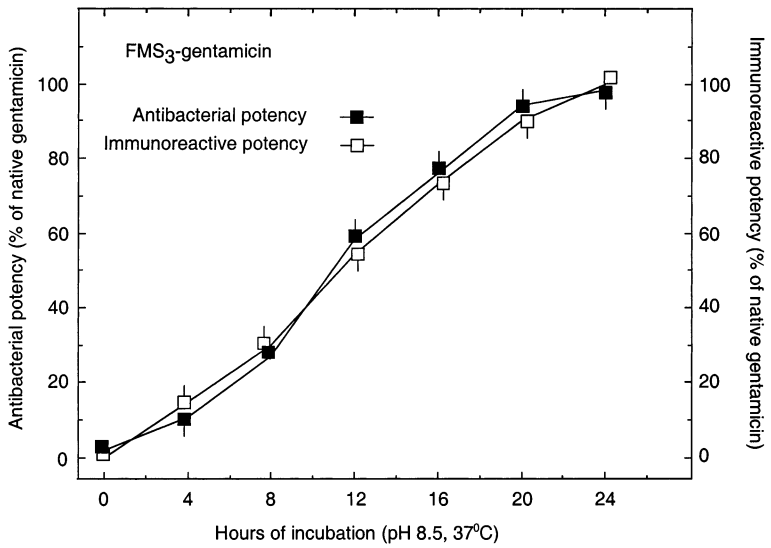

Figure 5. Correlation between immunoreactivity and antibacterial potency of FMS_3-gentamicin upon hydrolysis. FMS_3-gentamicin (0.14 μmol/mL) was incubated in 0.1 M $NaHCO_3$ (pH 8.5, 37 °C). Aliquots were drawn at the indicated time points and were analyzed for their antibacterial (■) and immunoreactive (□) potencies. Results are expressed as percent activity of native gentamicin.

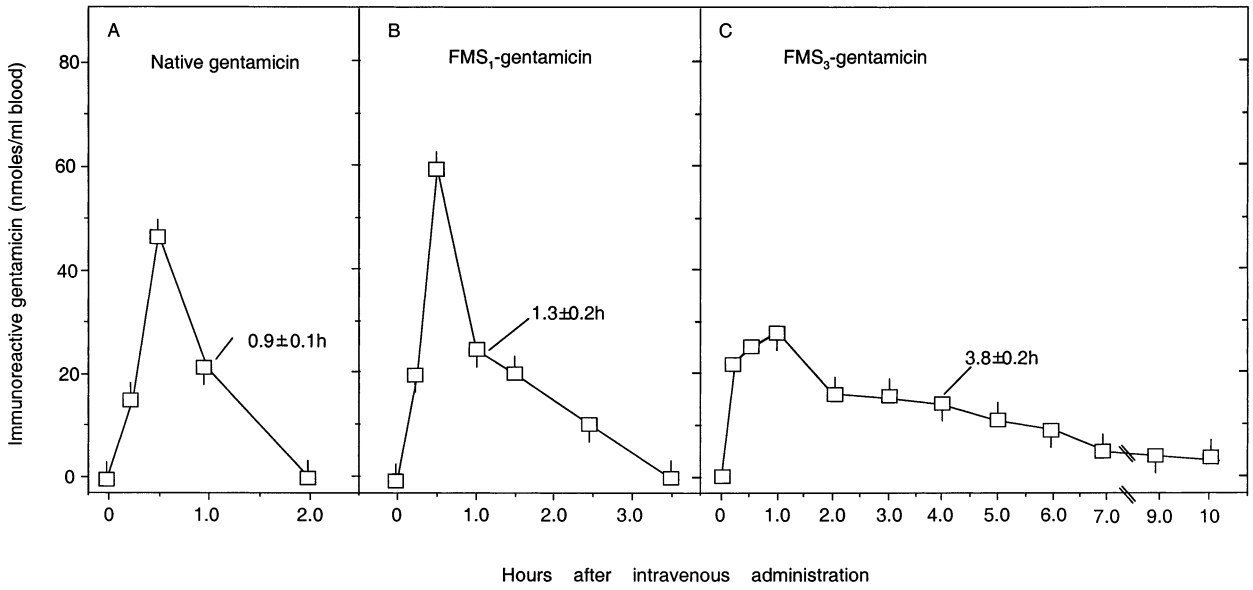

Figure 6. Intravenously administered FMS_3-gentamicin in rats facilitates prolonged circulating levels of immunoreactive gentamicin. Groups of rats ($n = 4$ for each group) were administered intravenously (1 μmol/rat) native gentamicin (A), FMS_1-gentamicin (B), or FMS_3-gentamicin (C) in 0.2 mL of PBS buffer, pH 7.4. Blood aliquots were drawn at the indicated time points and assayed for immunoreactive gentamicin. Each point is the arithmetic mean of four aliquots drawn (at the same time) from four rats.

circulatory level of >1 nmol/mL where bacterial killing continues, was recently recommended. Our pharmacological profile obtained in rats following FMS_3-gentamicin administration appears to fit well with this pattern (Figures 5 and 6C). The initial peak of >10 nmol/mL is followed by a "through" circulating level of >1.0 nmol/mL immunoreactive gentamicin that persists over a period of 9 h, as opposed to a period shorter than 2 h in native-gentamicin-administered rats.

Finally, it is of interest to compare our findings with those made on insulin−fatty acid conjugates that associate with albumin.[15] Insulin−FFA conjugates, having a K_d value toward albumin of 40−60 μM, showed protracted action in vivo. In our system, about a 5-fold higher affinity toward albumin (K_d = 7.6−9.2 μM) appeared to be required to obtain significant prolongation in situ following administration. These findings suggest that, unlike with proteins, with regard to low-molecular-weight compounds, a higher affinity toward albumin, namely, for $K_d = 8-10$ μM, must be engineered to tackle the rapid rate of clearance characterizing this drug category.

In summary, we found that our prodrug technology is applicable to the low-molecular-weight drug gentamicin as well as to the polypeptide insulin and interferon-α2.[2,3] On the basis of this study, two or three FMS per molecule should be introduced for sufficient associating affinity toward albumin to obtain a significant lifetime in situ. The loss of pharmacological potency usually accompanying derivatization is rather advantageous with this technology, provided reactivation in vivo proceeds with desired rates and profiles. This feature allows the administration of higher dosages with no fear of desensitization or intoxification. This and other

aspects brought forth in this study are currently under investigation.

Acknowledgment. We thank Elana Friedman for typing the manuscript and Yigal Avivi for editing this paper. M.F. is the Lester Pearson Professor of Protein Chemistry. Y.S. is the incumbent of the C. H. Hollenberg Chair in Metabolic and Diabetes Research, established by the friends and associates of Dr. C. H. Hollenberg of Toronto, Canada.

Supporting Information Available: Figure showing rate of hydrolysis of FMS_3-gentamicin. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Gershonov, E.; Fridkin, M.; Shechter, Y. Long-acting drugs and pharmaceutical compositions comprising them. Israel Patent WO97−IL265, 1997.
(2) Gershonov, E.; Goldwaser, I.; Fridkin, M.; Shechter, Y. A novel approach for a water-soluble long-acting insulin prodrug: Design, preparation, and analysis of [(2-sulfo)-9-fluorenylmethoxycarbonyl]$_3$-insulin. *J. Med. Chem.* **2000**, *43*, 2530−2537.
(3) Shechter, Y.; Patt, L. P.; Schreiber, G.; Fridkin, M. Prolonging the half-life of interferon-α2 in circulation: Design and analysis of [2-sulfo-9-fluorenylmethoxycarbonyl]$_7$-interferon-α2 [FMS_7-interferon-α2]. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1212−1217.
(4) Gershonov, E.; Shechter, Y.; Fridkin, M. New concept for long-acting insulin. Spontaneous conversion of an inactive modified insulin to the active hormone in circulation: 9-fluorenylmethoxycarbonyl derivative of insulin. *Diabetes* **1999**, *48*, 1437−1442.
(5) Benett, L. Z.; Mitchell, J. R.; Scheiner, L. B. In *The Pharmacological Basis of Therapeutics*; Goodman Gilman, A., Rall, T. W., Nies, A. S., Taylor, P., Eds.; Pergamon Press: New York, 1990; pp 3−32.
(6) Edson, R. S.; Terrell, C. L. The aminoglycosides. *Mayo Clin. Proc.* **1999**, *74*, 519−528.
(7) Carter, D. C.; Ho, J. X. Structure of serum albumin. *Adv. Protein Chem.* **1994**, *45*, 153−203.
(8) Merrifield, R.; Bach, A. E. 9-(2-Sulfo)fluorenylmethoxy-carbonyl chloride, a new reagent for the purification of synthetic peptides. *J. Org. Chem.* **1978**, *43*, 4808−4816.
(9) Koh, S. W.; Means, G. E. Characterization of a small apolar anion binding site of human serum albumin. *Arch. Biochem. Biophys.* **1979**, *192*, 73−79.
(10) Hunter, W. M.; Greenwood, F. C. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature (London)* **1962**, *194*, 495−496.
(11) Jolley, M. E.; Stroupe, S. D.; Wang, C.-H. J.; Panes, H. N.; Keegan, C. L.; Schmidt, R. L.; Schwenzer, K. S. Fluorescence polarization immunoassay I. Monitoring aminoglycoside antibiotics in serum and plasma. *Clin. Chem.* **1981**, *27*, 1190−1197.
(12) Habeeb, A. F. S. A. *Anal. Biochem.* **1966**, *14*, 328−333.
(13) Peters, J., Jr. Ligand Binding to Albumin. *All About Albumin: Biochemistry, Genetics and Medical Applications*; Academic Press Inc.: San Diego, CA, 1996; Chapter 3, pp 76−132.
(14) Shechter, Y. Studies on insulin receptors: Implications for insulin action. *The Receptors*; Academic Press: Orlando, FL, 1987; Vol. II, pp 221−244.
(15) Markussen, J.; Havelund, P.; Karzhals, A. S.; Anderson, J.; Halsbrom, E.; Hasselager, V. D.; Larsen, V.; Ribel, L.; Schaffer, K.; Jonassen, V. I. Soluble fatty acid acylated insulins bind to albumin and show protracted action in pigs. *Diabetologia* **1996**, *39*, 281−288.

JM020042T