Synthesis of EF24−Tripeptide Chloromethyl Ketone: A Novel Curcumin-Related Anticancer Drug Delivery System

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The blood coagulation cascade includes a step in which the soluble protein, factor VIIa (fVIIa), complexes with its transmembrane receptor, tissue factor (TF). The fVIIa/TF protein−protein complex is subsequently drawn into the cell by endocytosis. The observation that TF is aberrantly and abundantly expressed on many cancer cells offers an opportunity to specifically target those cells with an effective anticancer drug. Thus, we propose a new drug delivery system, drug-linker-Phe-Phe-Arg-mk-fVIIa, which can associate with TF on the surface of cancer cells, but release the cytotoxic agent in the cytoplasm. Synthetic procedures have been developed for the preparation of phenylalanine-phenylalanine-arginine chloromethyl ketone, (FFRck) followed by coupling with the cytotoxin EF24 and subsequently fVIIa to give EF-24-FFRmk-fVIIa. When breast cancer cells (MDA-MB-231) and human melanoma cells (RPMI-7951) are treated with the complex, the cells are arrested to a greater extent than EF24 alone by comparison with controls.

Introduction

Cancer chemotherapy is one of the important life-extending methods of treatment for cancer patients. However, the nonselective actions of many antitumor agents can cause serious side effects and great patient distress by damaging normal cells along with the target cancer cells. A large number of patients who undergo chemotherapy suffer from hair and weight loss, nausea, depression and a range of related physical and mental stress. In attempts to reduce such side effects, specific drug delivery systems (DDS) have been investigated. One promising approach employs a macromolecular prodrug,[1] a polymer−drug combining a homing device and an antitumor agent.[2] Alternatively, the use of avidin and biotin as vehicles for antibody pretargeting and drug targeting has been studied extensively in the last 10 years.[3] However, in most cases, synthesis of a well-defined macromolecular prodrug requires many reaction steps. Ouchi and co-workers recently developed a new type of DDS by formation of a complex between avidin and two separate derivatives of biotin, a fluorescent drug model and a galactose homing device conjugate. The three-component model system was shown to be internalized into hepatoma cells by means of a receptor-mediated endocytosis mechanism.[4]

Our strategy for selective anticancer drug delivery is similar to the one used by Ouchi et al., but differs in both the homing device and carrier protein. It takes its inspiration from two sets of observations: (1) In cancers, tissue factor (TF) is aberrantly expressed on endothelial cells of the tumor vasculature as well as on tumors,[5,6] and (2) TF is the cellular receptor for factor VIIa (fVIIa) in the blood coagulation cascade. Upon formation of a strong and specific TF/fVIIa complex, the blood clotting sequence is initiated,[7] and the protein−protein complex undergoes endocytosis.[8] A recent study using baby hamster kidney (BHK) cells stably transfected with TF demonstrated that about 50% of cell surface-bound fVIIa was internalized in 1 h, and a majority of the internalized VIIa was degraded shortly thereafter.[9] The un-degraded fVIIa (∼20%) is recycled back to the cell surface as an intact and functional protein. The same work also provided evidence that endocytosois of the TF/VIIa complex is an active process independent of general turnover of bulk membrane components. Other studies have shown that variants of fVIIa, either mutated at the active site[10] or inactivated with a tripeptide-chlormethyl ketone,[6,11,12] retain their binding affinity to TF, but lack the functionality to initiate clotting. Importantly, the methyl ketone FFRmk-fVIIa[13] exhibits a 5-fold greater affinity to TF relative to the native fVIIa.[11]

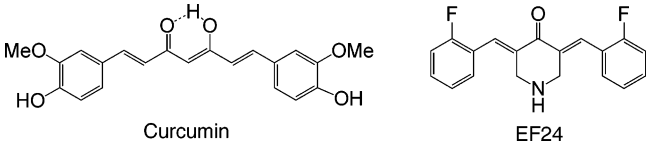

Figure 1. Structures of curcumin and EF24.

On the basis of the above observations, we contemplated using fVIIa as an anticancer drug carrier. By using fVIIa as a specific carrier, antiangiogenic and chemotherapeutic drugs can be selectively delivered to TF-expressing cancer cells as well as tumor-associated blood vessels (vascular endothelial cells) by conjugating the agents to FFRmk-fVIIa. We recently synthesized over 100 curcumin analogues and tested them for cytotoxic and antiagiogenic properties.[14] Many of the compounds are considerably more active than curcumin itself. One of the leading compounds, EF-24 (Figure 1), is not only more efficacious in anticancer screens but also considerably less toxic than cisplatin, a commonly used chemotherapeutic agent. We found that the cytotoxic effect of EF24 against MDA-MB-231 human breast and DU-145 human prostate cancer cells arises, at least in part, from the induction of cell cycle arrest and subsequent apoptosis by means of a redox-dependent mechanism.[15]

The Homing Device and Target. Banner and co-workers have reported the crystal structure of D-Phe-L-Phe-L-Arg-mk-fVIIa−TF. The fVIIa component adopts an extended confirmation that wraps around TF with the catalytic domain of the serine protease distal to the cell membrane.[16] It requires coupling of a tripeptide chloromethyl ketone (e.g. FFRck) to the protein. The use of peptide chloromethyl ketones as the coupling reagent to generate irreversible inhibitors of serine proteases was pioneered

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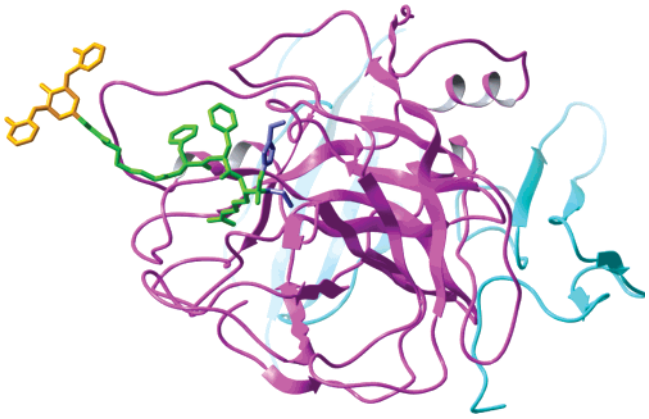
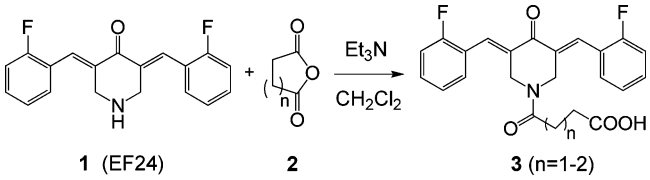


Figure 2. A heuristic model of EF24−FFRck covalently linked to fVIIa via His57 and Ser195 to give EF24−FFRmk-fVIIa; EF24, gold; linker-FFRmk, green; fVIIa, magenta; His57 and Ser195, blue; part of tissue factor in background, cyan; constructed from the fVII−TF X-ray structure.[16]

Scheme 1



by Shaw and co-workers.[17] A variety of fluorescent chloromethyl ketone reagents were also synthesized to discriminate between zymogen (inactive enzyme precursor) and the corresponding enzyme.[18] The key reaction is alklylation of the histidine in the serine protease catalytic triad. Thus, the chloromethyl carbon undergoes nucleophilic attack by the lone electron pair of the imidazole nitrogen atom, thereby anchoring the inhibitor to fVIIa (Figure 2). This complex structure serves as a model for our closely related drug delivery system.

Herein, we report the synthesis of EF24−phenylanaline-phenylanaline-arginine chloromethyl ketone (EF24−FFRck), which can be coupled with fVIIa to give the methyl ketone homing agent EF24−FFRmk-fVIIa. This protein−drug conjugate was predicted to combine with tissue factor on the surface of cancer cells to effect EF24 cancer cell targeting. A model of EF24 (gold) bound to the FFRmk linker (green) in turn bound to fVIIa is presented in Figure 2. The linker is covalently coupled to both His57 and Ser195 (blue) of fVIIa (magenta). The model structure was derived by grafting EF24 and its succinic acid tether onto the phenylalanine terminus of the FFRmk-fVIIa−TF X-ray structure[16] with the aid of computer graphics. No computational optimization has been performed. The 3-D representation is meant only to illustrate that the appended EF24 is accessible to enzymes in the cytoplasm. Not shown are the protein−protein interactions between fVIIa and tissue factor (cyan) portrayed in the crystal structure.[16]

Synthesis of EF24−FFRck. The EF24−Peptide Route. Synthesis of the precursor to the fVIIa−drug molecule conjugate, EF24−FFRck, proceeded in three steps. First, an appropriate derivative of EF24 was selected to permit attachment of the cytotoxic agent to the tripeptide chloromethyl ketone linker (FFRck). Succinic acid (**3**, $n = 1$, Scheme 1), glutaric acid (**3**, $n = 2$), and 3-methylglutaric acid (**3**, $(CH_2)_n = CH_2-CH(CH_3)$) were examined. Of these, **3** ($n = 1$, 86% yield) was chosen, since it shows two-thirds the activity of EF24 in cytotoxicity assays (see below).

The first attempt to obtain the desired product **13** was conceived as a route through FF intermediate **5**. Thus, the EF24−succinic acid derivative **3** (EF24−FFRck) was coupled with phenylalanine-phenylalanine ethyl ester **4** (Scheme 2). A variety of coupling conditions and reagents were explored as listed in Table 1. The classic coupling agents DCC or DCC with HOBt in different solvents failed to provide the desired coupling product (entries 1, 2, 3).

Compound **3**, when treated with *tert*-butyl chloroformate followed by **4**, likewise failed to achieve coupling (entry 4). However, the coupling reagent di(2-pyridyl) thionocarbonate (DPT)[19] in benzene solvent in the presence of a catalytic amount of DMAP afforded the coupling product **5** in 42% yield (entry 7). A number of other solvents were examined, but none of them proved to be superior to benzene.

As a prelude to combining the EF24−Phe-Phe unit in **5** with arginine-chloromethyl ketone **8**, it was necessary to hydrolyze ethyl ester **5** to the corresponding carboxylic acid. Unfortunately, hydrolysis under either acidic or basic conditions, at best, provided only very low yields of the desired product. It may well be that the EF24 moiety is unstable to the conditions employed.

The FFRck Route. An alternative strategy was adopted. Thus, synthesis of the tripeptide chloromethyl ketone linker **8** was performed first followed by coupling with EF24 (Scheme 3). Preparation of the chloromethyl ketone linker began with the commercially available Boc-D-Arg(Mtr)-OH, in which the arginine side chain was protected by the methoxytrimethylphenylsulfonyl group (Mtr) (**6**). The compound was treated with diazomethane to afford Boc-D-Arg(Mtr)-CHN_2 **7**. Using the modification of Kettner's method,[20] isopropyl chloroformate was employed instead of isobutyl chloroformate. Subsequently, Arg-(Mtr)-chloromethyl ketone **8** was prepared as its hydrochloride salt by treatment of diazomethyl ketone **7** with HCl in ethanol (72%) (Scheme 3).

Compound **8** was further coupled with *N*-Boc-Phe-Phe-OH (**9**) again using isopropyl chloroformate as the coupling agent and *N*-methylmorpholine as base. The reaction gave the desired tripeptide (*N*-Boc-D-Arg(Mtr)-Phe-Phe) chloromethyl ketone **10** in 72% yield. Deprotection of the Boc-group in **10**, followed by $NaHCO_3$ neutralization of the corresponding hydrochloride salt, afforded the FFR compound **11**. The latter was combined with the EF24 analogue **3** using DCC as coupling reagent and dichloromethane as solvent. The reaction delivered compound **12** in 49% yield. DPTC and isopropyl chloroformate coupling agents were also examined in this reaction, but neither resulted in improved yields by comparison with DCC. Removal of the Mtr protecting group on the arginine side chain was accomplished with 95% aqueous TFA and a small amount of anisole (Scheme 4). The final product was purified by recrystallization to afford pure **13** as a light yellow solid characterized by MS, 600 MHz NMR, and elemental analysis.

EF24−FFRmk-fVIIa Inhibits Cell Growth in Vitro. EF24−FFRmk-fVIIa was prepared by coupling **13** to fVIIa.[21] SELDI-TOF mass spectroscopic analysis showed that the conjugate carries up to two molecules of drug. We presume the chloromethyl ketone binds covalently to the histidine of the fVIIa catalytic triad as well as to a second histidine elsewhere in the protein. To demonstrate the cytotoxicity of the EF24−FFRmk-fVIIa conjugate and its TF-dependence, we examined its effect on both TF-expressing and non-TF-expressing cells. The TF-expressing cells included the MDA-MB-231 human breast cancer cell line[22] and the RPMI-7951 human melanoma cell line,[23] both of which produce high levels of VEGF and TF. We also examined human umbilical vascular endothelial

Scheme 2

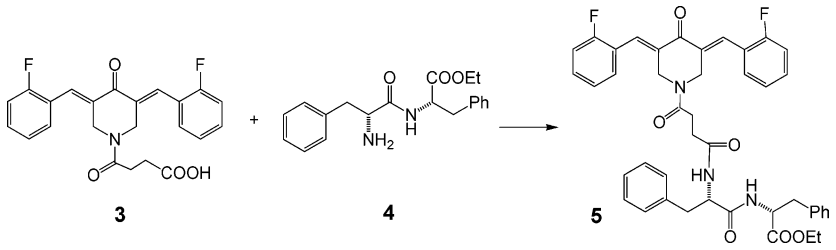
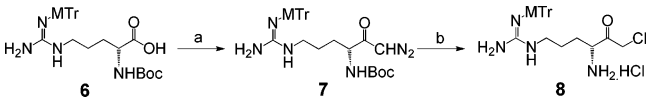


Table 1. Various Conditions Explored for the Coupling of **3** and **4** To Give **5**

entry	conditions	yield (%)[a]
1	DCC, CH_2Cl_2, rt, 24 h	0
2	DCC, HOBt, CH_2Cl_2	0
3	DCC, HOBt, THF	0
4	$ClCOOBu^t$, CH_2Cl_2	trace[b]
5	DPT[c], DMAP, CH_2Cl_2, rt	4
6[d]	DPT, DMAP, benzene, refluxing	trace[b]
7[d]	DPT, DMAP, benzene, Et_3N, rt, 10 h	42

[a] Isolated yield. [b] Results according to TLC or HPLC. [c] DPT. [d] HCl salt of **4**.

Scheme 3[a]



[a] Mtr = 2,3,6-trimethyl-4-methoxybenzenesulfonyl; reagents and conditions: (a) (i) isopropyl chloroformate, 4-methylmorpholine, (ii) CH_2N_2/ether; (b) HCl/EtOH.

cells (HUVECs) treated with phorbol ester for induction of TF. The non-TF-expressing cells employed were the normal human breast cell line MCF10, melanocytes, and HUVECs. Results from the cell viability assay demonstrate that the EF24−FFRmk-fVIIa conjugate significantly decreases the cell viability of the TF-expressing cells, MDA-MB-231 and RPMI-7951, in a dose-dependent manner, whereas the conjugate has very little effect on the non-TF-expressing cells, normal breast cells, and normal melanocytes (Figures 3a and 3b).

The conjugate also showed improved cytotoxicity against HUVECs treated with phorbol ester compared to (normal) untreated HUVECs (data not shown). However, while EF24 alone manifested the nonspecific nature of a cytotoxic drug, it is still more cytotoxic to cancer cells than to normal cells. For example, Figure 3b shows that EF24 elicits the same % cell survival against RPMI-7951 cells (i.e. 2 μM) as twice the concentration (4 μM) against normal non-TF-expressing melanocytes. EF24−FFRck, unconjugated to fVIIa, has no effect on cell viability for the cell lines tested, since it cannot bind to cell-surface TF (Figures 3a and 3b).

In another experiment, when normal non-TF-expressing MCF-10 breast cells are treated either with vehicle control or EF24−FFRck, more than 95% of the cells survive the treatments (filled circles, Figure 4). A similar effect was observed when treating human breast cancer cells (MDA-MB-231) with EF24−FFRck, indicating lack of cell penetration by the complex lacking fVIIa.

In sharp contrast, when MDA-MB-231 cells were treated with EF24 alone or EF24−FFRmk-fVIIa, most of the cells were eliminated at a concentration of 1.5 μM. They are decisively eliminated within the 1−5 μM range (diamonds and open circles, respectively, Figure 4). In the latter figure, we have chosen drug concentrations (0.8−4.0 μm) such that the benign effect on MCF-10 cells brackets the concentrations necessary to reduce % survival of cancer cells to 10−30% (1−3 μM).

For the cytotoxicity experiments with EF24−FFRmk-fVIIa, we postulate that proteases in the cytoplasm of tumor cells cleave the drug from its succinic acid linker to release EF24. It is also possible that proteolysis cleaves the distal amide bond of the succinic linker to give **3** ($n = 1$) as the active agent. Since this compound is nearly as cytotoxic as EF24 (see Experimental Section), either cleavage generates an efficacious warhead within target cells. Further studies are underway to shed light on this matter.

Conclusions

The synthesis of EF24−Phe-Phe-Arg chloromethyl ketone (EF24−FFRck, **13**) has been achieved in seven steps in 10% overall yield. The compound can be conjugated with fVIIa to produce EF24−FFRmk-fVIIa. Treatment of breast cancer and melanoma cells with EF24 and EF24−FFRmk-fVIIa shows that both compounds are effective cytotoxic agents.[21] By contrast, EF24−FFRck alone, without a mechanism for crossing the lipophilic cell membrane, is ineffective. The results strongly suggest that EF24−FFRmk-fVIIa combines with tissue factor at the cell surface, but releases its EF24 drug cargo following endocytosis and degradation similar to that observed for FFRmk-fVIIa.[9] Detailed biological results on the action of EF24−FFRmk-fVIIa in other cell lines and in vivo will be published elsewhere.[21] Additional work is underway to demonstrate that the reagent is both a selective targeting agent for a range of cancer cells and active in animal models.

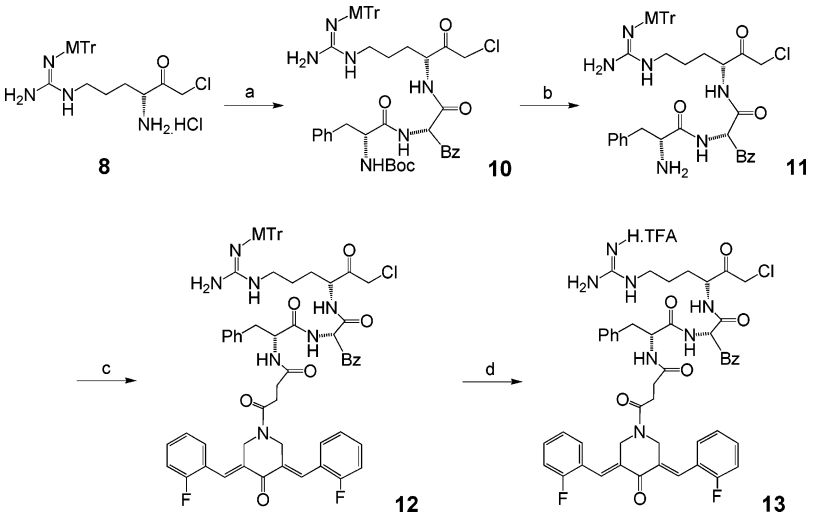
Experimental Section

General. Routine proton and carbon NMR spectra measured during synthesis were obtained on Varian Inova-400 (400 MHz) or Varian Inova-600 (600 MHz) spectrometers. Solvent for NMR was deuteriochloroform with residual chloroform (δ 7.26 for proton and δ 77.7 for carbon) taken as internal reference and reported in parts per million (ppm). TLC and preparative thin-layer chromatographies (PTLC) were performed on precoated, glass-backed plates (silica gel 60 F_{254}; 0.25 mm thickness) from EM Science and were visualized by UV lamp. Column chromatography was performed with silica gel (230−400 mesh ASTM). Elemental analyses were performed by Atlantic Microlab Inc., Norcross, Georgia. All solvents and other reagents were purchased from Aldrich Chemical Co., Milwaukee. The reagents were used as received. All reactions were performed under anhydrous nitrogen atmosphere in oven-dried glassware. Products and intermediates were stored under N_2 in the cold.

General Procedure for Preparation of EF24 Derivatives 3. To a solution of **1**[14] (0.5 mmol) in anhydrous CH_2Cl_2 (6 mL) were added anhydride **2** (0.5 mmol) and Et_3N (1 mmol). The mixture was stirred at room temperature for 3 h, diluted with CH_2Cl_2, washed twice with sat. $NaHCO_3$ (2 × 10 mL) and brine, dried over anhydrous Na_2SO_4, and relieved of solvent by evaporation. The resulting solid was purified by flash chromatography using benzene/acetone/acetic acid (27:10:0.5) as the eluent to obtain compounds **3.**

EF24 Succinic Acid Derivative (**3**, $n = 1$). To a solution of **1** (0.16 g, 0.5 mmol) in anhydrous CH_2Cl_2 (6 mL) were added

Scheme 4[a]



[a] Reagents and conditions: (a) (i) 4-methylmorpholine, isopropyl chloroformate, −20 °C, (ii) *N*-Boc-Phe-Phe-OH; (b) (i) HCl/ MeOH, (ii) $NaHCO_3$ (sat. aq); (c) 1,3-dicyclohexylcarbodiimide, CH_2Cl_2; (d) TFA/anisole.

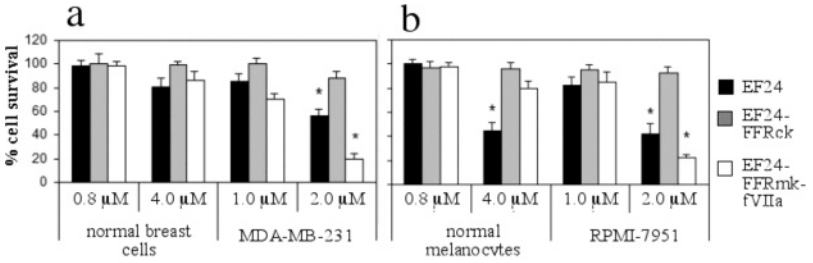


Figure 3. In vitro effects of EF24, EF24−FFRck and the EF24−FFRmk-fVIIa conjugate on normal non-TF-expressing cells and TF-producing cells; % cell survival vs control; all treatments 48 h. (a) Normal human breast cells MCF10 and the MDA-MB-231 human breast cancer cell line; (b) normal melanocytes and the RPMI-7951 human melanoma cell line; $N = 3$, $p < 0.05$ for the starred (∗) bars (Student's *t*-test).

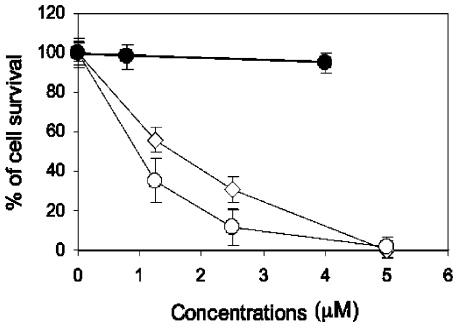


Figure 4. Filled circles: Non-TF-expressing normal MCF-10 breast cells (2×10^4/well) treated with EF24 alone, EF24−FFRck, or EF24−FFRmk-fVIIa; or MDA-MB-231 breast cancer cells exposed to EF24−FFRck; diamonds and open circles: EF24 alone and EF24−FFRmk-fVIIa treatment, respectively, of TF-expressing MDA-MB-231 breast cancer cells (2×10^4/well); $N = 3$, $p < 0.05$ (Student's *t*-test).

succinic anhydride **2** (0.057 g, 0.5 mmol) and Et_3N (0.14 mL, 1 mmol). The mixture was treated as described by the general procedure to give a yellow solid (852 mg, 86% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.43 (2H, t, $J = 6.4$ Hz), 2.56 (2H, t, $J = 6.0$ Hz), 4.59 (2H, s), 4.80 (2H, s), 7.10−7.44 (8H, m), 7.91 (2H, d, $J = 18$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): 186.41, 178.07, 170.84, 133.80, 133.66, 132.35, 132.14, 131.42, 125.13, 124.95, 117.11, 116.89, 116.78, 116.57, 46.97, 44.36, 29.62, 28.09. Anal. Calcd for $C_{23}H_{19}F_2NO_4$: C, 67.15; H, 4.66; N 3.40. Found: C, 66.88; H, 4.59; N, 3.29.

EF24 Glutaric Acid Derivative (**3** ($n = 2$)). Following the general procedure for **3** ($n = 1$), compound **3** ($n = 2$) was obtained as yellow solid in almost yield. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 1.64 (2H, t, $J = 7.2$ Hz), 2.15 (2H, t, $J = 7.6$ Hz), 2.28 (2H, t, $J = 7.4$ Hz), 4.82 (4H, d, $J = 7.6$ Hz), 7.44−7.49 (4H, m), 7.62−7.68 (4H, m), 7.83 (2H, d, $J = 10$ Hz). Anal. Calcd for $C_{24}H_{21}F_2NO_4$: C, 67.76; H, 4.98; N 3.29. Found: C, 67.58; H, 4.91; N, 3.25.

EF24 3-Methylglutaric Acid Derivative (**3** (($CH_2)_n = CH_2CH(CH_3)$). Following the general procedure for **3**, compound **3** (($CH_2)_n = CH_2CH(CH_3)$) was obtained as a yellow solid in quantitative yield. Anal. Calcd for $C_{25}H_{23}F_2NO_4$: C, 68.34; H, 5.28; N 3.19. Found: C, 68.04; H, 5.22; N, 3.18.

EF24−Phe-Phe-OEt (5). Di(2-pyridyl) thionocarbonate (DPT) (60 mg, 0.26 mmol) and DMAP (3.15 mg, 0.026 mmol) were added to **3** ($n = 1$) (107 mg, 0.26 mmol) in benzene (5 mL) at room temperature. The mixture was stirred at this temperature for 30 min during which time the solution turned yellow. A solution of Phe-Phe-ethyl ester hydrochloride (100 mg, 0.26 mmol) was added to the reaction mixture followed by triethylamine (0.26 mmol) in CH_2Cl_2 (2 mL). After stirring at room temperature for 8 h, evaporation, and purification by chromatography using ethyl acetate as eluent compound **5** was obtained as a yellow solid (80 mg, 42%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.13 (2H, t, $J = 7.2$ Hz), 2.27−2.51 (4H, m), 2.83−3.81 (4H, m), 4.03 (2H, dq, $J = 2$ Hz, 7.2 Hz), 4.45−4.84 (6H, m), 6.31 (1H, d, $J = 8$ Hz), 6.80 (1H, d, $J = 8$ Hz), 6.92−7.45 (18H, m), 7.88 (2H, d, $J = 7.9$ Hz). MS Calcd for $C_{43}H_{41}F_2N_3O_6$ 733.3, Found 740.3 $[M + Li]^+$.

Boc-Arg(MTr)CHN$_2$ (7). Commercially available Boc-Arg-(Mtr)-OH (**6**, 486 mg, 0.25 mmol) was dissolved in THF (2 mL) and allowed to react with isopropyl chloroformate (1.0 M in toluene, 0.25 mL, 0.25 mmol) in the presence of *N*-methylmorpholine (0.027 mL, 0.25 mmol) for 4 h at −20 °C. The mixture was filtered, and the filtrate was added to 4 mL of ethereal diazomethane. After stirring the reaction solution for 1 h at 0 °C, the solvent was evaporated to obtain the crude product as white needles. These were purified by chromatography using EtOAc as the eluent to obtain **7** as a white solid (60 mg, 47% yield):^{1}H NMR (400 MHz, $CDCl_3$)

δ 1.41 (9H, s), 1.50−1.76 (4H, m), 2.12 (3H, s), 2.60 (3H, s), 2.67 (3H, s), 3.16−3.28 (2H, m), 3.82(3H, s), 5.52(1H, d, $J = 8.8$ Hz), 5.54 (1H, s), 6.18−6.31(3H, m), 6.53 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): 159.14, 157.02, 156.65, 139.20, 137.23, 134.06, 125.53, 112.38, 80.92, 61.09, 56.12, 54.80, 41.42, 36.20, 30.86, 28.99, 25.84, 24.81, 21.74, 19.01, 14.87, 12.63. IR(cm $^{-1}$): 3443, 3343, 2976, 2937, 2108, 1710, 1625, 1586, 1556, 1463, 1366, 1309, 1258, 1170, 1119. Anal. Calcd for $C_{22}H_{34}N_6O_6S$: C%, 51.75; H%, 6.71; N%, 16.46. Found: C%, 52.09; H%, 6.86; N%, 15.56.

H-Arg(Mtr)CH_2Cl·HCl (8). Boc-Arg(MTr)CHN_2 (**7**, 51 mg, 0.1 mmol) was dissolved in a minimum volume of THF (0.1 mL) and allowed to react with ethanolic HCl (0.5 mmol; from a 1:1 mixture of acetyl chloride and ethanol) at room temperature until nitrogen evolution ceased (30−40 m). The solvent was removed by evaporation at room temperature, and the residue was taken up in 0.93 mL of 1.8 N ethanolic HCl under N_2. After stirring the solution for 30 min at room temperature, 50 mg of a white solid, **8**, was obtained by evaporating the solvent. The solvent was removed in vacuo over a 24 h period, and the resulting product was used in subsequent reactions without further purification (80−100%).

***N*-Boc-Phe-Phe-Arg(Mtr)-CH_2Cl (10).** *N*-Boc-Phe-Phe-OH **9** (197 mg, 0.4 mmol) was allowed to react with *N*-methylmorpholine (0.04 mL, 0.4 mmol) and isopropyl chloroformate (1.0 M in toluene, 0.4 mL, 0.4 mmol) for 10 min at −20 °C. Cold THF (5.72 mL) containing Et_3N (0.056 mL, 0.4 mmol) was added to the mixture which was immediately added to Arg(Mtr)CH_2Cl.HCl **8** (200 mg, 0.4 mmol) dissolved in DMF (0.92 mL). After stirring for 1 h at −20 °C and 2 h at room temperature, THF (5.6 mL) was added and the mixture was filtered. The filtrate was evaporated and the solid residue purified by column using EtOAc/hexanes (4:1) as the eluent. Compound **10** was obtained as a white solid (0.18 g, 53% yield): Anal. Calcd for $C_{40}H_{53}ClN_6O_8S$: C, 59.06; H, 6.57; N, 10.33. Found: C, 58.29; H, 6.59; N, 9.95.

Compound **10** (0.05 mmol, 42.5 mg) was dissolved in EtOAc (0.16 mL) and allowed to react with methanolic HCl (0.85 mmol) at room temperature for 3.5 h. The reaction mixture was then poured into ice−water, neutralized with $NaHCO_3$ (s), extracted with $CHCl_3$ (3×10 mL), dried with $MgSO_4$, and concentrated in vacuo to furnish a white solid, FFRck **11** (40 mg), which was used for the next step without further purification.

EF24−Phe-Phe-Arg(Mtr) Chloromethyl Ketone (12). To a mixture of **11** (24 mg, 0.032 mmol) and **3** (12 mg, 0.03 mmol) in CH_2Cl_2 (0.6 mL) was added fresh solid DCC (6.18 mg, 0.03 mmol). A precipitate of *N*,*N*′-dicyclohexylurea started to separate after a period of time. After stirring overnight, evaporation of the solvent, and purification by column chromatography (silica gel; ethyl acetate and hexane, 4:1), **12** was obtained as a light yellow solid. (10 mg, 49% yield). The compound was deprotected as described below to give **13** without further characterization.

EF24−Phe-Phe-Arg Chloromethyl Ketone·TFA (13). Compound **12** (34 mg, 0.03 mmol) was dissolved in 95% aqueous TFA (0.95 mL) and treated dropwise with thioanisole (0.05 mL). The resulting dark solution was stirred for 48 h at room temperature and then concentrated under vacuum. The resulting solid was triturated with ether, recrystallized with $CHCl_3$/ether, and dried under a vacuum to supply compound **13** as a light yellow solid (12 mg, 45% yield). Anal. Calcd for $C_{50}H_{51}ClF_5N_7O_8$: C, 58.71; H, 4.99; N, 9.59. Found: C, 58.91; H, 5.20; N, 8.89; *m*/*z* (FAB^+) 894.4 $(M + H)^+$.

Conjugation of EF24−FFRck (13) and Factor VIIa. Details of the method will be published elsewhere.[21]

Characterization of EF24−FFRmk-fVIIa. The concentrate was tested for cytotoxic activity as described below. The drug conjugate composition was estimated at 1−2 mol of EF24 drug/fVIIa based on mass spectroscope analysis (performed by the Micro-chemical Facility, Winship Cancer Institute, Emory University). Thus, unmodified fVIIa and the EF24−FFRck-modified fVIIa protein were mass analyzed (0.2% mass accuracy) using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)[24] with bovine serum albumin as an external standard for calibration. The mass difference found (+1978 Da) between the base peak of unmodified protein (52,392 Da) and modified protein (54322 Da) is consistent with the attachment of up to two EF24−FFR molecules per molecule of fVIIa (expected mass difference of 1714 Da).

Cell Culture.[14,22,23] Human breast cancer cells (MDA-MB-231), human melanoma cells (RPMI-7951), and human mammary gland cells (MCF-10) were purchased from American Type Cell Collection (ATCC: Rockville, MD). Human umbilical vascular endothelial cells (HUVECs), its medium EGM-2 BlueKit System (CC-3162), human melanocytes, its medium, and the MGM-2 BlueKit System (CC-3143) were obtained from Clonetics (San Diego, CA). MEM-alpha medium, RPMI 1640 medium, MEM with Earle's BSS, D-MEM/F12 (a 1:1 Dulbecco's modified Eagle's medium: Ham′ F12) medium, penicillin, streptomycin, and L-glutamine were purchased from GIBCO−BRL/Invitrogen (Rockville, MD) and Mediatech (Herndon, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA). The MDA-MB-231 cells were maintained in MEM-alpha medium and RPMI-1640, respectively. RPMI-7951 cells were maintained in MEM (Eagle) with 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and Earle's BSS. All media contained 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and 2 mM L-glutamine. MCF-10 mammary gland cells were maintained in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium and 5% horse serum. HUVECs and human melanocytes were maintained in EGM-2 BlueKit System (CC-3162) and MGM-2 BlueKit System (CC-3143) containing 2% FBS and no FBS, respectively, and both containing penicillin (100 units/mL) and streptomycin (100 μg/mL), respectively. Cells were incubated at 37 °C in 5% CO_2, 95% air in a humid atmosphere.

NR and SBR Viability Assays. Neutral Red (NR) Dye Cell Viability Assay. Cell viability following 48 h drug treatment was assayed by using the NR dye.[14,15,25] The latter is taken up by viable cells. Briefly, at the termination of culture, existing medium was removed and 200 μL of fresh, warm medium containing 50 μg of NR/mL was added to each well in a 96-well plate. Cells were incubated at 37 °C for 30 min, followed by two washes with 200 μL of PBS. The NR taken up by cells was dissolved by adding 200 μL of 0.5 N HCl containing 35% ethanol. Then, the amount of the dye in each well was read at 570 nm with an EL800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Tustin, CA). Each experiment was performed in triplicate and analyzed by Student's *t*-test; $N = 3$, $p < 0.05$. As applied to EF24 and derivatives **3**, dose−response curves using MDA-MB-231 breast cancer cells provide EC_{50}s of 2.0 (EF24), 3.0 (**3** ($n = 1$)), 3.7 (**3** ($n = 2$)), and 3.8 μM (**3**, $(CH_2)_n = CH_2CH(CH_3)$; i.e., 3-methylglutaric acid analog). For the curves expressing % cell survival against concentration, see Figure S1 in the Supporting Information.

Sulforhodamine B (SRB) Cell Viability Assay.[26] Cells were treated with drugs for 48 h and then fixed in TCA at 4 °C for 1 h, washed with tap water five times, and air-dried. Then, the cells were incubated in SRB solution (100 μL) at 0.4% (w/v) in 1% acetic acid for 10 min at room temperature. The unbound dye was removed by washing five times with 1% acetic acid, and the cells were air-dried. Bound dye was subsequently solubilized with 200 μL of 10 mM trizma base, and the absorbance was read on a 96-well plate reader at a wavelength of 490 nm by an EL800 Universal Microplate Reader. Each experiment was performed in triplicate and analyzed by Student's *t*-test; $N = 3$, $p < 0.05$.

Acknowledgment. We are grateful to Andrew Prussia for preparing the graphical model shown in Figure 2.

Supporting Information Available: Dose−response curves for the % cell survival for EF24 and compound series **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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