Role of Cross-Linking Agents in Determining the Biochemical and Pharmacokinetic Properties of Mgr6−Clavin Immunotoxins

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Several immunotoxins (ITs) were synthesized by the attachment of clavin, a recombinant toxic protein derived from *Aspergillus clavatus*, to the monoclonal antibody Mgr6 that recognizes an epitope of the gp185^{HER-2} extracellular domain expressed on breast and ovarian carcinoma cells. Conjugation and purification parameters were analyzed in an effort to optimize the antitumor activity and stability of the ITs in vivo. To modulate the in vitro and in vivo properties of the immunotoxins, different coupling procedures were used and both disulfide and thioether linkages were obtained. Unhindered and hindered disulfide with a methyl group linkage ethyl *S*-acetyl 3-mercaptopropionthioimidate ester hydrochloride (AMPT) or ethyl *S*-acetyl 3-mercaptobutyrothioimidate ester hydrochloride (M-AMPT) were obtained by reaction with recombinant clavin, while the monoclonal antibody Mgr6 was derivatized with ethyl 3-[(4-carboxamidophenyl)dithio]propionthioimidate ester hydrochloride (CDPT). To achieve higher hindrance (a disulfide bond with a geminal dimethyl group), Mgr6 was derivatized with the *N*-hydroxysuccinimidyl 3-methyl-3-(acetylthio)butanoate (SAMBA) and clavin with CDPT. To evaluate the relevance of the disulfide bond in the potency and pharmacokinetic behavior of the ITs, a conjugate consisting of a stable thioether bond was also prepared by derivatizing Mgr6 with the *N*-hydroxysuccinimidyl ester of iodoacetic acid (SIA) and clavin with AMPT. The immunotoxins were purified and characterized using a single-step chromatographic procedure. Specificity and cytotoxicity were assayed on target and unrelated cell lines. The data indicate that the introduction of a hindered disulfide linkage into ITs has little or no effect on antitumor activity and suggest that disulfide cleavage is essential for activity; indeed, the intracellularly unbreakable thioether linkage produced an inactive IT. Analysis of IT stability in vitro showed that the release of mAb by incubation with glutathione is proportional to the presence of methyl groups and increases exponentially with the increase in steric hindrance. Analysis of the pharmacokinetic behavior of ITs in Balb/c mice given intravenous bolus injections indicated that ITs with higher in vitro stability were eliminated more slowly; i.e., the disulfide bearing a methyl group doubled the β-phase half-life (from 3.5 to 7.1 h) compared with that of the unhindered, while a geminal dimethyl protection increased the elimination phase to 24 h. The thioether linkage showed its intrinsic stability with a β-phase half-life of 46 h. The thioether linkage also increased the distribution phase from 17 to 32 min. The in vitro characteristics and in vivo stability of Mgr6−clavin conjugates composed of a methyl and dimethyl steric hindered disulfide suggest clinical usefulness.

INTRODUCTION

The potent and highly specific cytocidal activity of conjugates between monoclonal antibodies and toxins has raised the promise of effective immunotherapy for tumors and graft-versus-host disease. ITs[1] have particular potential in the treatment of leukemias and lymphomas (*1*, *2*), although some evidence suggests they are effective against solid tumors as well (*3*, *4*).

Extremely active ITs have been constructed by covalently linking mAb to bacterial toxins such as *Pseudomonas* exotoxin or to plant toxins such as the ricin A chain, saporin, and gelonin (*5*−*8*).

Other interesting ribosome-inactivating proteins (RIPs) with potent antitumor activity such as α-sarcin, restrictocin, and mitogillin have been isolated from fungi and used to prepare ITs (*9*−*11*). RIPs of fungi are extensively homologous in their primary structure (>80%) and have the same catalytic activity, i.e., hydrolysis of the phosphodiester linkage between the G residue at position 4325 and the A residue at position 4326 in 28S rRNA (*12*).

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[1] Abbreviations: ITs, immunotoxins; AMPT, ethyl *S*-acetyl-propionthioimidate; M-AMPT, ethyl *S*-acetyl-3-mercaptobutyrothioimidate; CDPT, 3-[(4-carboxamidophenyl)dithio]propionthiomidate; SAMBA, *N*-hydroxysuccinimidyl 3-methyl-3-(acetylthio)butanoate; SIA, *N*-hydroxysuccinimidyl ester of iodoacetic acid; RIP, ribosome-inactivating protein; DTNB, Ellman's reagent; DTT, dithiothreitol; TNB, 5-mercapto-2-nitrobenzoic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GSH, reduced glutathione; SMPT, 4-[(succinimidyloxy)carbonyl]-α-methyl-α-(2-pyridyldithio)toluene.

Recently, Parente et al. (*13*) described the cloning and expression of a new RIP, clavin, from *Aspergillus clavatus*. Clavin was highly active on free ribosomes, inducing low and transient systemic toxicity and a late, low-level antibody response in mice. These characteristics and its availability in large amounts identify clavin as a good candidate for IT preparation.

To selectively target clavin to breast and ovarian carcinoma cells, we used a mAb (Mgr6) that recognizes an epitope of the $gp185^{HER-2}$ extracellular domain (*14*). The tissue distribution of $gp185^{HER-2}$ in normal human tissues is restricted, and overexpression of the HER-2 oncogene is associated with poor prognosis in breast and ovarian carcinoma patients (*15*, *16*). Existing ITs directed to $gp185^{HER-2}$ have already shown immunotherapeutic promise (*17*–*19*).

In this study, we analyzed the conjugation and purification parameters that might yield efficient and stable ITs suitable for preclinical studies.

We prepared various ITs and characterized them with respect to coupling systems (disulfide or thioether linkage). In fact, most of the ITs prepared to date have a reducible disulfide bond which is required for the intracellular release of the toxic moiety (*20*). The major in vivo limitations of such ITs rest in the instability of the chemical linkage, which restricts the amount of conjugate that is able to bind to target cells (*21*, *22*), and in the potential competition of the released antibody with the intact conjugate since the mAb persists longer in circulation. Previously released antibody may mask tumor antigens, thereby compromising IT potency during multiple-dose treatment regimens.

In an effort to prolong the in vivo half-life of ITs and enhance their antitumor potency, we prepared Mgr6–clavin conjugates using the thioimidate cross-linking system which preserves the positive charge on the derivatized proteins and allows modulation of the hindrance on the chemical linkage between clavin and the mAb.

EXPERIMENTAL PROCEDURES

Materials. Anhydrous dichloromethane, Ellman's reagent (DTNB), and all other reagents were from Aldrich (Milwaukee, WI).

Diethyl ether was distilled from lithium aluminum hydride, and dry tetrahydrofuran was obtained by distillation from sodium.

General Procedures. Melting points were determined with a Reichert Kofler apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a JEOL PMX-60 spectrometer, operating at 60 MHz, with tetramethylsilane as the internal standard. IR spectra were obtained as KBr disks on a Shimadzu FT-IR 8101 M spectrophotometer; wavelengths are given in inverse centimeters. Mass spectra were obtained with a FINNIGAN-MAT TSQ-700 or with a VG Analytical 70-70 EQ-HF spectrometer. Ultraviolet spectra were recorded on a Beckman DU-70 spectrophotometer.

Reactions were checked on F_{254} silica gel precoated sheets (Merck, Milan, Italy). Purification was carried out by column flash chromatography on silica gel 60 (Merck, 230–400 mesh).

Synthesis of Cross-Linkers. *Preparation of Thioimidate Ester Hydrochlorides (CDPT, AMPT, and M-AMPT).* Hydrogen chloride gas was bubbled through ice-cold ethanethiol (3.25 mL, 0.0435 mol) for 1 h. The as described by Arpicco (*23*), were diluted in anhydrous diethyl ether or dry tetrahydrofuran and quickly added to the cold solution with stirring, and the reaction mixture was left overnight at 0 °C. Anhydrous cold diethyl ether was then added, and the reaction mixture was left at −20 °C until a crystalline solid formed. The supernatant was decanted, and the precipitate was washed with anhydrous diethyl ether under argon and dried under reduced pressure at room temperature.

Ethyl 3-[(4-carboxamidophenyl)dithio]propionthioimidate ester hydrochloride (CDPT): yield 0.64 g (65%); mp 110–115 °C; ^{1}H NMR (CD_3OD) δ 7.85 (q, 4H, Ar-H), 3.4–3.15 (m, 6H, SCH_2CH_3 and CH_2CH_2), 1.47 (t, 3H, SCH_2CH_3); IR (KBr) 3500–2200 (NH_2 and NH_2^+), 1650 (C=O), 1620 (C=N); MS (FAB^+) 301 ($M^+ + 1$).

Ethyl S-acetyl 3-mercaptopropionthioimidate ester hydrochloride (AMPT): yield 0.87 g (88%); mp 64–66 °C; ^{1}H NMR ($CDCl_3$) δ 3.48 (q, 2H, SCH_2CH_3), 3.26 (m, 4H, CH_2CH_2), 2.38 (s, 3H, SAc), 1.45 (t, 3H, SCH_2CH_3); IR (KBr) 3300–2400 (NH), 1690 (C=O), 1620 (C=N), 1360 (SAc).

Ethyl S-acetyl 3-mercaptobutyrothioimidate ester hydrochloride (M-AMPT): yield 0.79 g (75%); mp 112 °C; ^{1}H NMR ($CDCl_3$) δ 3.4–3.2 (m, 5H, SCH_2CH_3, CH, and CH_2), 2.43 (s, 3H, SAc), 1.6 (d, 3H, CH_3), 1.43 (t, 3H, SCH_2CH_3); IR (KBr) 3300–2400 (NH), 1690 (C=O), 1620 (C=N), 1360 (SAc); MS-EI *m/z* (relative intensity) 206 (M^+, 7), 162 (100), 145 (53), 130 (20), 102 (65), 89 (20), 75 (33), 61 (52), 43 (100).

Preparation of N-Hydroxysuccinimidyl 3-Methyl-3-(acetylthio)butanoate (SAMBA). The method of Carrol (*24*) was used with minor modifications. Briefly, 3-methyl-3-(acetylthio)butanoic acid (1.5 g, 0.0085 mol) dissolved in 12 mL of dry dichloromethane was mixed with 1.3 g of NHS (0.011 mol) in 5 mL of dichloromethane, and dicyclohexylcarbodiimide (2.3 g, 0.011 mol) dissolved in 1 mL of dry dichloromethane was added dropwise. The reaction mixture was stirred at room temperature for 22 h. After filtration and evaporation, the mixture was purified by flash chromatography with elution in hexane/EtOAc (80/20). The ester was obtained as a pale yellow oil that crystallized rapidly at room temperature: yield 1.6 g (70%); mp 63 °C; TLC (80/20 hexane/EtOAc) R_f = 0.15; ^{1}H NMR ($CDCl_3$) δ 3.23 (s, 2H, CH_2), 2.80 (s, 4H, NHS ester), 2.25 (s, 3H, SAc), 1.6 (s, 6H, CH_3); MS-EI *m/z* (relative intensity) 273 (10), 159 (90), 117 (30), 75 (15), 43 (55).

The *N*-hydroxysuccinimidyl ester of iodoacetic acid (SIA) was prepared as described (*25*).

Clavin and Mgr6 Production. For clavin production, a 100 mL culture of *Escherichia coli* HB101 cells carrying the pMRS38 plasmid was used to start the final cultivation step in a Chemap bioreactor with a working volume of 1.5 L. The cultivation was performed in complex medium [20 g/L glucose, 25 g/L yeast extract, 40 g/L casamino acid (Difco), 0.5 g/L NaCl, 5 g/L KCl, 2.6 g/L K_2SO_4, 0.86 g/L $MgCl_2 \cdot 6H_2O$, 6.6 mg/L $CaCl_2 \cdot 6H_2O$, and trace amounts of oligo elements], supplemented with 100 mg/mL ampicillin. Cultivation parameters such as temperature, pH, and oxygen dissolved in tension (DOT) were computer-controlled. The culture supernatant was recovered by centrifugation and filtered on a Millipore 0.22 μm filter. Clavin was purified as described (*13*).

Mgr6 hybridoma, directed against the extracellular domain of $gp185^{HER-2}$, was obtained by immunization of Balb/c mice with the adenocarcinoma cell line CaLu3, and

hollow fiber bioreactor (Acusyst R, Endotronics) using a serum-free medium. Medium, prepared in our facilities, consisted of a basal mixture (1/1) of RPMI 1640 and DMEM supplemented with transferrin, insulin, albumin, and Ex-Cite (Miles Inc.) as a lipid source. Additional ingredients were 2-mercaptoethanol, ethanolamine, and sodium selenite.

Cell cultivation was performed as continuous perfusion for approximately 1 month, during which parameters such as glucose and lactic acid concentrations, pH, oxygen and carbon dioxide levels, and antibody production were monitored along with operator responses.

Conditioned media harvested during cultivation were pooled after thawing and adjusted to pH 5.5. Material was diluted and filtered on 4.5 μm filters (Millipore Co., Bedford, MA) before being loaded on a Bakerbond Abx column (Mallinckrodt Baker B. V., Deventer, The Netherlands). The eluted protein peak was desalted on a Sephadex G-25 (Pharmacia-Biotech, Uppsala, Sweden) column and loaded onto a Q-Sepharose FF column (Pharmacia-Biotech). The eluted peak was desalted as indicated above and subjected to the final purification step on a S-Sepharose FF column (Pharmacia-Biotech). Elution was obtained by applying a linear gradient from 20 to 200 mM NaCl in 20 mM phosphate buffer.

Preparation of Immunotoxins. *Disulfide Bridge.* In a standard preparation, a recombinant clavin solution (357 μM, 1 mL) was stirred with AMPT (122 mM, 30 μL dissolved in ethanol), M-AMPT (98 mM, 30 μL dissolved in ethanol), or CDPT (139 mM, 40 μL in dry dimethylformamide) for 30 min at 25 °C to incorporate an average of 1−1.3 linkers per mole of clavin. The mixture was purified by gel filtration on a 1 × 20 cm Bio-Gel P6-DG column (Bio-Rad, Hercules, CA) eluted with HBS (0.1 M, 0.2 M NaCl and 1 mM EDTA disodium salt at pH 7.4) at 20 °C. The protein solution was concentrated to 1 mL with an Amicon concentrator (Amicon, Beverly, MA) equipped with a Y10 membrane.

mAb Mgr6, dissolved in HBS, was separately reacted with two different cross-linkers, CDPT and SAMBA. The reaction of Mgr6 with CDPT proceeded as follows: CDPT in dry dimethylformamide (13 mM, 40 μL) was added to the mAb in solution (33 μM, 1 mL), and the mixture was stirred for 30 min at 25 °C. The Mgr6/CDPT molar ratio was 1/1.1. Derivatization of Mgr6 with SAMBA (11 mM, 30 μL) dissolved in ethanol was carried out for 30 min at 25 °C to introduce two acetylthio groups. In both cases, the mixture was purified by gel centrifugation on a 15 × 55 mm Bio-Gel P6-DG column preequilibrated in HBS at 20 °C.

The number of thioacetylated groups linked to the protein was calculated spectrophotometrically by reaction of the sample with the deacetylating reagent hydroxylamine hydrochloride (0.5 M, 12.5 mM EDTA, pH 7.4) followed by thiol disulfide exchange with DTNB as described (*26*).

The number of aryldithio groups linked to Mgr6 or clavin was determined following the release of the thiolated anion at 313 nm, after incubation of the protein sample (1 mL) with 2-mercaptoethanol in PBS/EDTA (11 mM sodium phosphate and 3 mM EDTA, 50 μL) and NaOH (1 M, 40 μL), to a final pH of 8.8−9.4. The molar absorptivity value of the 4-carboxamidophenylthiolate anion under these conditions at 313 nm was 15 200 ± 300 M^{-1} cm^{-1}.

All conjugations were performed by mixing the derivatized mAb and RIP in the presence of a solution of hydroxylamine (1/10, v/v). The conjugation reactions proceeded for 18 h at 4 °C; at the end of the reaction, a solution of *N*-ethylmaleimide (20 mM, 20 μL) was added to block free thiol groups.

Thioether Bridge. Clavin (357 μM, 1 mL) was derivatized with AMPT as described above; Mgr6 (33 μM, 1 mL) was reacted with SIA (5.7 mM, 30 μL) dissolved in ethanol, for 30 min at 25 °C. The iodoacetyl groups inserted on Mgr6 were identified by reaction with the TNB (5-mercapto-2-nitrobenzoic acid) reagent previously prepared; briefly, 25 mg of dithiothreitol (DTT) was added to 50 mg of DTNB dissolved in 300 μL of 1 M aqueous NaOH under an inert atmosphere. After 30 min, the reaction mixture was acidified with 1 M HCl and the mixture was extracted with diethyl ether under an inert atmosphere. The organic layers were then anhydrificated and evaporated under reduced pressure. The residue was washed several times with ethanol to obtain TNB as a dark yellow product.

The Mgr6 derivatization degree was determined by reaction of mAb (3.3 μM, 500 μL) with TNB dissolved in ethanol (3.4 mM, 10 μL) for 90 min at 25 °C. After purification by gel centrifugation, the number of iodoacetyl groups was determined spectrophotometrically (TNB molar absorption coefficient was 8800 M^{-1} cm^{-1} at 339 nm). The mean derivatization degree was 1.7.

The derivatized mAb and RIP were mixed in the presence of a solution of hydroxylamine (1/10, v/v). The conjugation reactions proceeded for 18 h at 4 °C; at the end of the reaction, a solution of *N*-ethylmaleimide (20 mM, 20 μL) was added to block free thiol groups.

Immunotoxin Purification. Conjugates were purified from unconjugated clavin and Mgr6 in a one-step procedure using CM MemSep cartridges (1000 or 1010) (Millipore) for analytical or preparative application and a Merck-Hitachi 655A-12 HPLC gradient system equipped with an L-5000 LC controller. The eluting fractions were monitored at 280 nm using a L4000 UV detector. Peak heights were recorded and processed on a CBM-10A Shimadzu interface. The mobile phase was sodium acetate buffer (20 mM at pH 5.5) flushed at 1 mL/min. Fractions containing immunoconjugates were dialyzed and concentrated. The purity of the immunotoxins was monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using 4−15% precast gels (Bio-Rad) under nonreducing conditions and Coomassie blue staining.

In Vitro Evaluation of Disulfide Bond Stability. The in vitro stability of the bond in the various conjugates was evaluated as follows. Samples (1 mg/mL, 3 μL) were incubated for 1 h at 37 °C with solutions of reduced glutathione (GSH, 3 μL) in increasing excesses (from 3 to 10000-fold), and the reaction was stopped by addition of excess iodoacetamide. Following SDS−PAGE under nonreducing conditions, the 7.5% precast gels (Bio-Rad) were stained with Coomassie blue, dried, and scanned on a Compact 4800 flatbed scanner using Twain compatible software. Band densities were analyzed using ImagePC (Scion Co., Frederick, MD) to calculate the amount of Mgr6 released.

Cell Lines. Human breast carcinoma cell line SKBr3 (ErbB2+) was purchased from ATCC (Rockville, MD), and human melanoma cell line MeWo (ErbB2−) was kindly provided by the late J. Fogh (Memorial Sloan-Kettering Cancer Center, New York). Both cell lines were grown in RPMI 1640 containing 10% fetal calf serum and gentamicin (100 μg/mL).

Binding Inhibition Assay. mAb activity after conjugation was assayed as the ability to inhibit binding of

dilutions of cold Mgr6 or IT, starting from a 100-fold molar excess. The mixture was added to SKBr3 fixed cells (adherent in 96-well plates) and incubated for 3 h at 37 °C. Cells were washed 10 times with PBS and incubated with 2 N NaOH (100 μL/well) for 20 min at room temperature. The supernatant was collected and radioactivity determined in a γ-counter (Beckman Instruments, Fullerton, CA).

Percent inhibition was calculated as follows:

$$\% \text{ inhibition} = (1 - C_i/C_{ni}) \times 100$$

where C_i is the average counts per minute in the presence of cold inhibitor and C_{ni} is the average counts per minute without inhibitor.

Inhibition of Protein Synthesis. The assay was carried out essentially as described (*13*). Briefly, SKBr3 and MeWo cells were suspended in culture medium containing the appropriate concentration of IT, toxin, or mAb alone and incubated for 3 h at 4 °C. Control cells were incubated with medium alone. Cells were centrifuged, resuspended in fresh culture medium, and seeded in 96-well plates (3×10^5 cells/well). After incubation at 37 °C for 48 h, [^{3}H]proline (1 μCi/well) was added. After 48 h, cells were washed and [^{3}H]proline incorporation was determined by liquid scintillation in a β-counter. Results are expressed as a percentage of [^{3}H]proline incorporation in control cells.

Radioiodination Procedure. ITs, clavin, and Mgr6 were ^{125}I-labeled using the Iodogen method according to the manufacturer's instructions, to a mean specific activity of 1.5 mCi/mg for ITs, 8.5 mCi/mg for Mgr6, and 4.5 mCi/mg for clavin. The integrity of the radiolabeled protein was tested by ascending paper chromatography in 10% trichloroacetic acid and by SDS−PAGE analysis.

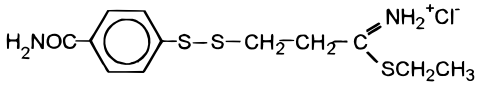
Pharmacokinetic Evaluation. Experiments were performed on 6-week-old female Balb/c mice (Charles River, Como, Italy) maintained according to the provisions of the European Economic Community Council directive 86/209 recognized and adopted by the Italian Government.

Mice received a Lugol solution (0.02% I_2) and 0.6 mg/mL $KClO_4$ in their drinking water 3 days before administration of radioiodinated ITs and throughout the experiments to block free iodine uptake by the thyroid gland and the stomach mucosa. Animals were injected intravenously with a single dose of ^{125}I-labeled IT, ranging from 1.7 to 2.5 μg/mouse. Blood samples were drawn from the retro-orbital sinus at fixed times after injection. Pharmacokinetic analysis was carried out on serum samples. Each serum sample was diluted 1/20 and run in SDS−PAGE under nonreducing conditions. Gels were fixed, dried, and exposed in a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis, performed using Image Quant software (Molecular Dynamics), was used to determine the percentage of intact immunoconjugates in the serum at each time point. Pharmacokinetic parameters were calculated using PCNONLIN software (SCI Software, Lexington, KY).

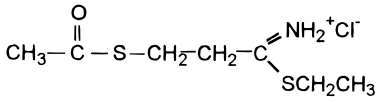
RESULTS

Chemistry. The cross-linkers, prepared as described (*23*−*25*), were characterized by NMR, IR, and MS. Before conjugation, the reactivity of the linkers toward the lysyl ϵ-amino groups of IgG as a model protein was

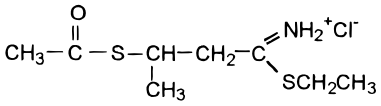
Scheme 1. Structures of Cross−Linkers Used to Produce Mgr6−Clavin ITs



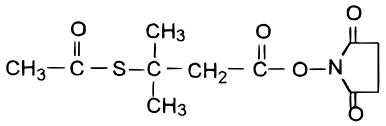
CDPT



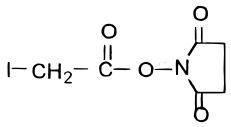
AMPT



M-AMPT



SAMBA



SIA

Preparation of Immunotoxins. Four different coupling procedures (Schemes 2 and 3) were followed to compare the relative activity, stability, and in vivo fate of the immunotoxins.

Disulfide Bridge Immunotoxins. Linkers AMPT and M-AMPT were reacted with recombinant clavin to introduce 1−1.3 *S*-acetylthio groups per protein molecule, whereas a mean value of 1.1 aryldithio residues was introduced into Mgr6 with CDPT; the derivatized proteins were coupled in the presence of hydroxylamine to deprotect the acetylthio groups (IT-1 and IT-2 in Scheme 2). Clavin modified with CDPT was reacted with the Mgr6 previously reacted with the hindered *N*-hydroxysuccinimidyl linker SAMBA (IT-4) (Scheme 3).

All coupling reactions were allowed to proceed for 5 h at 25 °C and for 18−35 h at 4 °C; aliquots of the reaction mixtures were analyzed by SDS−PAGE.

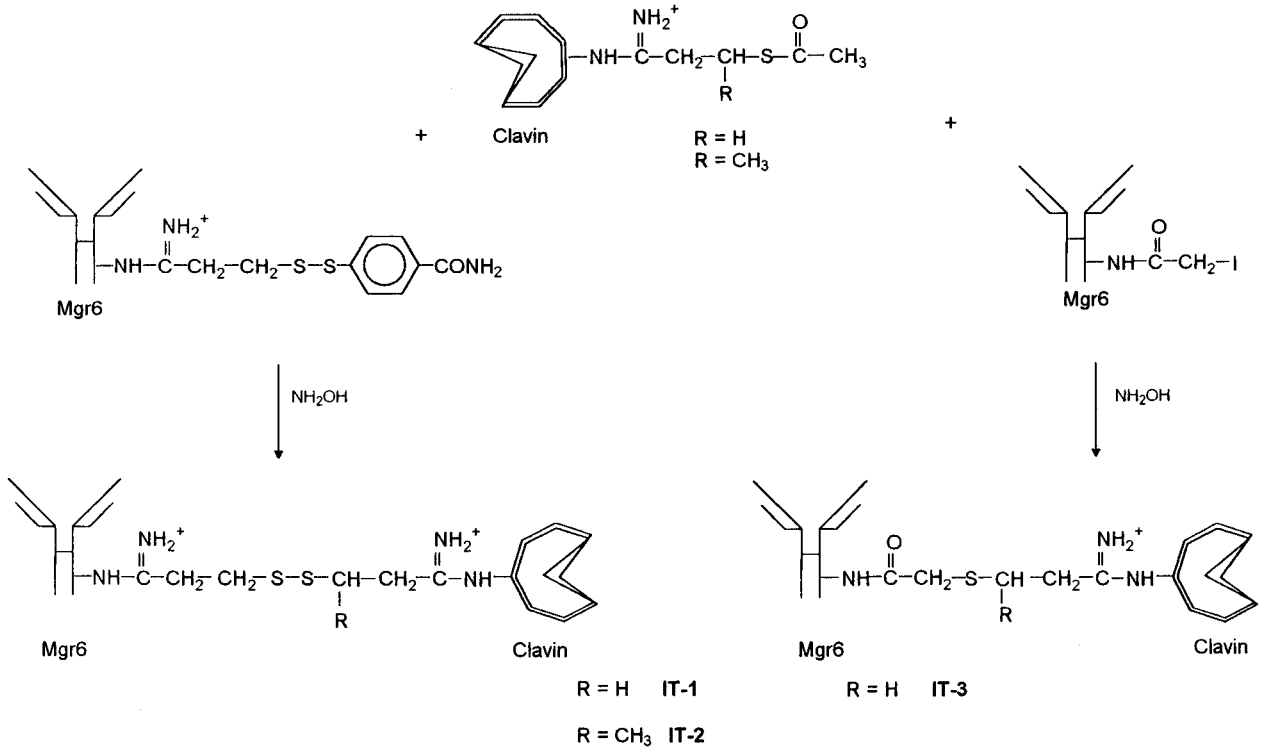
Thioether Bridge Immunotoxin. To obtain a thioether linkage, Mgr6 was derivatized with SIA (1.7 iodoacetyl groups) and clavin with AMPT as described above. After addition of hydroxylamine, the reaction proceeded for 18 h at 4 °C (IT-3 in Scheme 2).

The yield of conjugation for the thioether-linked IT was 12%, while for the disulfide-ITs, the yield was approximately 10% for IT-1 and IT-2 and 5% for IT-4. This suggested an inverse correlation between disulfide bond stability and conjugation efficiency. The low yield can be attributed in part to the low (range of 1−1.7) number of reactive groups per protein.

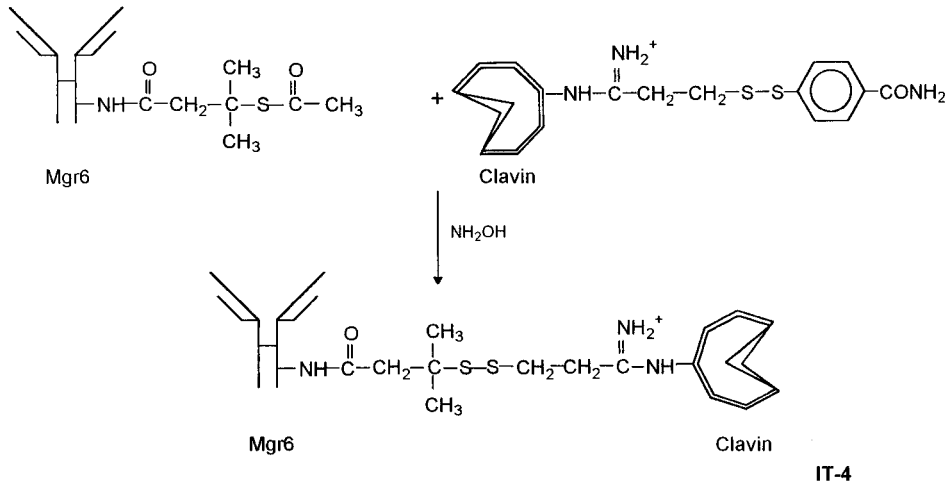
Purification of Immunotoxins. All ITs were purified from the reaction mixture in a single step by HPLC−ion exchange chromatography using a 1010 CM MemSep cartridge.

Figure 1A shows an example of the elution profile of the crude mixture eluted using a discontinuous gradient of sodium chloride ranging from 0 to 250 mM. Fractions

Scheme 2. Preparation of Mgr6−(CDPT)SS(AMPT)−Clavin (IT-1), Mgr6−(CDPT)SS(M-AMPT)−Clavin (IT-2), and Mgr6−(SIA)CS(AMPT)−Clavin (IT-3)



Scheme 3. Preparation of Mgr6−(SAMBA)SS(CDPT)−Clavin (IT-4)



PAGE analysis (Figure 1B). By comparison with purified Mgr6 and clavin electrophoresed in parallel, peak 1 was composed only of unreacted Mgr6, peak 2 was 80% IT with a molecular mass of 167 kDa, peak 3 was >99% IT (167 kDa), and peak 4 contained only unreacted clavin (17 kDa).

Disulfide Bond Stability of Conjugates in Vitro. To test the in vitro stability of the immunotoxins, samples were incubated with reduced GSH in different excesses, subjected to SDS−PAGE, and assessed densitometrically (Figure 2). The profile of the disulfide rupture as a function of GSH excess used is shown in Figure 3. An increase in disulfide stability in relation to the steric hindrance around the disulfide of the conjugates was observed. In comparison, the presence of a thioether bond precluded the breakage by GSH. The increase in stability was exponential, i.e., 10-fold for one methyl cleavage of the mAb disulfide bond was measurable (data not shown).

In Vitro Activity of ITs. To determine whether Mgr6 retained its binding capacity after derivatization and conjugation, competitive inhibition experiments were conducted with the four ITs. A small, reproducible drop in binding capacity was observed after derivatization that was independent of the cross-linker used (see Figure 4, Mgr6−CDPT curve). All ITs competed with the native [^{125}I]mAb, with a titration similar to that of cold derivatized Mgr6, and no significant differences due to the linkage method adopted were evident (Figure 4).

Each IT was tested for its ability to inhibit protein synthesis in ErbB2-positive SKBr3 cells compared with the inhibition by unconjugated clavin. All ITs specifically inhibited protein synthesis in the SKBr3 cells, whereas none did so in ErbB2-negative MeWo cell. As shown in

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Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

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Sync your system to PACER to automate legal marketing.