Toxin-Targeted Design for Anticancer Therapy. II: Preparation and Biological Comparison of Different Chemically Linked Gelonin–Antibody Conjugates

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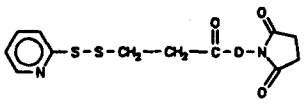
Received June 10, 1992, from the *Istituto di Chimica Farmaceutica Applicata, University of Turin, C.so Raffaello, 33-10125 Turin, Italy.* Accepted for publication November 5, 1992.

Abstract □ To obtain more potent immunotoxins for anticancer therapy a gelonin–AR3 antibody immunoconjugate was prepared with different new linkers and coupling procedures. The gelonin was derivatized with the heterobifunctional thioimidate linkers ethyl-acetyl-3-mercaptopropionthioimidate (AMPT) and 3-(4-carboxamidophenyldithio)propionthioimidate (CDPT), and with the succinimidyl type reagents *N*-succinimidyl-3-(4-carboxamidophenyldithio)propionate (SCDP) and *N*-succinimidyl-*S*-acetyl thiolacetate (SATA). The biological activity of gelonin modified with different linkers (AMPT, CDPT, SCDP, SATA) was determined by a rabbit reticulocyte assay. We found that AMPT was the molecule of choice to derivatize the toxin, confirming the preferability of thioimidate linkers. The monoclonal antibody Mab was derivatized with CDPT and SCDP. Then the following immunoconjugates were prepared with different procedures: Mab–CDPT with gelonin–AMPT; Mab–CDPT with gelonin–CDPT; Mab–SCDP with gelonin–SATA. To verify whether selection of the most suitable coupling procedure could affect the antitumoral activity of the gelonin–AR3 immunoconjugate, the three immunotoxins were tested on target HT-29 human colon carcinoma cells versus nontarget MeWo cells. The gelonin immunoconjugate linked via the AMPT–CDPT thioimidate reagents showed highest antitumoral activity as well as best selectivity for the target cells.

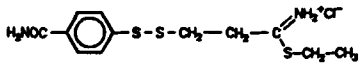
Immunotoxins (ITs) are molecular conjugates formed by monoclonal antibodies (Mabs) linked to toxic agents and are capable of inactivating the cellular synthesis of proteins.[1–5] Unlike currently available drugs that usually do not differentiate between tumor and normal cells, ITs, like the "magic bullets" described by Erlich,[6] are designed to deliver their toxin to the targeted tumor cells.

ITs are usually obtained with a heterobifunctional linker or by gene fusion. The most commonly used toxic agent is the ricin A chain, a ribosome inactivating protein (RIP) that inactivates protein synthesis in eukaryotic cells by specific covalent modification of ribosomal RNA.[7] ITs have also been synthesized with a large group of RIPs (type 1) of plant origin, which resemble the ricin A chain in size and which inactivate ribosomes by an identical mechanism.[8,9] One of these is gelonin, a 30 kDa, single-chain glycoprotein obtained from the seed of *Gelonium multiflorum*, which is able to inhibit protein synthesis once inside the eukaryotic cell.[10–12] Unlike two-chain protein RIPs (type 2), such as abrin and ricin, gelonin cannot enter the cells; therefore, it is much less toxic to cells in vivo unless it is provided with a mechanism of entry. Moreover, gelonin is more stable to chemical and physical treatment than ricin A chain[8] and is not toxic at up to 80 mg/kg when injected into mice.[13] IT made with gelonin has generally displayed similar or better specific toxicity than IT prepared with ricin A chain.[14–18]

To prepare gelonin-containing IT, heterobifunctional agents such as *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, **1**)[19] or 2-iminothiolane[20] (**2**, see structure) were used



N–succinimidyl 3–(2–pyridyldithio)propionate (SPDP)



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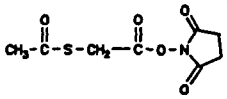
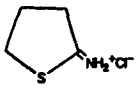
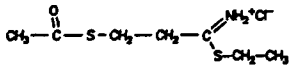


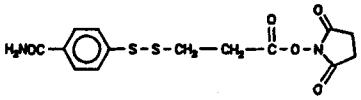
N–succinimidyl–acetylthioacetate (SATA)



2–iminothiolane (2–IT)



Ethyl S–acetyl–propionthioimidate (AMPT)



N–succinimidyl 3–(4–carboxamidophenyldithio)propionate (SCDP)

to introduce a thiol group into the toxin.[21,22] The thiolated gelonin was then added to an SPDP-derivatized Mabs until a stable disulfide bond was formed by a disulfide exchange reaction. Some authors have reported that gelonin is not affected by derivatization with 2-iminothiolane in its ability to inhibit protein synthesis, while it is inactivated to ~90% by modification with SPDP.[14,15] Recently,[23,24] a comparison of SPDP and 2-iminothiolane used to derivatize gelonin and some other type 1 RIPs has reported analogous results. 2-Iminothiolane has been considered the reagent of choice to covalently link PAP (pokeweed antiviral protein)[25] or abrin A chain to Mabs[26]; in this case the 2-iminothiolane-linked ITs were found to be much more effective than the SPDP-linked ITs as antitumoral agents in mice with murine lymphoma.[26] All these results can be explained by the fact that with SPDP the lysine is transformed into an uncharged amido group,

whereas 2-iminothiolane (and in general thioimidate reagents) forms an amidinium bond. The maintaining of the positive charge of the protein could be an important factor that influences the biological activity.

In a preceding paper[27] we reported the preparation of two new acyclic thioimidate cross-linking reagents, 3-(4-carboxamidophenyldithio)propionthioimidate (CDPT, **3**) and ethyl-acetyl-3-mercaptopropionthioimidate (AMPT, **4**). Here, we report using these reagents to prepare new ITs formed by gelonin linked to the AR-3 Mab that recognizes the CAR-3 antigen widely expressed in stomach and colon adenocarcinomas.[28] To get the optimal coupling procedure, we evaluated both the ITs made with AMPT and CDPT, and the immunoconjugates prepared with both a new reagent, *N*-succinimidyl-3-(4-carboxamidophenyldithio)propionate (SCDP, **6**) and the commercial *N*-succinimidyl ligand SATA (**5**, *N*-succinimidyl-*S*-acetylthioacetate). Among those tested, we found that ITs prepared with heterobifunctional thioimidate reagents showed the best selectivity for the target cells as well as the highest antitumoral activity.

Experimental Section

Mab, Toxin, and Tumor Cell Lines—The Mab AR3 (a mouse IgG1 that recognizes the CAR-3 antigen widely expressed among human adenocarcinomas of the stomach, colon, pancreas, ovary, and uterus) was obtained experimentally as described by Prat et al.[28] and was purified from mouse ascitic fluid by affinity chromatography on protein A-Sepharose CL-4B (Sigma, St. Louis, MO). The toxin gelonin,[1] purified as described by Stirpe et al.,[9] was from Sigma (St. Louis, MO). The tracer used for conjugation and purification was ^{125}I-labeled gelonin by the iodogen method[29] and then purified by high-performance liquid chromatography (HPLC) gel-filtration on TSK G2000 SW to a specific activity of 40 mCi/mg. The cell lines used were the HT-29 human colon adenocarcinoma, expressing the CAR-3 antigen, as target line and the MeWo human melanoma as control.

***N*-Succinimidyl Esters Preparation**—*SATA*—This ligand was prepared according to the method of Duncan et al.[29]

SCDP—The preparation of this ligand is a two-step process. First, 3-(4-carboxamidophenyldithio)propionic acid (**1a**) was formed as follows. 4-Carboxamidobenzensulfenyl chloride[27] was reacted with 3-mercaptopropionic acid in glacial acetic acid at reflux for 2 h. After cooling to room temperature, the resulting precipitate was removed by filtration, and the filtrate was diluted with distilled water. The aqueous solution was extracted with ethyl acetate (3 × 50 mL) and the organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure. The crude product was purified by flash chromatography (diethyl ether:ethyl acetate:CH_3COOH, 99:1:0.5), giving pure **1a** as a colorless crystalline powder (43% yield), mp 170–175 °C; IR (KBr): 3400, 3300, 3200, 1700, 1630, 1580, and 830 cm^{-1}.

Next, SCDP was formed as follows: **1a** (1.27 g, 4.9 mmol) was dissolved in 150 mL of refluxing anhydrous tetrahydrofuran (THF). After cooling to room temperature, *N*-hydroxysuccinimide (0.8 g, 7 mmol) in 5 mL of anhydrous THF was added to the solution. The coupling reaction was achieved by the addition of dicycloexylcarbodiimide (1.44 g, 7 mmol) in 5 mL of anhydrous THF. The solution was stirred at room temperature for 10 h, and the precipitate dicyclohexylurea was separated by filtration. The filtrate was then evaporated to dryness under reduced pressure, and the residue was crystallized from diethyl ether:petroleum ether (50:50) to give SCDP (**6**) in a 50% yield (0.85 g), mp 132 °C; IR (KBr): 3400–3200, 1740, 1630, 1590, and 810 cm^{-1}; ^{1}H NMR (CD_3COCD_3): 7.8 (q, 4H), 3.1 (m, 4H), 2.9 (s, 4H) ppm; Mass: 354 (M^+).

Immunoconjugate Preparation—*Conjugation of AR3 to Gelonin via Thioimidate Esters CDPT (3) and AMPT (4): General Procedure*—To a solution of gelonin (8 mg, 0.267 μmol) in 4.5 mL of phosphate-buffered saline (PBS)-EDTA containing 10 μCi of [^{125}I]gelonin, was added a solution of AMPT (100 μL, 88 mM) in absolute ethanol. After stirring for 40 min at 4 °C, the reaction mixture was dialyzed for 16 h at 4 °C. The number of thioacetilated groups linked to the protein was calculated by a method described elsewhere[31]; the gelonin:AMPT molar ratio was 1:1.2. The CDPT ligand (43 μL, 16 μM) in dry dimethylformamide (DMF) was added to a solution of AR3 Mab in PBS-EDTA (8 mg, 890 μL). The mixture was stirred for 30 min at room temperature and was dialyzed as before. The number of aryldithio groups linked to the protein were calculated as described in a preceding paper[31]; the AR3:CDPT molar ratio was 1:1.2. The derivatized proteins were mixed, and a solution of hydroxylamine (0.5 M plus 12.5 mM EDTA), neutralized to pH 7.4 with NaOH, was added (1:10, v/v). After stirring for 24 h at 4 °C, the mixture was treated with *N*-ethylmaleimide in dry DMF (2 mg, 50 μL).

Conjugation of AR3 to Gelonin via CDPT (3)—The CDPT ligand (434 μL, 20 mM) in dry DMF was added to a solution of gelonin in PBS-EDTA (8 mg, 4.5 mL) containing 10 μCi of [^{125}I]gelonin. After stirring for 30 min at 15 °C, the reaction mixture was dialyzed and the gelonin:CDPT molar ratio was determined to be 1:1.3. At the same time, a solution of AR3 in PBS-EDTA (8 mg, 890 μL) was derivatized with CDPT as previously described. The CDPT-derivatized antibody was then mixed with a 10-fold molar excess of dithiothreitol (DTT, 10 mM) in PBS-EDTA. After 20 min at room temperature, DTT was separated from the thiolated antibody by gel filtration on a Bio-Gel P6DG column that was pre-equilibrated in PBS-EDTA at 4 °C. The protein fraction was directly added in a dropwise manner to the CDPT-derivatized gelonin. The reaction mixture was stirred at 4 °C for 12 h, and *N*-ethylmaleimide was then added to block any free thio group.

Conjugation of AR3 to Gelonin via N-Succinimidyl Esters SATA (5) and SCDP (6): General Procedure—The SATA ligand (27 μL, 50 mM) in dry DMF was added to a solution of gelonin in PBS-EDTA (8 mg, 4.5 mL) containing 10 μCi of [^{125}I]gelonin. After stirring for 15 min at 20 °C, the reaction mixture was dialyzed and the gelonin:SATA molar ratio was calculated to be 1:1.3. The SCDP ligand (3.8 μL, 28 mM) in dry DMF was added to a solution of AR3 in PBS-EDTA (8 mg, 890 μL). The mixture was stirred for 30 min at room temperature and was then dialyzed. The Mab:SCDP molar ratio was 1:1.3. The two protein solutions were mixed in the presence of hydroxylamine as previously described. The conjugation time was reduced with respect to the conjugate procedure with CDPT and AMPT to 20 h at 4 °C to reduce loss of protein due to flocculation.

Purification of Immunoconjugates—The reaction mixtures were centrifuged and the supernatants applied to a HPLC gel filtration column (TSK G3000 SW, 7.5 × 600 mm) in several steps and eluted in a potassium phosphate buffer at pH 7.4 (50 mM phosphate, 300 mM NaCl; Figure 1). The elution was monitored spectrophotometrically and with a counter on line. The fractions containing the unreacted antibody and the conjugate were pooled, concentrated, and loaded onto an Affi-Gel Blue column (7 × 100 mm) that was pre-equilibrated at 4 °C in 50 mM sodium phosphate buffer (pH 7.4, 50 mM NaCl; Figure 1). Unconjugated AR3 did not bind to the solid phase under these conditions of ionic strength and was initially eluted from the column. The AR3–gelonin conjugates were then eluted with the same buffer containing 500 mM NaCl. The fractions containing conjugates were pooled and finally dialyzed. The purity and the molecular weight of the conjugates were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli.[32] Protein concentrations were determined by measuring the absorbance at 280 nm, with extinction coefficients ($E^{1\,cm}_{1\%}$) of 14.0 for AR3 and 7.0 for gelonin. The purified conjugates with Mab:toxin ratios of 1:1 and 1:2 had, respectively, estimated $E^{1\,cm}_{1\%}$ values of 13.0 and 12.0.

Determination of Antigen Binding Activity of Antibody and Conjugates—The binding activity of derivatized AR3 and immunoconjugates **7**, **8**, and **9** was measured by enzyme-linked immunosorbent assay (ELISA). Peroxidase-labeled goat antimouse IgG and ELISA kit were from KPL (Kirkegaard & Perry Lab. Inc, Gaithersburg, MD).

Determination of Toxin Residual Activity after Derivatization and Conjugation—*Inhibition of Protein Synthesis Assay on a Rabbit Reticulocyte Cell-Free System*—The Translation Kit Reticulocyte Type II from Boheringher Mannheim (Germany) was used. The reaction mixture contained the following in a final volume of 28 μL:2.8 μL of translation reaction mixture without leucine, 1.4 μL of potassium thioacetate, 2.1 μL of magnesium thioacetate, 0.56 μL of RNAse inhibitor (Boheringher Mannheim, Germany), 7.14 μL of [^{3}H]leucine (Amersham International, Buckinghamshire, U.K.), and 14 μL of a lysate of rabbit reticulocytes. After addition (3.5 μL each) of the last samples, water, and mRNA of tobacco mosaic virus (Amersham International, Buckinghamshire, U.K.) and rapid mixing, incubation was performed at 30 °C with and without preincuba-

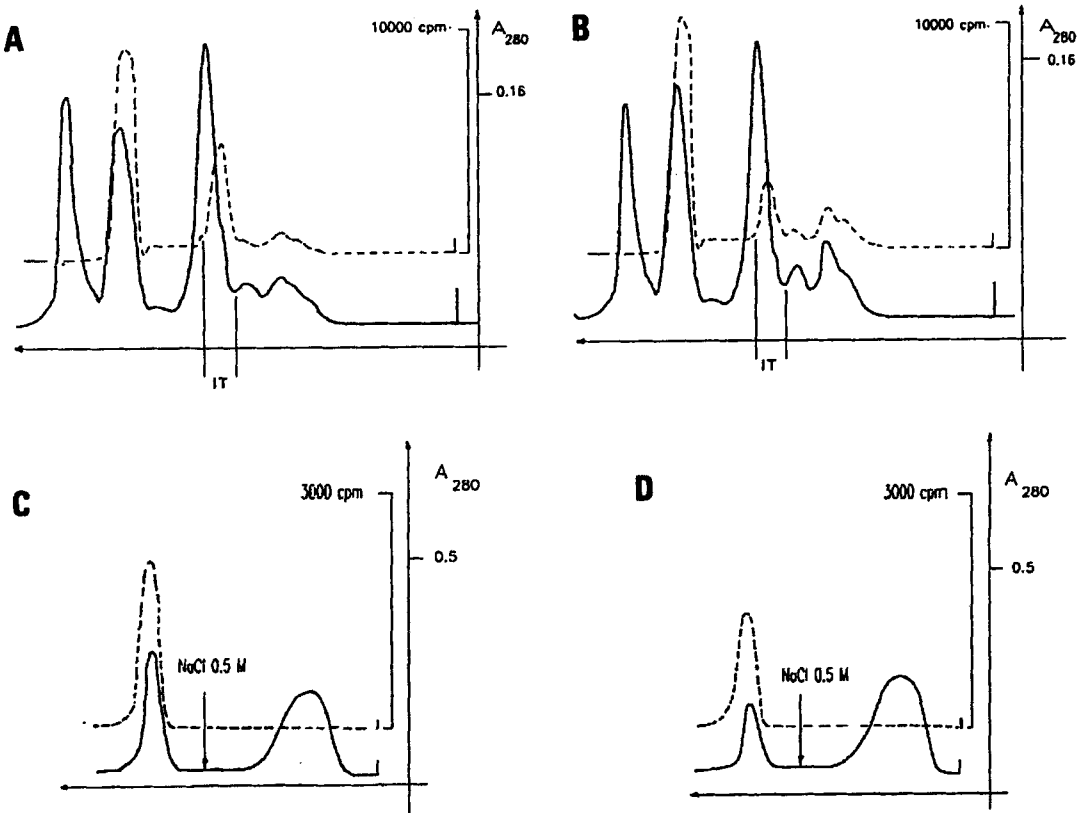


Figure 1—Purification of ITs. (*A*) HPLC gel filtration of CDPT–AMPT IT. The reaction mixture was based on a TSK G 3000 SW column, eluted with PBS over several cycles (flow rate, 0.5 mL/min). The eluate was monitored spectrophotometrically at 280 nm (——) and with an on-line gamma counter (- - -). Fraction IT was collected for the next purification step. (*B*) HPLC gel filtration of CDPT–CDPT IT as described above. (*C*) Affinity chromatography on an Affi-Gel Blue column of CDPT–AMPT gel filtrated mixture, containing immunoconjugate and unreacted AR-3. The AR-3 was eluted with PBS; adding NaCl (0.5 mol) in the medium the immunotoxin eluted. (*D*) Affinity chromatography purification of the CDPT–CDPT IT.

tion with 1% 2-mercaptoethanol. Samples of the reaction mixture (3 μL) were spotted after 30, 60, 90, and 120 min on Whatman 3 MM paper. Radioactivity incorporated into protein was determined by precipitation with trichloroacetic acid and counting in a Beckman liquid scintillation spectrometer, with Packard Filter Count Scintillation liquid (Packard Company).

Inhibition of Cellular Protein Synthesis—HT-29 and MeWo cell lines were tested in the presence of serial dilutions of intact gelonin, derivatized gelonin, and ITs 7, 8, and 9, according to the method reported by Cattel et al.[31] Results are expressed as percentages of [^{3}H]leucine incorporation compared with control cultures (background values were subtracted).

Results and Discussion

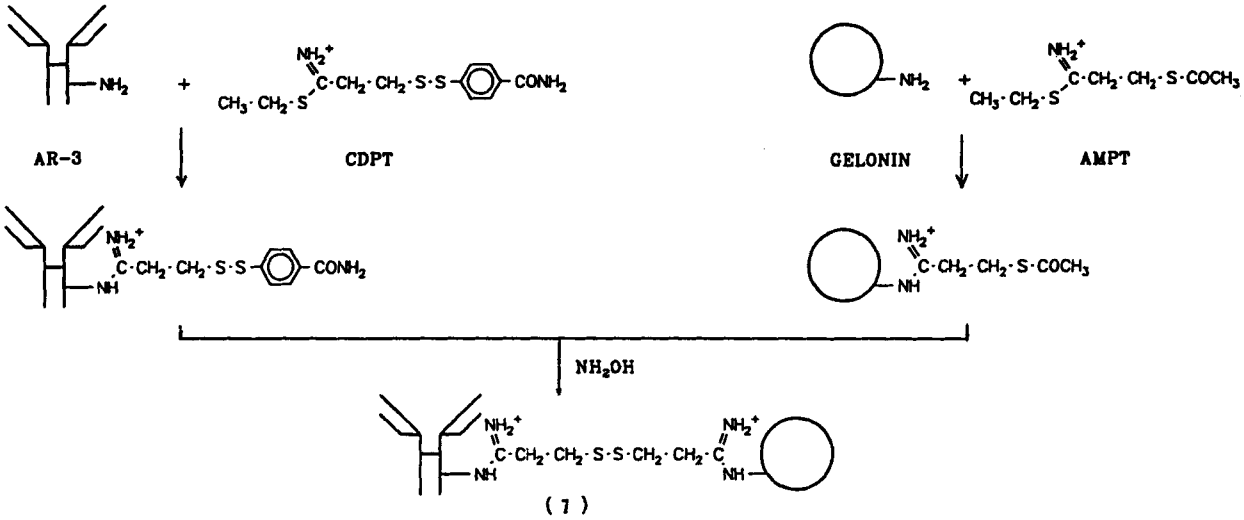
The AR3–gelonin IT was prepared with the thioimidate linkers AMPT (4) and CDPT (3)[27] (Schemes I and II) or the corresponding *N*-succinimidyl ester reagents SATA (5) and SCDP (6) (Scheme III). SCDP is a new heterobifunctional linker possessing an *N*-succinimidyl ester function on one side and a carboxamidophenyldithio group on the other side of the molecule. This reagent was prepared following the known procedure used to synthesize SPDP.[19]

The thioimidate-type immunoconjugate was prepared by two different coupling procedures (A and B, Schemes I and II, respectively). (*A*) CDPT was first reacted with the Mabs to introduce 1.2 aryldithio groups per protein molecule (Scheme I), whereas 1.2 acetylthio residues were introduced into gelonin with AMPT at 4 °C. The thioacetylated gelonin was coupled to the CDPT-derivatized Mabs with NH_2OH to deprotect the acetylthiogroups. (*B*) Both the Mab and the toxin were derivatized with CDPT by introducing 1.2 and 1.3 aryldithio groups, respectively (Scheme II). The modified Mab was then reduced with DTT and directly coupled to the disulfide-containing gelonin by a thiol–disulfide exchange reaction.

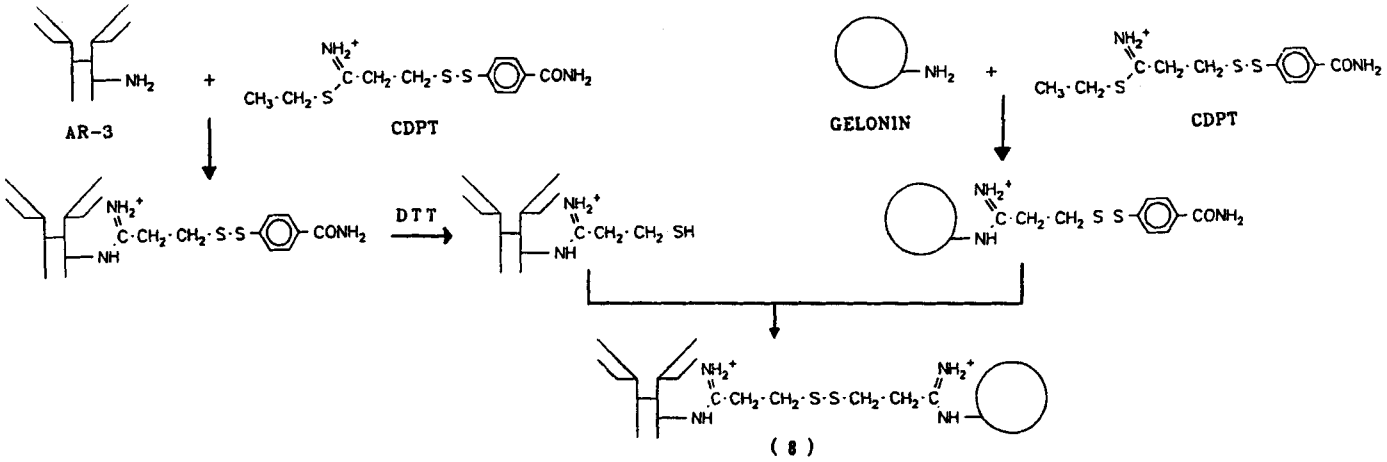
The coupling procedure A (CDPT-derivatized Mabs versus AMPT-thiolated gelonin) could offer some advantages because, during the conjugation reaction, the thiol group introduced into the toxin, which is protected as a thioacetyl derivative, was liberated in situ. This would prevent spontaneous oxidation of the free thiol group as well as the formation of a heteroaggregated polymer.

The *N*-succinimidyl ester-type immunoconjugate was prepared by coupling the SCDP-derivatized AR3 Mab to the gelonin previously thioacetylated with SATA (Scheme III, Method C).

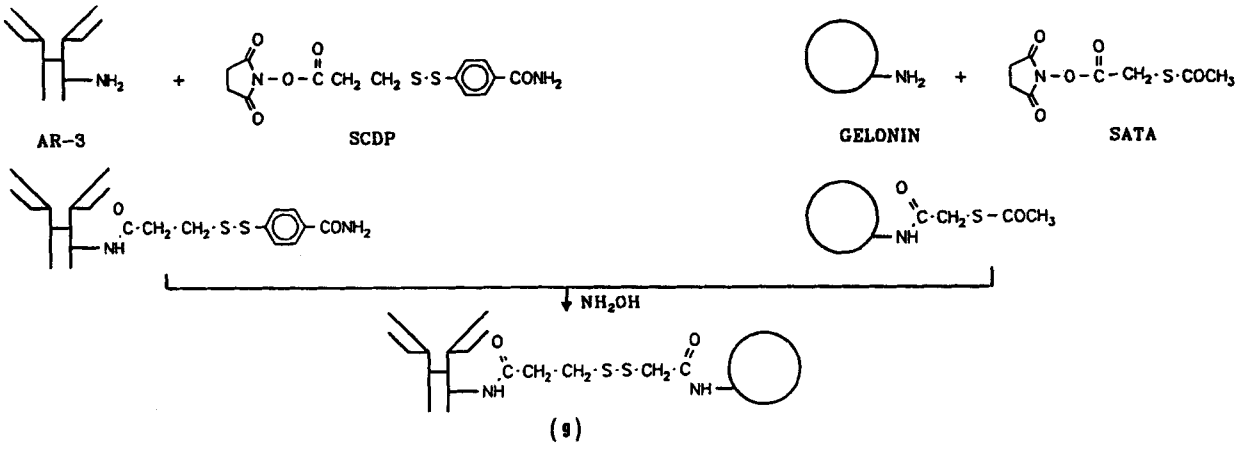
The crude disulfide-linked IT prepared following the procedure described in Schemes I–III (Methods A, B, and C, respectively) was purified by HPLC-gel filtration to remove the aggregates and unreacted proteins. By comparing the elution and SDS-PAGE electrophoresis profiles under nonreducing conditions, unreacted gelonin and high molecular weight compounds (≥300 kDa) most abundantly in the IT 8 prepared via the CDPT–CDPT procedure (Method B) were observed. After Affi-Gel Blue affinity chromatography to completely remove the free Mab from the conjugates, the final yields of the purified IT were 12% for the AMPT–CDPT coupling procedure (Method A), 4.5% for the CDPT–CDPT (Method B), and 10% for the SATA–SCDP (Method C) with respect to the molar concentration of Mab used. The relatively small yield of the conjugate obtained by Method B was probably due to the large amount of intermolecular cross-linked products arising from air oxidation of the free sulfydryl



Scheme 1—Coupling procedure A.



Scheme 2—Coupling procedure B.



Scheme 3—Coupling procedure C.

groups introduced into the Mabs molecule. Because this procedure is analogous to that followed for preparing ITs with SPDP,[22] one could relate the low yield obtained by Method B with those observed producing a gelonin-containing IT with SPDP.[18] In contrast, the very good results obtained with the AMPT–CDPT procedure (Method A) were in line with those already found with mixed 2-iminothiolane–SPDP linkers to prepare ITs on a large scale.[25]

To confirm the supposed superiority of the thioimidate ligands versus *N*-succinimidylesters analogues in making highly active ITs, we tested the biological activity of gelonin modified with different linkers in the cell-free system. We

found that the thioimidate ligands did not significantly reduce gelonin toxicity until 0.6–1.2 groups were introduced into the protein. AMPT was the molecule of choice because gelonin toxicity was completely maintained (0.8–1.2 mol of ligand/mol of protein) whereas SCDP and SATA caused a loss of activity (Figure 2). As expected, by further linking the Mab to the toxin, the activity of gelonin in a cell-free system was strongly reduced for all the tested ITs (Table I). However, by addition of 2-mercaptoethanol, gelonin toxicity was completely restored only in the case of ITs prepared with thioimidate reagents.

It is clear that thioimidate heterobifunctional linkers are indeed the best of the tested reagents for protein derivatization because they did not affect the biological activities of such proteins. This has been already found for 2-iminothiolane.[20,33,34] Among them, AMPT was superior to 2-IT (2) in maintaining gelonin activity; this also demonstrated the superiority of the acetylthio-type reagent over disulfide-type for thiolation of proteins.[31,35]

Finally, the antitumor activity of the differently linked gelonin–AR3 immunoconjugates was compared by incubating the IT with the target HT-29 human colon carcinoma cell lines and checking the respective inhibition of protein synthesis in the presence of [^{3}H]leucine. Highest activity was shown by the IT made with AMPT-CDPT [50% inhibitory concentration (IC_{50}), 0.2 nM]. This was followed by the IT constructed with CDPT–CDPT (IC_{50}, 0.3 nM), and the lowest activity was shown by the SATA–SCDP IT (IC_{50}, 13 nM). In contrast, free gelonin or gelonin modified with linkers had slight toxicity (Figure 3). To test the specificity of the IT, more experiments were performed with MeWo melanoma cells that do not carry target CAR-3 antigen. All the conjugates were much less effective on MeWo cells than on the targeted HT-29 cell-lines (data not shown). Among the molecules tested, the gelonin-IT linked via the AMPT–CDPT thioimidate reagents showed the highest antitumoral activity and the best selectivity, being >100 times less toxic to control cells compared with target cells.

In conclusion, as can be confirmed by other studies, the choice of the most suitable heterobifunctional reagent as well as of the best coupling procedure seems of fundamental importance for achieving both a good conjugation method and for obtaining ITs with the highest activity and specificity. Different factors may be involved in the superior protein conjugating quality of thioimidate reagents AMPT and

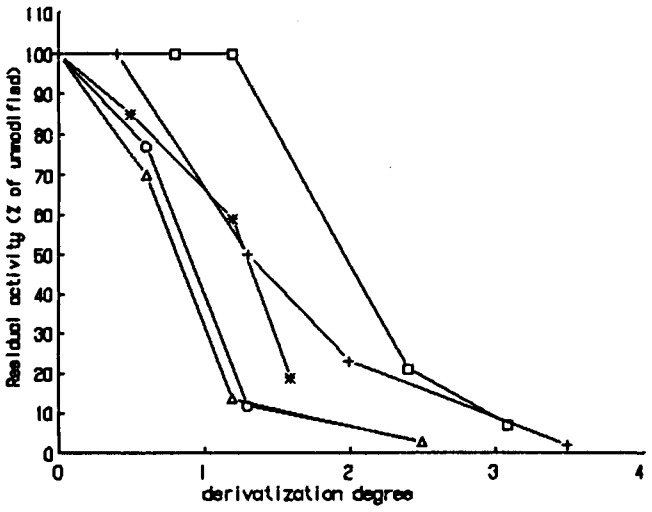


Figure 2—Residual gelonin toxicity after derivatization in cell-free system. Key: (□) AMPT; (○) SATA; (△) SCDP; (*) CDPT; (x) 2-IT.

Table I—Inhibition of Protein Synthesis in Cell-Free System by Differently Linked ITs[a]

Coupling Procedure	IC_{50}, pM[b]	IC_{50}, pM[c]
A: CDPT–AMPT	220 ± 15	20 ± 9
B: CDPT–CDPT	230 ± 20	25 ± 5
C: SCDP–SATA	450 ± 20	120 ± 20

[a] IC_{50} free gelonin, 20 pM. [b] Tested on intact ITs. [c] Tested after addition of 2-mercaptoethanol.

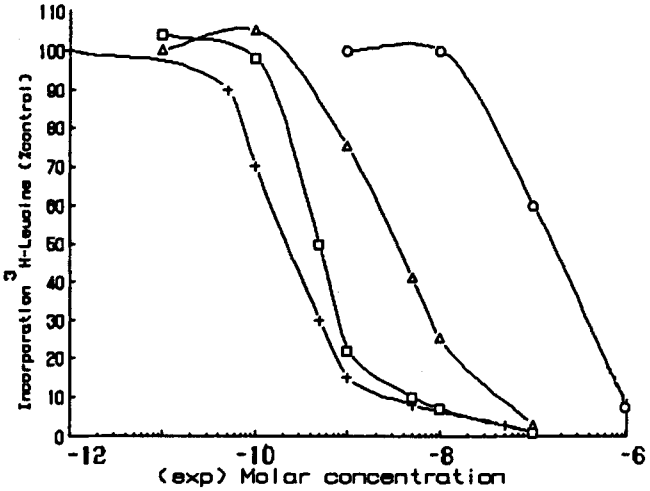


Figure 3—Antitumor activity of gelonin (○), immunotoxin **9** (SCDP–SATA, △), immunotoxin **8** (CDPT–CDPT, □), and immunotoxin **7** (CDPT–AMPT, x) against target cells.

CDPT. One of these is certainly the ability of thioimidate linkers to amidinate proteins (instead of creating an amidic bond as SPDP does), thus preserving a positive charge on the molecule.[20,33] A second factor could be the highest specificity of thioimidates to react with the lysyl groups of proteins.[34] These conditions and the greater polarity connected with the positive charge born by the thioimidate ester linkers could make the reaction of the linker with the external lysine group more selective without causing alteration of the tertiary structure of the protein. It is also possible, as recently suggested by Thorpe,[26] that ITs provided with an amidinium bond could be more resistant than those containing an amide bond (such as SPDP) to the enzymes capable of splitting the linkage. Clearly, a more extensive investigation is needed to elucidate the mechanism by which AMPT–CDPT-linked ITs possess the highest activity in inhibiting protein synthesis in human carcinoma cell lines.

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