Conjugation of Folate via Gelonin Carbohydrate Residues Retains Ribosomal-inactivating Properties of the Toxin and Permits Targeting to Folate Receptor Positive Cells*

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Conjugation of folate to proteins permits receptor-mediated endocytosis via the folate receptor (FR) and delivery of the conjugate into the cytoplasm of cells. Since many cancers up-regulate the FR it has enabled the targeting of toxins to tumor cells resulting in specific cell death. However, current conjugation methods rely on chemistries that can affect certain catalytic subunits, such as the A-chain of the plant toxin gelonin. As a result many folate-targeted toxins are a compromise between receptor/ligand interaction and toxin activity. We describe the first example of folate conjugated to a protein via carbohydrate residues, using a novel SH-folate intermediate. The folate-gelonin conjugate retains over 99% of toxin activity in a cell-free translational assay compared with unmodified gelonin and is able to bind the FR at the same affinity as free folic acid (10^{-10} M). Additionally, the conjugate exhibits prolonged inhibition of protein synthesis in FR positive cell lines *in vitro*. Folate linked to gelonin via amino conjugation exhibits the same affinity for FR as free folic acid but the toxin is 225-fold less active in a cell-free translational assay. The effect of different conjugation methods on toxin activity and the implications for folate targeting of other glycoproteins are discussed.

The plant toxins have been employed in targeted therapies for the treatment of cancer for many years (1). The main reason for their use is their extreme potency; for example, it has been shown that a single plant toxin molecule injected into the cytoplasm can kill a cell (2). Therefore a selectively targeted plant toxin has the potential to be a powerful anti-cancer therapy. Gelonin is a member of the type I ribosome-inactivating plant toxin family (3). Members of this family possess the catalytic A-chain necessary for protein synthesis inhibition but lack the B-chain that is characteristic of the type II toxins (*e.g.* ricin (1)). The B-chain is required for cell binding and endosomal translocation of the type II toxins into the cytoplasm of cells where protein synthesis is then inhibited (4). Since gelonin has no active mechanism of cell entry it is relatively non-toxic to intact cells, relying instead on nonspecific endocytosis

and fluid phase uptake for cellular entry (3). However, in cell-free translational systems gelonin is extremely efficient at inhibiting protein synthesis (IC$_{50}$ 10^{-9} M), acting by preventing the association of elongation factors 1 and 2 with the 60 S ribosomal subunit (1). As a result gelonin is a potential candidate for use as a cytoplasmic targeted toxin, and previous studies have shown it to be useful for targeting using transferrin (5), gonadotrophin (6), and antibodies (7).

The use of such a potent toxin as a targeted therapy is limited unless sufficient selectivity can be incorporated into the drug, thus reducing the effect of nonspecific cell death. Therefore, targeting moieties that exhibit high affinity for cell surface receptors selectively up-regulated on tumor cells are required for this approach. One such targeting ligand is folate, which exhibits high affinity for the folate receptor (FR)[1] (10^{-10} M). The FR is overexpressed on a range of cancers (8), in particular epithelial ovarian cancer where 90% of cases exhibit up-regulation (9). In its unconjugated state folate is non-immunogenic, it is also small in size (441.4 Da) and retains its affinity for the FR upon conjugation to various proteins (8). Additionally, it has been shown to be a useful targeting moiety for the selective delivery of proteins into the cytoplasm of tumor cells *in vitro* (10, 11). Therefore, folate is a potential candidate ligand for the targeted delivery of gelonin into the cytoplasm of tumor cells.

However, one difficulty with the synthesis of targeted toxins is the effect of ligand conjugation upon toxin activity. For example, it has been shown that modification of gelonin amino groups by a heterobifunctional cross-linking reagent (12–14) and conjugation of targeting agents to the toxin (3, 13) can result in up to 99% reduced toxin activity. Therefore the method of ligand conjugation is critical for retaining toxin activity while attaining sufficient selectivity for target cells. In this study, we have compared two strategies for the synthesis of folate-gelonin conjugates. The first exploits conjugation of SH-folate to gelonin carbohydrate residues (folate-S-gelonin) using 3-(2-pyridyldithio)propionyl hydrazide (PDPH), a carbohydrate-selective cross-linker. The second uses *N*-hydroxysuccinimide-folate to conjugate the ligand directly to amino groups present in gelonin (folate-CO-gelonin). We demonstrate by competitive inhibition of [³H]folic acid binding to HeLa cells that both conjugates bind to the FR with the same affinity as free folic acid. However, folate-CO-gelonin is 225-fold less active at inhibiting translation than both folate-S-gelonin and

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[1] The abbreviations used are: FR, folate receptor; PDPH, 3-(2-pyridyldithio)propionyl hydrazide; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.
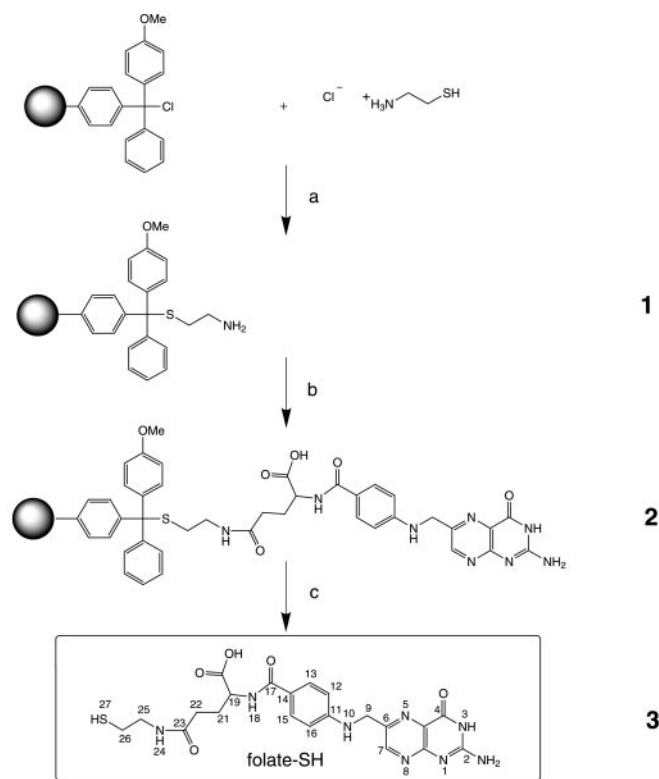
FIG. 1. **Solid phase synthesis of folate-SH.** Reagents: (*a*) CH_2Cl_2: CH_3OH, 3:1, pyridine 1 eq., room temperature, overnight; (*b*) Me_2SO, folic acid 5 eq, DIEA 5 eq., PyBOP^{TM} 5 eq., 30 °C, o/n; (*c*) CH_2Cl_2: trifluoroacetic acid, 97:3, room temperature, 3 × 30 min.

gelonin in a cell-free rabbit reticulocyte lysate assay. Folate-S-gelonin also exhibits prolonged inhibition of protein synthesis in HeLa and Skov3 cell lines *in vitro* compared with gelonin.

EXPERIMENTAL PROCEDURES
Solid Phase Synthesis of SH-folate

A glass column with a join connection and glass frit (porosity number 2) on the upper and lower end, respectively, was used as a reaction vessel. This allowed stirring of the resin on a rotary evaporator and washing after each reaction step. After each step, the resin was stirred (20 min) and rinsed successively with CH_2Cl_2 (5 × 10 ml) and CH_3OH (5 × 10 ml).

Cysteamine Grafting to the Resin via Thioether Bond Formation—4-Methoxytrityl chloride poly(styrene) (1% divinyl benzene) resin (200 mg, 0.346 mmol of chloride group; Novabiochem, Meudon, France) suspended in CH_2Cl_2/CH_3OH (2 ml; 3/1) and pyridine (24 μl; 0.346 mmol) was poured into the glass column. Two molar excess of cysteamine hydrochloride (78.6 mg, 0.692 mmol; Fluka, St. Quentin Fallavier, France) was then added to the resin. The column was connected to a rotary evaporator and stirred overnight at room temperature. The solvent was filtered off and the resin washed as described above, followed by two washes with CH_2Cl_2/pyridine (100/5) to avoid the resin sticking to the glass wall. At this stage the resin turned yellow. A positive Kaiser test (15) showed the presence of amino groups on the resin, and Ellmans' reagent (16) showed no presence of free thiol groups.

Reaction of Cysteamine Bound onto the Resin with Folic Acid—Folic acid (763 mg, 1.73 mmol; Fluka) was slowly added in Me_2SO (2 ml) heated to 50 °C, and diisopropylethylamine (303 μl, 1.73 mmol) was added to the dry resin. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate was then added (PyBOP^{TM}; 900 mg, 1.73 mmol; Novabiochem) and the mixture was stirred overnight at 30 °C. The mixture was filtered and the resin washed with Me_2SO (5 × 10 ml), N,N-dimethylformamide (5 × 10 ml), CH_2Cl_2 (5 × 10 ml), and CH_3OH (5 × 10 ml) resulting in an orange resin.

Cleavage of the Folic Acid-Cysteamine Conjugate from the Resin—A mixture of CH_2Cl_2/trifluoroacetic acid (10 ml; 97/3) was added to the resin and stirred for 30 min at room temperature. The mixture was filtered and the resin washed with CH_2Cl_2 (10 ml) followed by CH_3OH

(10 ml). After 3 cycles of cleavage/wash the combined organic fractions were concentrated under vaccum in the presence of toluene. The crude product was precipitated by addition of 40 ml of acetonitrile, centrifuged, and washed twice with diethyl ether before drying under vaccum. An orange powder was obtained (183 mg) which was identified by ^{1}H NMR and mass spectroscopy. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75 (s, 1H, C7-H_1), 7.64 (d, 2H, J = 8, C13-H_1/C15-H_1), 6.62 (d, 2H, J = 8, C12-H_1/C16-H_1), 4.52 (s, 2H, C9-H_2), 4.28 (dd, 1H, C19-H_1), 3.18 (m, 2H, C25-H_2), 2.8–2.4 (m, 4H, C21-H_2/C22-H_2), 2.50 (m, 2H, C26-H_2), 1.25 (s, 1H, S27-H_1). Mass spectroscopy (fast atom bombardment): $C_{21}H_{24}N_8O_5S$, m/z [M-H]$^-$ 499.54, found 499.2.

Synthesis of Folate-S-Gelonin

Gelonin (500 μl of 1 mg/ml in water; Sigma, Dorset, United Kingdom) was mixed with sodium periodate (500 μl of 1 mg/ml in phosphate-buffered saline, pH 7.4, Sigma) and incubated at room temperature for 1 h. Free sodium periodate was removed from the solution by size exclusion chromatography (PD-10 column; Amersham Pharmacia Biotech, Little Chalfont, UK) and fractions containing gelonin were pooled. The solution was treated with PDPH (40 μl of 0.1 M PDPH in ethanol to 1 ml of gelonin solution; Perbio, Tattenhall, UK) and incubated stirring at room temperature for 5 h. Free PDPH was removed from the solution by size exclusion chromatography (PD-10 column) and fractions containing gelonin were pooled. The gelonin solution was then mixed, under reduced conditions, with SH-folate (10-fold molar excess of SH-folate:gelonin) and HEPES buffer (pH 8.3) to give an overall HEPES molarity of 50 mM. The solution was incubated stirring overnight at 4 °C. Free folate was removed from the solution by size exclusion chromatography (PD-10 column) and fractions containing gelonin were pooled. The protein content was determined using the BCA protein assay (Sigma) and the number of folate moieties per gelonin was calculated using spectroscopic analysis ($\epsilon_{365\ nm} = 9120.1$ M$^{-1} \times$ cm^{-1}). The molar incorporation of folate:gelonin was calculated to be ~1:1.

Synthesis of N-Hydroxysuccinimide-folate (NHS-folate)

NHS-folate was synthesized according to the method of Lee and Low (17). Folic acid (5 g, 11.3 mmol; Sigma) was dissolved in Me_2SO (100 ml) and triethylamine (2.5 ml) and reacted with N-hydroxysuccinimide (2.6 g, 22.6 mmol) and dicyclohexylcarbodiimide (4.7 g, 22.7 mmol) overnight at room temperature. The solution was filtered, concentrated under reduced pressure at 37 °C, and NHS-folate precipitated in diethyl ether (yellow-orange precipitate). The NHS-folate was washed three times in anhydrous ether, dried under vacuum, and stored as a powder at −20 °C. ^{1}H NMR analysis confirmed the presence of N-hydroxysuccinimide on the γ-carboxyl (67%) and α-carboxyl (33%) groups of folic acid (data not shown).

Synthesis of Folate-CO-Gelonin

Folate-CO-gelonin was prepared by incubating gelonin (1 mg/ml in 50 mM HEPES buffer, pH 8.5) with NHS-folate (8:1 molar ratio of NHS-folate:gelonin) stirring at 4 °C overnight. The solution was spun to remove precipitates and free folate removed by size exclusion chromatography (PD-10 column). The protein content was determined using the BCA protein assay and the number of folate moieties per gelonin was calculated using spectroscopic analysis ($\epsilon_{365\ nm} = 9120.1$ M$^{-1} \times$ cm^{-1}). The molar incorporation ratio of folate:gelonin was calculated to be ~1:1.
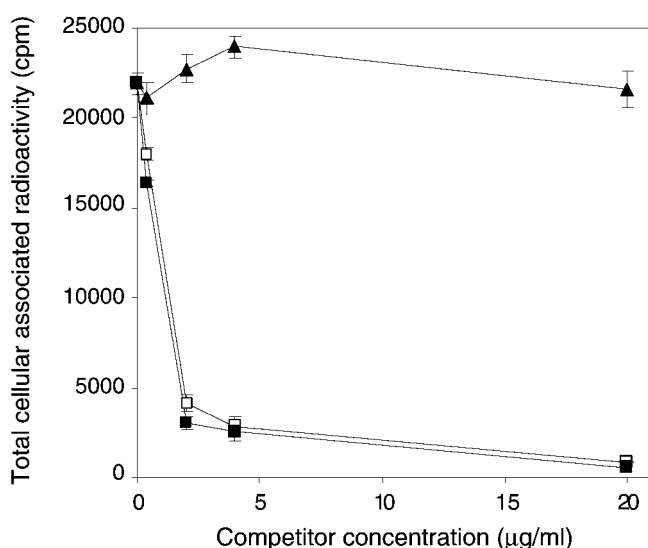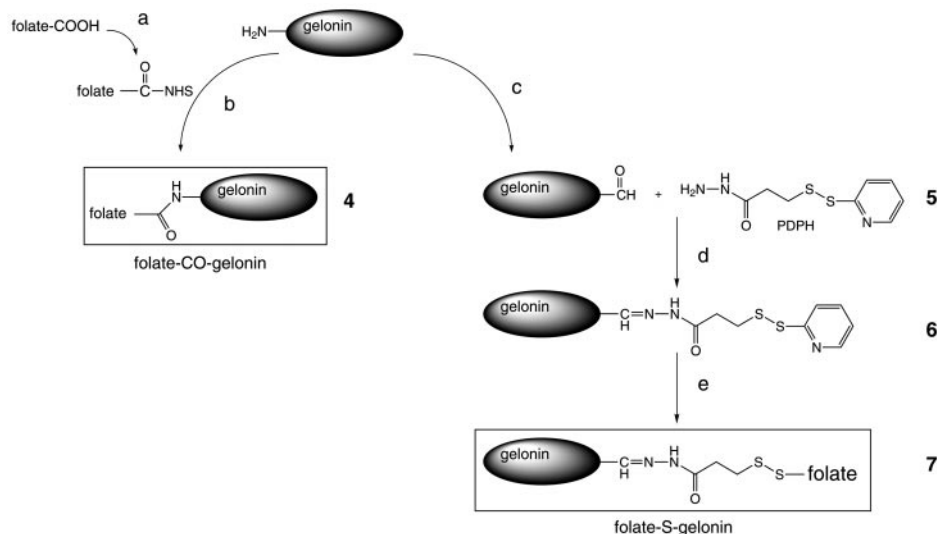
Competitive Inhibition of [³H]Folic Acid Binding to Cells by Folate-Gelonin

A sterile tube containing PBS (0.5 ml, pH 7.4), HeLa cells (100,000 total), [³H]folic acid (approximately 10^{-9} M; Amersham Pharmacia Biotech), and either folate-conjugated or unconjugated compounds (concentration ranging from 0 to 20 μg/ml) was incubated at 4 °C for 30 min. Cells were pelleted by centrifugation (2200 × g, 30 s), washed twice in PBS, and dissolved in urea buffer (9 M urea, 50 mM Tris-HCl, 0.15 M β-mercaptoethanol, pH 7.5). The samples were diluted in Ultima Flo AF scintillation fluid (4 ml) and assayed for radioactivity in a Packard 1900TR liquid scintillation analyser (Packard, Berkshire, UK).

Cell-free Translational Reticulocyte Lysate Assay

Gelonin, folate-S-gelonin, or folate-CO-gelonin (concentrations ranging from 10^{-11} to 10^{-7} M) was added to a sterile vial containing rabbit reticulocyte lysate reagent (17 μl) and SP6 luciferase mRNA-*luc* (1 μg; Promega, Southampton, UK). The samples were incubated at 37 °C for 90 min. An aliquot of each sample (1 μl) was assayed for luciferase activity by the following method. Luciferin (250 μl of a stock solution

FIG. 2. **Synthesis of folate-gelonin conjugates.** Formation of folate-gelonin conjugates via amino group (*4,* folate-CO-gelonin) or carbohydrate (*5–7,* folate-S-gelonin) modification. Reagents for folate-CO-gelonin: (*a*) Me$_2$SO, NHS 2 eq., DCC 2 eq., Et$_3$N 2 eq., room temperature, o/n; (*b*) 50 mM HEPES, pH 8.5, 1 mg/ml gelonin (gelonin:folate-NHS, 1:8), 4 °C, o/n. Reagents for folate-S-gelonin: (*c*) PBS, pH 7.4, 1 mg/ml gelonin, 1 mg/ml NaIO$_4$, room temperature, 1 h; (*d*) room temperature, 5 h; (*e*) HEPES 50 mM, N$_2$, folate-SH 10 eq., 4 °C, o/n.

FIG. 3. **Competitive inhibition of [³H]folic acid binding to HeLa cells by folate-gelonin.** HeLa cells grown in folate-free Dulbecco's modified Eagle's medium were incubated with [³H]folic acid and gelonin (▲), folate-S-gelonin (■), or folate-CO-gelonin (□) at 4 °C for 30 min. Cells were pelleted, washed twice in PBS, and dissolved in urea buffer. Radioactivity was assayed in a liquid scintillation analyzer. *Error bars* show the ± S.D. of three independent experiments.

TABLE I
*Inhibitory concentration (IC$_{50}$) of folate-protein conjugates in a
[³H]folic acid cell binding assay*

Folate-albumin-FITC is known to exhibit an affinity for the folate receptor of 10^{-10} M. The method described in the legend to Fig. 3 was used except HeLa cells were incubated with folate-albumin-FITC, folate-S-gelonin, or folate-CO-gelonin (10^{-10} to 10^{-6} M protein conjugate). Errors are ± S.D. of three independent experiments. Values are for the molarity of the protein.

	IC$_{50}$
	$\times 10^{-8}$ M
Folate-albumin-FITC	3.6 ± 0.3
Folate-S-gelonin	3.5 ± 0.16
Folate-CO-gelonin	3.7 ± 0.3

consisting of: 10 mg of beetle luciferin; 0.47 ml of 1 M glycylglycine, pH 8.0; 15 ml water) was added to luciferase assay reagent (5 ml: consisting of 1 M glycylglycine, pH 8.0 (2.0 ml), 100 mM MgCl$_2$ (1 ml), 500 mM EDTA (20 μl), dithiothreitol (50.8 mg), ATP (27.8 mg), coenzyme A (21.3 mg), water (99.0 ml); final pH 8.0). This luciferin/luciferase reagent (100 μl) was added to the sample (1 μl) and the luminescence intergrated over 10 s in a luminometer (Lumat LB 9507; Berthold, Pforzheim, Germany).

Protein Synthesis Inhibition in HeLa and Skov3 Cells in Vitro

HeLa or Skov3 cells were grown in a 96-well plate (10,000 cells/well) in folate-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.). Gelonin or folate-S-gelonin (10^{-7} M) was added to each well and the cells incubated at 37 °C, 5% CO$_2$ for various lengths of time. Media was removed, the cells washed in PBS (pH 7.4, 2 washes) and methionine and cysteine-free media (Sigma, Dorset, UK) was added to the cells for 30 min at 37 °C, 5% CO$_2$. [³⁵S]Methionine/cysteine mixture (5 μCi/well; Amersham Pharmacia Biotech) was added to the media and incubated at 37 °C, 5% CO$_2$ for 1 h. Cells were then washed in ice-cold PBS. Lysis buffer was added to each well (50 μl; Promega) and incubated on ice for 30 min. Bovine serum albumin (50 μl of 10 mg/ml in water; Sigma) was added to the lysis buffer followed by ice-cold trichloroacetic acid (100% w/v; Sigma) and incubated on ice for 30 min. The precipitate was centrifuged (1,000 × *g*; 1 min) and washed twice in trichloroacetic acid (10% w/v). The precipitate was resuspended in water and assayed for radioactivity in a Packard 1900TR liquid scintillation counter (Packard, Berkshire, UK).
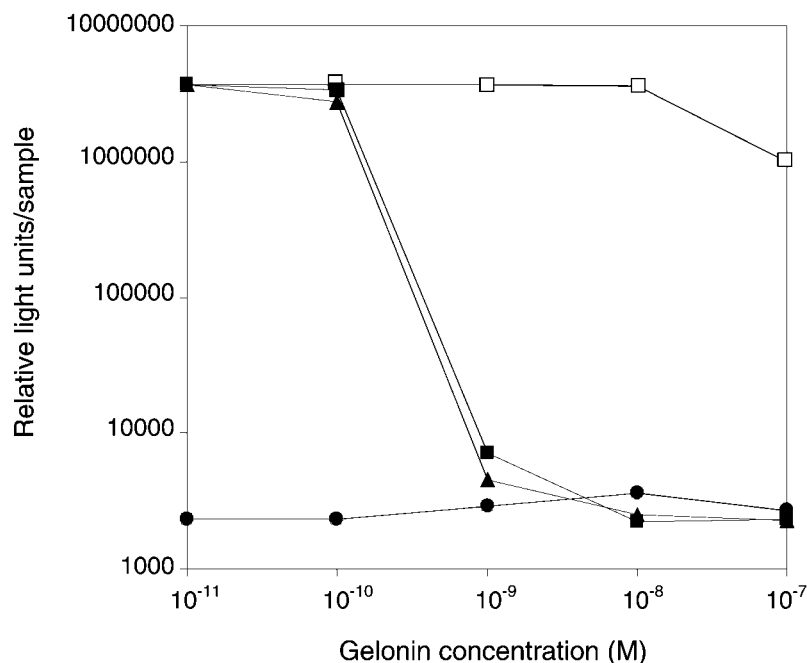
RESULTS

Synthesis of Thiol-derivatized Folate (SH-folate)—We have developed solid phase chemistry for the preparation of thiolated folic acid. Folic acid was functionalized with cysteamine to introduce a thiol group and the hydrochloride cysteamine was bound to the resin via the formation of a thioether linkage (Fig. 1, *1*). Under these conditions only the thiol can react with the chlorotrityl moieties, as the amino group is protonated. After cysteamine grafting onto the resin a Kaiser test showed the presence of amino groups. No thiol groups were detected with Ellman's reagent, proving the formation of a thioether bond. Folic acid was reacted with PyBOP™ to generate an active ester *in situ* and grafted onto the resin via formation of an amide bond (Fig. 1, *2*). The final step consisted of the cleavage of the functionalized folic acid from the resin under acidic conditions (Fig. 1, *3*). The use of a methoxy chlorotrityl resin allows the cleavage of thiol derivatized folic acid from the resin with a mildly acid solvent (3% trifluoroacetic acid in CH$_2$Cl$_2$). Strong acidic conditions were avoided as this lead to the breakage of the folic acid amide bond (data not shown). The global yield for the conversion of folate acid to the folate-thiol (Fig. 1, *3*) was 63% (*i.e.* 86% per step) as determined by thiol titration with Ellman's reagent (16).

Synthesis of Folate-S-Gelonin—Folate-S-gelonin was prepared with initial treatment of gelonin with sodium periodate to oxidize the carbohydrate residues. The recovered protein yield after treatment with NaIO$_4$ was 95%. PDPH (Fig. 2, *5*) is a heterobifunctional cross-linker containing an oxidized carbohydrate-specific hydrazide and a pyridylthio reactive group. Addition of PDPH to NaIO$_4$-treated gelonin enabled conjuga-

FIG. 4. **Effect of folate-gelonin conjugates on the inhibition of protein synthesis in a cell-free translational assay.** Gelonin (▲), folate-S-gelonin (■), folate-CO-gelonin (□), or water (●) was added to a rabbit reticulocyte lysate assay containing SP6 luciferase mRNA-*luc* and incubated at 37 °C for 90 min. An aliquot of each sample was assayed for luciferase activity in a luminometer. *Graph* shows the results from a single experiment.

tion of PDPH to oxidized carbohydrate residues and incorporation of a sulfhydryl-reactive group into the conjugate (Fig. 2, *6*). The recovered protein yield from this step was 94%. Folate was conjugated to gelonin by reacting SH-folate with the pyridyldithio group of PDPH (Fig. 2, *7*). The recovered protein yield was 87% and the folate:gelonin ratio was ~1:1.

Inhibition of [³H]Folic Acid Binding to HeLa Cells by Folate-Gelonin Conjugates—The effect of two different conjugation chemistries on the ability of folate-gelonin to bind cellular folate receptors were investigated using a [³H]folic acid binding assay. The addition of folate conjugates to samples containing [³H]folic acid has been previously shown to inhibit the binding of the radioisotope to folate receptor positive cell lines (18, 19). Both folate-CO-gelonin and folate-S-gelonin inhibited [³H]folic acid binding to HeLa cells (Fig. 3), with the latter showing slightly higher inhibition levels at lower concentrations of the conjugate (0.4 μg/ml). Higher levels of folate conjugates (20 μg/ml) almost totally inhibit radioisotope binding to the cells. In contrast, unmodified gelonin at all concentrations was unable to inhibit [³H]folic acid binding to HeLa cells, showing that the folate-gelonin conjugates were binding to folate receptors present on the HeLa cells. The folate-gelonin conjugates also inhibited the binding of [³H]folic acid to the same level as a folate-albumin-FITC conjugate (Table I). This conjugate has an affinity for the FR of 10^{-10} M, as measured by Scatchard analysis (data not shown). Therefore the ability of folate to bind to its receptor is not impeded by either of the conjugation procedures employed.

Inhibition of Protein Synthesis by Folate-Gelonin Conjugates in a Cell-free Translational Assay—The effects of different conjugation chemistries on the ribosomal-inactivating properties of folate-gelonin were investigated using a rabbit reticulocyte lysate assay (Fig. 4). In this assay mRNA encoding for luciferase is translated into protein which can be detected using luminometry. Ribosomal inhibition by gelonin leads to decreased mRNA translation, and thus lowered luciferase levels, which can be quantified in the linear scale of the luminometer. Folate-S-gelonin exhibited a similar translational inactivating profile compared with unmodified gelonin, with ribosomal inactivation apparent at 10^{-10} M and optimal at 10^{-8} M for both samples. There was no significant difference between the inhibitory concentration (IC$_{50}$) of these samples (Table II), show-
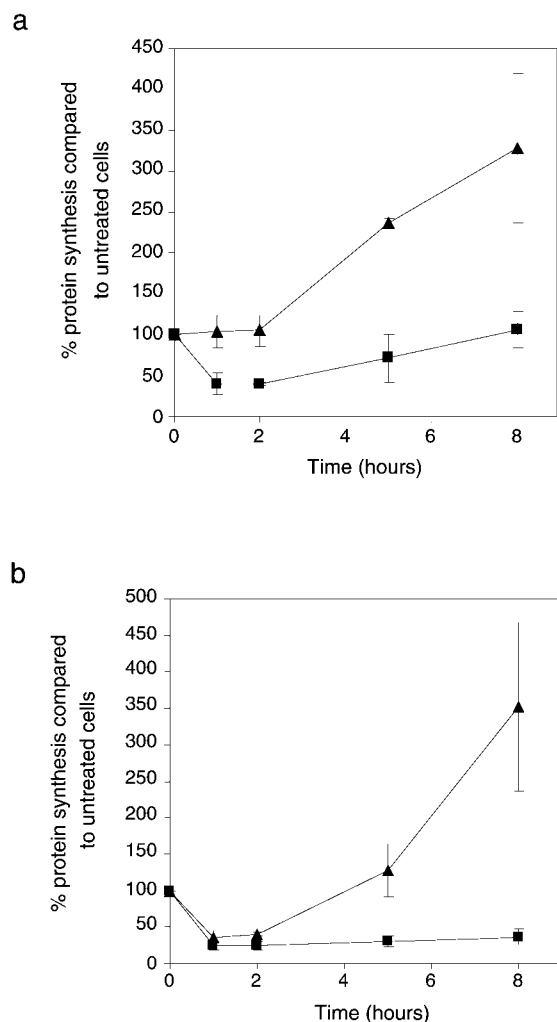
TABLE II
Inhibitory concentration (IC$_{50}$) of gelonin conjugates in a cell-free translational assay

The method as described in the legend of Fig. 4 was used. Errors are the $\pm$ S.D. of three independent experiments.

	IC$_{50}$
	$\times$ 10^{-9} M gelonin
Gelonin	3.1 ± 0.3
Folate-S-gelonin	3.3 ± 0.55
Folate-CO-gelonin	700 ± 0.32

ing that conjugation of folate via gelonin carbohydrate residues has no detrimental effect on the ribosomal inactivating properties of the toxin. In contrast, folate-CO-gelonin displayed a significantly altered translational inactivating profile compared with both gelonin and folate-S-gelonin, with ribosomal inactivation apparent at 10^{-7} M. The IC$_{50}$ of folate-CO-gelonin was over 225-fold higher than folate-S-gelonin (Table II), showing that the amino conjugation procedure drastically reduces the ribosomal inactivating property of the toxin.

Inhibition of Protein Synthesis by Folate-S-Gelonin in HeLa and Skov3 Cell Lines in Vitro—The effect of folate-S-gelonin on protein synthesis within intact cells was quantified by incorporation of radiolabeled amino acids into cellular protein. HeLa cells treated with 10^{-7} M folate-S-gelonin (Fig. 5*a*) exhibited over 50% inhibition of protein synthesis after 1 h compared to untreated cells. The protein synthesis inhibition remained at this level up to 2 h and subsequently increased to ~75% after 5 h. Protein synthesis levels were restored to the same level as untreated cells after 8 h. In contrast, cells treated with gelonin showed no change in protein synthesis after incubation with the toxin for 2 h. However, after 5 h protein synthesis levels were increased and reached almost 300% compared with untreated cells after 8 h. Skov3 cells treated with 10^{-7} M of folate-S-gelonin (Fig. 5*b*) exhibited a substantial decrease in translational levels after 1 h. After 8 h the level remained below 40% of protein translation compared with untreated cells. Skov3 cells treated with gelonin also exhibited decreased protein translation after 1 h. However, by 2 h there was evidence of a recovery of protein synthesis, and by 8 h the level was almost 400% compared with untreated cells. Therefore the folate-S-gelonin is causing a decrease in protein synthesis in

Novel Folate-targeted Gelonin Conjugate

a

b

FIG. 5. **Effect of folate-S-gelonin on protein synthesis levels in HeLa and Skov3 cell lines *in vitro*.** HeLa (*a*) or Skov3 (*b*) cells lines in a 96-well plate (10,000 cells/well) were exposed to either gelonin (▲) or folate-S-gelonin (■) (10^{-7} M) for various lengths of time at 37 °C, 5% CO_2. Cells were washed in PBS and methionine/cysteine-free media was added for 30 min at 37 °C, 5% CO_2. [^{35}S]Methionine/cysteine mixture was then added to the cells for 1 h at 37 °C, 5% CO_2. Cells were washed in ice-cold PBS, lysed, and total protein precipitated in trichloroacetic acid. Radioactivity was assayed in a liquid scintillation analyzer. *Error bars* show the ±S.D. of three independent experiments.

both cell lines compared with the gelonin control, probably due to increased cellular uptake and/or cytoplasmic delivery of the toxin. We found that 0.01 μM of either folate-S-gelonin or gelonin had no significant effect on HeLa or Skov3 protein synthesis over 8 h (data not shown). Therefore the minimum concentration of folate-S-gelonin that inhibits protein synthesis in these cell lines is between 0.01 and 0.1 μM. Higher concentrations (1 μM) of either folate-S-gelonin or gelonin resulted in significant inhibition of protein synthesis in both cell lines over 8 h (data not shown).

DISCUSSION

The formation of targeted toxins presents several problems of a chemical and biochemical nature. First, the binding of the ligand to the toxin must enable relatively simple and reproducible conjugation. Second, the conjugation must occur at a site that will not impede receptor-ligand interactions nor affect the activity of the toxin. Most targeted toxins cause a decrease in at least one of these areas (3, 13, 14, 20, 21), therefore there is a pressing need for the design of alternative conjugation chemistries. In the results presented here we show that attachment

of folate via gelonin carbohydrate residues retains both toxin activity and ligand binding affinity. This work has implications for the targeting of other glycoproteins via the FR and also for other ligands where similar chemistries can be performed.

One advantage of using folate as a targeting ligand is the selective up-regulation of the FR on many epithelial tumors (8). Indeed, Toffoli and others (9) have shown that the folate receptor is up-regulated on 90% of ovarian carcinomas, making the FR a potential target for this high mortality cancer. It has recently been described that tumor cells isolated from the ascitic fluid of ovarian cancer patients internalize folate-conjugated albumin via the FR (19). Therefore folate-targeted toxins have the potential to be useful within a clinical setting. The targeting of gelonin to the cytoplasm of cells using other targeting ligands, such as transferrin (5) and antibodies (23), has been shown to lead to target cell death. However, the receptors for these ligands are expressed on a range of neoplastic and non-neoplastic tissues. Therefore the use of these ligands may give rise to toxin uptake in non-target cells, a phenomenon not observed with folate-targeted conjugates (19, 24, 25).

It has been reported that folate linked to a variety of proteins via amino conjugation has no effect on the binding of the conjugates to the FR (26). We show that this is also true of folate-CO-gelonin and folate-S-gelonin. It has recently been shown, contrary to popular belief, that folate linked to proteins via the α- or γ-carboxyl group retain both receptor affinity and the ability to trigger endocytosis of the FR (27). Additionally, removal of the remaining unconjugated carboxyl group on folate-protein conjugates had no effect on their cell binding or uptake (27). Therefore folate is a versatile molecule that can withstand various conjugation chemistries. Such versatility may be advantageous for the formation of alternative folate-targeted proteins for use as cancer therapies.

The effect of the two conjugation procedures on the activity of folate-gelonin in a cell-free translational assay is striking. Folate-S-gelonin exhibits the same IC_{50} as unmodified gelonin, implying that the presence of folate on the toxin has no detrimental effect. Since carbohydrate modification of plant toxins have not been reported before it is difficult to conclude the reasons for the retention of toxin activity, however, it is possible that the small size of folate is a factor. For example, it has recently been shown that the attachment of antibodies via terminal galactose residues of cobra venom factor leads to a 25% reduction in cobra venom factor activity (20). The large size of the antibody (150 kDa) compared with cobra venom factor (137 kDa) may lead to steric hindrance or physical obstruction of the cobra venom factor activity. Thus folate may not be large enough to exert steric hindrance on the toxin. Alternatively the folate may be located in a region of the toxin that is not dependent on activity, although the conjugation of a larger ligand to the same site could lead to steric hindrance.

Conjugation of folate to gelonin via amino modification results in a 225-fold decrease in activity of the toxin. The effect of amino modification of gelonin on the ribosomal inactivating properties of the toxin has been previously reported, with the attachment of conconavilin A (3) or amino modification of the toxin (13) both leading to decreased ribosomal inactivating properties. The ribosomal inactivating properties of the toxin are extremely dependent on lysine residues within the protein (13). Since NHS-folate reacts with lysines it is likely that this is the reason for the loss of toxin activity in the folate-CO-gelonin conjugate. Trypsin digest of folate-CO-gelonin followed by mass spectroscopy analysis revealed no evidence of folate binding to a single amino residue (data not shown). Therefore, it is likely that folate is linked to a number of sites within the toxin, resulting in a heterogeneous conjugate population.

Whether the activity of folate-CO-gelonin at 10^{-7} M (Fig. 4) is due to a small population of conjugates that retain 100% activity or an overall reduction in activity of the total population has yet to be established. The activity of folate-CO-gelonin at 10^{-7} M may also be due to the presence of unmodified gelonin within the sample.

It has been previously shown that gelonin is relatively nontoxic to HeLa cells (3), and we show that 10^{-7} M gelonin does not lead to decreased protein synthesis in this cell line. However, the ovarian cancer cell line Skov3 is more sensitive to gelonin, with decreased protein synthesis apparent at 10^{-7} M of toxin. These differential effects may be due to altered uptake of the unconjugated toxin or reflect its differential intracellular trafficking within these cell lines. An unexpected effect of unmodified gelonin in both cell lines is the increase in protein synthesis above the control following toxin challenge. This could be due to increased translation of mRNA already present in the cytoplasm in an attempt to equilibrate cellular protein levels. Alternatively, it could reflect an increase in stress response factors stimulated by the presence of the toxin or its downstream by-products. This effect is also seen with folate-S-gelonin, although it is most prominent in the HeLa cell line.

The prolonged protein synthesis inhibition exhibited by folate-S-gelonin in both cell lines shows that the conjugation of folate to the toxin is having an enhanced effect on the toxin activity. This may reflect increased uptake and cytoplasmic delivery of folate-S-gelonin compared with unmodified gelonin. Alternatively the half-life of folate-S-gelonin may be increased upon conjugation to folate, perhaps due to the folate moiety inhibiting cellular protein degradation processes. HeLa and Skov3 cell lines exhibit the same level of uptake of folate-protein conjugates over 8 h.[2] If this is also true of folate-S-gelonin it suggests that the increased protein inhibition in Skov3 compared with HeLa cells is due to the increased sensitivity of Skov3 to the toxin, rather than increased uptake of the conjugate.

In a clinical setting it is desirable to administer the lowest level of drug possible to achieve a therapeutic effect, thus decreasing any side effects to the patient and the overall cost of the treatment. There are several approaches that may be useful for increasing the potency of folate-S-gelonin. Conjugation of folate to multiple carbohydrate residues on gelonin is possible by increasing the stringency of the sugar oxidation reaction. Alternatively, the use of a branched peptide, such as di- or tri-lysine, would present several connecting points allowing the grafting of 2 or more folates per sugar residue. Such approaches may increase the avidity of folate-S-gelonin for the FR, as demonstrated by the attachment of multiple folate moieties to liposomes (8). We have recently shown that the uptake of folate-albumin-FITC conjugates in tumor cells freshly isolated from the ascitic fluid of ovarian cancer patients is higher than that seen in both HeLa and Skov3 cell lines (up to 22-fold higher (19)).[2] Therefore, the level of toxin required to inhibit protein synthesis in tumor cells within a clinical setting may be much lower than the levels used in this study. This, along with the optimization of the folate-S-gelonin conjugate, may enable the administration of much lower levels of the toxin to patients to achieve a therapeutic effect.

Deglycosylated plant toxins exhibit increased half-lives in the blood (28–30), probably due to decreased phagocytosis by scavenging receptors in the liver (28, 29, 31). Therefore it is possible that the conjugation of folate to gelonin carbohydrate residues will lead to an increase in the blood circulation of the conjugate compared with toxin alone. Intravenous administration of folate-targeted conjugates to mice has been shown to lead to tumor accumulation of the conjugates, with minimal accumulation in other tissues (24, 25). Therefore folate-S-gelonin may be a suitable candidate as an intravenous administered cancer therapy. Alternatively, the conjugate could be administered by intraperitoneal or intratumoral injection, thus providing an additional means of selectivity. The use of the conjugate as an intraperitoneal administered therapy is promising since it has already been shown that folate conjugates are internalized by tumor cells isolated from ascitic fluid of ovarian cancer patients (19).

We have shown that the conjugation of folate to gelonin carbohydrate residues enables targeting of active toxin to intact cells. The application of this method of ligand conjugation to other plant toxins or glycoproteins may enable the formation of more potent anti-cancer therapies. Additionally, where similar chemistries are possible the conjugation of alternative ligands to glycoproteins may enable protein targeting via other cellular receptors up-regulated on cancer cells. It will be interesting to see if this method of conjugation can be translated to peptides capable of binding and internalizing via cell surface receptors (22). The small size of these targeting ligands and the recent increase in the availability of such peptides may allow the development of therapeutic proteins targeted to a variety of cell types. Such studies are currently under investigation within our laboratory.

REFERENCES

1. Pastan, I., and Kreitman, R. J. (1998) *Adv. Drug Deliv. Rev.* **31,** 53–88
2. Yamaizumi, M., Mekada, E., Uchida, T., and Okada, Y. (1978) *Cell.* **15,** 245–250
3. Stirpe, F., Olsnes, S., and Pihl, A. (1980) *J. Biol. Chem.* **255,** 6947–6953
4. Eiklid, K., Olsnes, S., and Pihl, A. (1980) *Exp. Cell Res.* **126,** 321–326
5. Yazdi, P. T., Wenning, L. A., and Murphy, R. M. (1995) *Cancer Res.* **55,** 3763–3771
6. Singh, V., and Curtiss, R. (1994) *Mol. Cell Biochem.* **130,** 91–101
7. Rosenblum, M. G., Zuckerman, J. E., Marks, J. W., Rotbein, J., and Allen, W. R. (1992) *Mol. Biother.* **4,** 122–129
8. Wang, S., and Low, P. S. (1998) *J. Control Release* **53,** 39–48
9. Toffoli, G., Cernigoi, C., Russo, A., Gallo, A., Bagnoli, M., and Boiocchi, M. (1997) *Int. J. Cancer.* **74,** 193–198
10. Leamon, C. P., and Low, P. S. (1992) *J. Biol. Chem.* **267,** 24966–24971
11. Turek, J. J., Leamon, C. P., and Low, P. S. (1993) *J. Cell Sci.* **106,** 423–430
12. Singh, V., and Sairam, M. R. (1989) *Biochem. J.* **263,** 417–423
13. Singh, V. (1991) *Biochem. Int.* **24,** 677–688
14. Singh, V., and Curtiss, R. (1991) *Biochem. Int.* **25,** 531–536
15. Sarin, V. K., Kent, S. B., Tam, J. P., and Merrifield, R. B. (1981) *Anal. Biochem.* **117,** 147–157
16. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) *Anal. Biochem.* **94,** 75–81
17. Lee, R. J., and Low, P. S. (1994) *J. Biol. Chem.* **269,** 3198–3204
18. Gabizon, A., Horowitz, A. T., Goren, D., Tzemach, D., Mandelbaum-Shavit, F., Qazen, M. M., and Zalipsky, S. (1999) *Bioconjug. Chem.* **10,** 289–298
19. Ward, C. M., Acheson, N., and Seymour, L. W. (2000) *J. Drug Target.* **8,** 119–123
20. Fu, Q., and Gowda, C. (2001) *Bioconjug. Chem.* **12,** 271–279
21. Sharma, S., Podder, S. K., and Karande, A. A. (1999) *Mol. Cell Biochem.* **200,** 133–141
22. Koivunen, E., Restel, B. H., Rajotte, D., Lahdenranta, J., Hagedorn, M., Arap, W., and Pasqualini, R. (1999) *Methods Mol Biol.* **129,** 3–17
23. Sforzini, S., Bolognesi, A., Meazza, R., Marciano, S., Tazzari, P. L., Stein, H., Stirpe, F., and Ferrini, S. (1995) *J. Hematother.* **4,** 429–432
24. Shinoda, T., Takagi, A., Maeda, A., Kagatani, S., Konno, Y., and Hashida, M. (1998) *J. Pharm. Sci.* **87,** 1521–1526
25. Wang, S., Luo, J., Lantrip, D. A., Waters, D. J., Mathias, C. J., Green, M. A., Fuchs, P. L., and Low, P. S. (1997) *Bioconjug. Chem.* **8,** 673–679
26. Leamon, C. P., and Low, P. S. (2001) *Drug Discov. Today.* **6,** 44–51
27. Leamon, C. P., DePrince, R. B., and Hendren, R. W. (1999) *J. Drug Target.* **7,** 157–169
28. Fulton, R. J., Tucker, T. F., Vitetta, E. S., and Uhr, J. W. (1988) *Cancer Res.* **48,** 2618–2625
29. Fulton, R. J., Uhr, J. W., and Vitetta, E. S. (1988) *Cancer Res.* **48,** 2626–2631
30. Blakey, D. C., Watson, G. J., Knowles, P. P., and Thorpe, P. E. (1987) *Cancer Res.* **47,** 947–952
31. Magnusson, S., and Berg, T. (1993) *Biochem. J.* **291,** 749–755

[2] C. M. Ward, unpublished data.