Radio sensitizer Conjugation to the Carcinoma 19-9 Monoclonal Antibody


ABSTRACT

Misonidazole was covalently conjugated (3-68 mol drug/mol antibody) to 19-9 monoclonal antibody directed against a colorectal carcinoma tumor-associated antigen as a method for targeting radiosensitizing agents. This attachment was accomplished by the mixed anhydride method using the hemisuccinate derivative of misonidazole. Evaluation of conjugates in vitro shows a loss of antibody binding activity with increasing loading levels; however, significant binding activity is retained even at relatively high sensitizer/antibody ratios. This observation was consistent in three binding assays: a competitive radioimmunoassay; an enzyme immunoassay; and an affinity column assay. From these studies, it was concluded that the optimal loading factor for misonidazole-antibody conjugates, when it is used for immunochemotherapy lies between 8 and 15. In vitro release studies indicated that conjugates are hydrolytically stable (t½ = 4 days) under physiological conditions.

INTRODUCTION

The search for effective electron affinic radiation sensitizers to enhance the lethality of ionizing radiation, especially in the radiotherapy of hypoxic tumors, has been extensively reviewed (1, 2). Misonidazole [1-(2-nitro-imidazole-1-yl)-3-methoxypropan-2-ol or Ro-07-0582] is one of the most promising sensitizers in radiation therapy of both animal and human malignancies (3). Its neurological toxicity, however, constitutes a severe limitation to the drug levels which can be used and consequently to the degree of radiosensitization achieved (4, 5).

Attempts have been made to prepare misonidazole analogues with altered lipophilicities but unaltered electron affinities to enhance tumor delivery and reduce uptake in normal tissues (6). These results appear to be only partially successful. An alternate approach to enhancement of tumor affinity of misonidazole would be to exploit monoclonal antibodies raised against tumor-associated antigens as transport vehicles. Monoclonal antibodies to tumor cell surface antigens have attracted considerable interest because of their possible value in cancer diagnosis and in targeting drugs, toxins, and other therapeutic agents to tumor cells (7-10).

In this study, misonidazole was linked to an anticolorectal carcinoma monoclonal antibody, 19-9, as a method for targeting radiosensitizing agents to tumors. Hybridoma 1116-NS-19-9 was produced by fusion of the 653 variant of P3X63 Ag8 murine myeloma cells with splenocytes from a mouse immunized with the human colorectal carcinoma cell line SW1116 (11). The hybridoma and its clone secrete 19-9 antibody which binds specifically to human colorectal carcinoma cell lines. The antigenic determinant recognized by 19-9 antibody on cultured tumor cells is also recognized by the antibody in the serum of patients with gastrointestinal cancer (12-15).

In the following discussion, the preparation and in vitro evaluation of misonidazole antibody conjugates are described. These preliminary investigations indicate that monoclonal antibody transport of radiosensitizers may be an effective way to enhance site-specific delivery.

MATERIALS AND METHODS

Synthesis of Misonidazole Hemisuccinate

Misonidazole was obtained from Hoffmann-La Roche, Nutley, NJ. To a stirred solution of misonidazole (2.01 g, 10.00 mmol) and triethylamine (0.73 g, 7.21 mmol) in dry tetrahydrofuran (30 ml) was added succinic anhydride (3.00 g, 30.00 mmol) during 15 min. The reaction solution was stirred and heated under reflux for 5 h. The reaction was monitored by thin layer chromatography using an ethyl acetate-methanol-acetone (45:10:10) solvent system, and compounds were detected by visual examination under UV. After the reaction was complete, the mixture was stirred overnight at room temperature, evaporated to dryness under reduced pressure, and the brown residue was dissolved in 100 ml of ethyl acetate and acidified with 50 ml of concentrated hydrochloric acid. The organic layer was separated, washed with two 25-ml portions of water, dried (anhydrous sodium sulfate), and evaporated to dryness under reduced pressure. The product was crystallized from a 95% ethanol-water mixture: yield, 1.55 g (52%) off-white crystals; m.p. 109-110°C; proton magnetic resonance (dimethyl sulfoxide-d6): δ 2.40 (s, 3H, OCH3), 3.29 (s, 4H, CH2-CH2); 3.50 (d, 2H, CH2-O; J = 4.5 Hz); 4.37-4.88 (m, 2H, N-CH2); 5.20-5.44 (m, 1H, CH-O); 7.14 (d, 1H, CH=CH; J = 0.6 Hz); 7.59 (d, 1H, CH3; J = 0.6 Hz); 12.20 (s, 1H, COOH). IR (KBr): 3050-2780, 1740, 1500, 1370 cm-1. UV (0.9% NaCl solution): λmax 325 (ε = 7428).

C18H15N3O5
Calculated: C 43.86, H 5.02, N 13.95
Found: C 43.93, H 5.05, N 13.66

Synthesis of Sensitizer-antibody Conjugates

Mixed Anhydride Method. Monoclonal antibody 1116-NS-19-9 (19-9) was supplied by Centocor, Malvern, PA. Misonidazole hemisuccinate was coupled to 19-9 using a modified procedure of Erlanger et al. (16). In all conjugation reactions the final concentration of antibody was 1.5 x 107 m and the total volume was 1.0 ml. Varying concentrations of misonidazole hemisuccinate (1.8 x 104 to 7.5 x 10-3 M) in dry acetone were reacted with equimolar triethylamine in dry acetone and incubated at 4-6°C. After 15 min, equimolar isobutyl chloroformate was added, mixed, and incubated at 4-6°C for 1 h. The resulting solution was then added to a solution of 19-9 and 0.9% saline solution (pH 7.5), gently mixed, and incubated at 4-6°C (1-18 h) with periodic maintenance of pH at 7.5 using 0.01 N NaOH solution until stable. The final concentration of acetone in conjugation reactions varied from 15-45%. Conjugates were purified by Sephadex G-25 gel filtration chromatography (1.5-× 35-cm column) using 0.9% NaCl solution.


Received 12/19/86; revised 4/24/87; accepted 5/1/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Supported by a fellowship from the Mildred Sched Endowment (Federal Republic of Germany). Present address: Department of Internal Medicine II, Klinikum Grosshadern, University of Munich, Marchionini Str. 15, D-8000 München 70, Federal Republic of Germany.

To whom requests for reprints should be addressed, at Center for Health Sciences, Lehigh University, Bethlehem, PA 18015.
solution as the eluting solution; eluant was monitored at 325 nm and collected in 1.0-mI fractions. Conjugate purity was assessed by gel filtration HPLC,\(^*\) (Toyosas-TSK 3000 column; 0.2 M phosphate buffer, pH 6.8; 1.0 ml/min) monitoring at 325 nm. Subsequent purification when necessary was accomplished by ultrafiltration.

Carbodiimide Method. Misonidazole hemisuccinate was coupled to 19-9 using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), a water soluble carbodiimide. Varying concentrations of misonidazole hemisuccinate (1.33–13.30 mmol) in 0.9% saline solution (pH 7.0) were reacted with two equivalents of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, incubated at room temperature for 30 min, and then titrated to pH 5.5–6.0 using 0.01 N NaOH solution. The reaction mixture was slowly added to 2 mg 19-9 antibody in 0.9% saline solution, stirred for 1 h maintaining the pH at 5.5–6.0, and then incubated at room temperature 18–48 h. The final concentration of antibody was 2.66 × 10⁻³ m, and the final total volume was 0.5 ml. Conjugates were purified as described above.

Determination of Loading Factor

The number of moles of misonidazole hemisuccinate per mole of 19-9 antibody (loading factor) was calculated by UV difference spectrometry. Standard curves were run at 280 nm (antibody \(\lambda_{max}\)) and at 325 nm (drug \(\lambda_{max}\)) to obtain Equations A and B

\[
A_{325} = 7.428 \text{ (mol MisHS/liter)} + 2.500 \text{ (mol 19-9/liter}) \quad (A)
\]

\[
A_{280} = 2.055 \text{ (mol MisHS/liter)} + 214,000 \text{ (mol 19-9/liter}) \quad (B)
\]

The molar concentrations of misonidazole hemisuccinate and antibody in a purified conjugate were calculated by measuring its absorbance (A) at 325 and 280 nm, then solving Equations A and B simultaneously. These values were then substituted into Equation C to obtain the loading factor

\[
\text{Loading factor} = \frac{[\text{M MisHS}]}{[\text{M 19-9}]} \quad (C)
\]

Evaluation of Antibody Activity

Radioimmunoassay. Polystyrene beads (Precision Ball Co., Chicago, IL) were coated with SW1116 cell supernatant. The beads were washed three times with ethanol, incubated with 19-9 IgG in pH 8.6 buffer (0.01 M sodium phosphate-0.1% sodium azide) for 16 h at room temperature, washed, and incubated with SW1116 cell supernatant for 17 h. Eight serial 10-fold dilutions starting at 0.2 mg/ml were prepared for each conjugate, 19-9 control, and a nonspecific antibody as a negative control in buffer consisting of 50 mm citrate (pH 4.0), 1% bovine serum albumin, and 0.001% thimerosal. Tracer was prepared by \(^{125}\)I labeling of 19-9 by the Bolton and Hunter method (17) to a level of 1–2 mol \(^{125}\)I/mo1 antibody and diluting to 500,000 cpm/ml with buffer. Equal volumes of tracer and antibody dilution were combined, and 200 μl of this solution were added to each of triplicate beads. The beads were incubated overnight at room temperature, then washed three times with distilled water to remove unbound tracer and counted in a gamma counter for 60 s.

Enzyme immunoassay. Enzyme-linked immunosorbent assay materials were from a Hybricolonal EIA Screening Kit (Kirckvard and Perry Laboratories, Gaithersburg, MD). A cell-free supernatant of nearly confluent SW1116 cell culture as above was incubated to dryness at room temperature overnight in microtiter plates (Linbro, Hamden, CT) at 50 μl/well. A 10-min incubation with 2% bovine serum albumin in PBS was carried out followed by a 5-min 0.1% glutaraldehyde fixation which was quenched with 0.1 M glycine for 5 min. After washing, 100 μl of native or conjugated 19-9 dilutions were incubated overnight at 4°C. The plate was again washed and incubated with 50 μl of peroxidase-labeled anti-mouse IgG antibody for 3 h. After an additional washing, substrate solution [2,2-azino-di-(3-ethylbenzthiazoline-sulfonate) containing cacodylic acid, buffer, and hydrogen peroxide] was added and incubated for 1–2 h, and absorbance at 405 nm was measured in a Titertek Multiscan (Flow Laboratories).

Affinity Chromatography. One ml of Sepharose-bound 19-9 antibody was washed with pH 4.0, 50 mM citrate-1% bovine serum albumin-0.001% thimerosal buffer; 3.0 ml of SW1116 cell supernatant were added to the column and washed with 4.0 ml of the above buffer. \(^{125}\)I-labeled antibody (native or misonidazole conjugate) was added to the column and eluted with 4.0 ml of the above buffer to remove unconjugate antibody. The column was then eluted with 4.0 ml of 3 M ammonium thiocyanate to remove bound antibody, and the percentage of active (bound) antibody was calculated.

Evaluation of Conjugate Hydrolytic Stability. A conjugate with a misonidazole/antibody ratio of 56 was incubated at 25° and 37°C in pH 7.4, 0.05 M phosphate-buffered saline, and in mouse serum. Aliquots were removed at appropriate times and chromatographed on an IBM LC9533 instrument with a Toyosas TSK3000 column using 0.2 M phosphate buffer, pH 6.8 and flow rate of 1.0 ml/min. Absorbance at 325 nm was monitored and the peak areas integrated. Conjugate purity was assessed by gel filtration HPLC,\(^*\) (Toyosas-TSK 3000 column; 0.2 M phosphate buffer, pH 6.8; 1.0 ml/min) monitoring at 325 nm. Subsequent purification when necessary was accomplished by ultrafiltration.

Table 1 Misonidazole hemisuccinate conjugation to 19-9

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Reaction ratio*</th>
<th>Loading factor*</th>
<th>% immunoreactivity retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed anhydride conjugates</td>
<td>12.5:1</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>12.5:1</td>
<td>3</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>15.0:1</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>25.0:1</td>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>5</td>
<td>50.0:1</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>50.0:1</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>100.0:1</td>
<td>24</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>165.0:1</td>
<td>56</td>
<td>0.3</td>
</tr>
<tr>
<td>9</td>
<td>500.0:1</td>
<td>68</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2 Hydrolytic stability of antibody misonidazole conjugate at 37°C

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Bound misonidazole</th>
<th>Free misonidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99</td>
<td>1.0</td>
</tr>
<tr>
<td>3.5</td>
<td>100</td>
<td>0.0</td>
</tr>
<tr>
<td>8</td>
<td>99.6</td>
<td>0.4</td>
</tr>
<tr>
<td>22.5</td>
<td>97.4</td>
<td>2.6</td>
</tr>
<tr>
<td>74</td>
<td>79.9</td>
<td>20.1</td>
</tr>
<tr>
<td>120</td>
<td>60.8</td>
<td>39.2</td>
</tr>
</tbody>
</table>

\(^*\) Mol misonidazole hemisuccinate per mol 19-9 in initial reaction.

\(^\dagger\) Mol misonidazole hemisuccinate per mol 19-9 in conjugate.
RADIOSENSITIZER CONJUGATION TO 19-9 MONOCLONAL ANTIBODY

Fig. 2. Radioimmunoassay evaluation of misonidazole-labeled 19-9 antibody conjugates. Varying dilutions of unmodified 19-9 (○), and misonidazole-antibody conjugate with a loading factor of 8 (△), 22 (■), and 56 (●) were incubated competitively with 125I-labeled antibody for binding to solid phase-bound antigen.

Fig. 3. Relationship between percentage of antibody-binding activity retained, as measured by radioimmunoassay (Table 1), and loading factor.

Table 3 Comparison of activity of three conjugates using three different assays
Results are with negative control activity subtracted.

<table>
<thead>
<tr>
<th>Loading factor</th>
<th>Affinity column</th>
<th>Radioimmunoassay</th>
<th>EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>36</td>
<td>32</td>
<td>63</td>
</tr>
<tr>
<td>22</td>
<td>24</td>
<td>16</td>
<td>25</td>
</tr>
<tr>
<td>56</td>
<td>5</td>
<td>0*</td>
<td>20</td>
</tr>
</tbody>
</table>

* Precipitate present when conjugate was added to radioimmunoassay buffer.

eluted with a retention time of 9–10 min, and unbound misonidazole had a retention time of 14–16 min. The amount of misonidazole and misonidazole hemisuccinate released from the conjugate was determined from the relative peak areas.

RESULTS

Synthesis of Misonidazole Hemisuccinate

Misonidazole was converted to its hemisuccinate by condensation in a basic medium with succinic anhydride. The technique used was similar to that developed for the preparation of steroidal conjugates (16). H-1 nuclear magnetic resonance and elemental analysis confirmed the structure of the hemisuccinate, which was isolated in the 52% yield.

Synthesis and Purification of Sensitizer-Antibody Conjugates

Two coupling methods, a mixed anhydride and a carbodiimide method, were developed to covalently attach the carboxyl group of misonidazole hemisuccinate to amino groups on the antibody. The loading factors (moles of drug bound per mole of antibody) obtained under various reaction ratios (drug to antibody molar ratio in initial reaction) are reported in Table 1.

Using the mixed anhydride technique and varying molar concentrations (12.5:1–500:1) of drug to antibody, 3–68 drug molecules could be incorporated per IgG molecule, respectively. In comparison to the mixed anhydride method, carbodiimide coupling at comparable reaction ratios resulted in a lower degree of protein substitution. For example, at a 100:1 reaction ratio, the mixed anhydride method yielded conjugates with loading factors of 20–24 compared with 9–10 via the carbodiimide procedure. The carbodiimide method also yielded more cross-linking of protein as indicated by HPLC, and noncovalently attached misonidazole hemisuccinate was found by HPLC in conjugates which had been extensively purified, indicating that the loading factors reported in Table 1 do not necessarily reflect covalently attached drug. Therefore, the mixed anhydride method as shown in Fig. 1 was selected for subsequent conjugations, and only these conjugates were evaluated in vitro.

Sephadex G-25 gel filtration chromatography gave good baseline separation between conjugated antibody and free misonidazole. However, HPLC evaluation indicated unbound misonidazole present in some conjugates following purification, suggesting noncovalent association. Further membrane ultrafiltration removed all unbound misonidazole, as indicated by HPLC.
Characterization of the Sensitizer-Conjugates

Hydrolytic Stability. The stability of the misonidazole-protein conjugate was evaluated and the results are shown in Table 2. The assay for cleavage of sensitizer from protein was an HPLC separation described in "Materials and Methods." A highly loaded conjugate (loading factor = 56) was incubated in pH 7.4 PBS. Additional studies showed no detectable difference between incubation in PBS and incubation in normal mouse serum. The drug-antibody complex showed good hydrolytic stability ($t_{1/2} = 4$ days at $37^\circ$C).

Antibody Activity. Conjugates were evaluated by in vitro binding assays to study the effect of bound drug on the ability of antibodies to bind to the corresponding antigen. All conjugates were tested using a competitive radioimmunoassay, and the percentage of activity retained as measured by this assay is reported in Table 1. These results are also shown graphically in Fig. 2 for three conjugates with various loading levels of sensitizer. This assay measures the ability of the modified antibody to compete with radiolabeled 19-9 antibody for binding to SW1116 antigen bound to solid phase-bound 19-9 antibody. The ability of unmodified control antibody to inhibit binding of $^{125}$I-labeled antibody was arbitrarily set at 100%. Relative binding activity for any given conjugate was calculated as

\[
\text{Relative Binding Activity} = \frac{\text{Unmodified antibody}}{\text{Modified antibody}} \times 100
\]

for 50% inhibition of $^{125}$I-labeled antibody binding.

In Fig. 3, the retained activity of conjugates is shown as a log plot versus loading factor. As can be seen, relative immunoreactivity of the antibodies was as high as 100% when 19 drug molecules were conjugated per molecule and decreased exponentially with increasing loading factor to less than 20% when approximately 20 drug molecules were conjugated.

For comparative analysis, the three conjugates shown in Fig. 1 were evaluated by two additional assays, an EIA and an affinity column assay. In both assays, a comparison was made between the conjugate and native 19-9 to determine the percentage of retained activity. The results are summarized in Table 3.

In the EIA, antigen is adsorbed directly on the solid phase. Dilutions of the antibody to be assayed are incubated with the antigen, then quantified by a second anti-mouse IgG labeled peroxidase. This sandwich assay serves as a measure of the antibody activity as well as its antigenicity toward the second antibody. The results are illustrated in Fig. 4.

An additional evaluation of these conjugates was performed using affinity column chromatography. The method used was the formation of an affinity column of 19-9 antigen (from SW1116 cell supernatant) bound to Sepharose-linked 19-9 antibody. Antibody not exhibiting appreciable affinity for the antigen will pass unretarded through the column, whereas those which recognize antigen will be retarded. The specific binding and elution of native 19-9 was compared to conjugates of various loading levels. The results of three conjugates are shown in Table 3, along with the data from the immunoassays. Additionally, a sample of the 22-ratio conjugate was applied to the affinity column, and the nonbinding portion was subsequently assayed by the radioimmunoassay procedure and found to have no more activity than a nonspecific control antibody.

As shown in Table 3, the fraction of immunoreactive antibody decreased as the loading factor increased. These results show that the antibody activity measured in the immunoassays cannot simply be attributed to a fraction of unmodified antibody.

In summary, the three assays show a comparable reduction in antibody immunoreactivity with increasing loading factor. Conjugates containing less than 10 mol of sensitizer/mol of antibody retained a high degree of activity, whereas conjugates with loading factors of greater than 20 showed severe loss of antibody binding activity.

DISCUSSION

Although the 19-9 antigen is shed by the tumor, the antibody has been successfully used as a carrier of tumor-imaging radioisotopes (18). Therefore, it is reasonable to propose that conjugates constructed from 19-9 antibody and therapeutic agents have potential for immunotherapy in patients with colorectal carcinoma.

Misonidazole is easily derivatized as the hemisuccinate, and a mixed anhydride coupling procedure can be readily controlled via reactant concentration to reproducibly obtain desired loading levels with 19-9 antibody.

These investigations demonstrate the feasibility of preparing stable misonidazole monoclonal antibody conjugates which retain, to some degree, antibody-cell binding reactivities. The degree of antibody substitution which can be achieved without significantly reducing target cell binding is restricted with 19-9, this being about 20 drug residues/antibody molecule.

The binding of the antibody to its antigen depends on both the fraction and the binding affinity of immunoreactive antibody. In both immunoassays, antibody binding activity is a relative measure of immunoreactivity, reflecting a decrease in the fraction of immunoreactive antibody and/or a decrease in the affinity constant. The results of the two immunoassays are similar, despite considerable differences in pH, binding methodology of the shed antigen to the solid phase, and the required recognition of the conjugate by a second antibody in the enzyme immunoassay.

The fact that the nonbinding portion of conjugate in the affinity column assay shows no binding in the radioimmunoassay suggests that some percentage of the conjugate molecules is totally inactivated. This percentage is presumably a fraction consisting of heavily loaded molecules with antigen binding sites inactivated. Data in Table 2 show that each conjugate contained a fraction of inactivated antibody and that the frac-
tion of immunoreactive antibody decreases with increasing loading levels.

An important issue addressed in this paper is the effect of conjugate loading factor on antibody activity. As shown in Fig. 3, antibody activity decreases exponentially with increasing loading factor. It is very important, therefore, to control the reaction concentrations in order to optimize the balance between the degree of conjugation and the activity of the modified antibody.

The amount of misonidazole delivered to a tumor site is expected to depend on the level loaded on the antibody as well as the ability of the modified antibody to bind to its relevant antigen. Thus the product of loading factor times fractional activity remaining may be used as an index of the therapeutic utility of a conjugate. This product is plotted as a function of loading factor in Fig. 5 to determine the optimal drug:antibody ratio. It can be seen that the parabolic curve reaches a maximum at a loading factor of approximately 11, with conjugates containing 8–15 mol of misonidazole hemisucone/mol of antibody being in the clinically useful range. From this plot, it was concluded that the optimal loading factor range for misonidazole-19-9 antibody conjugates, when used for immunotherapy, lies between 8 and 15. In addition, their potential might be substantially enhanced by removing the fraction of inactive antibody by affinity chromatography.

REFERENCES


Radiosensitizer Conjugation to the Carcinoma 19-9 Monoclonal Antibody


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/15/4071

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.