pounds were studied at a level of 10% (v/v). The results of experiments carried out in triplicate agreed and growth inhibitory action at the end of 3 weeks are presented in Table I.

C. Tubes containing varying concentrations of test compounds in Youman's media were inoculated with 14-day-old cultures of INH and streptomycin-resistant strains of *M. tuberculosis*. Controls containing only the Youman's medium, solvent (ethylene glycol), INH (various concentrations), and streptomycin (various concentrations) were also inoculated in the same way. All the tubes were then sealed with paraffin and incubated at 37 °C for 4 weeks. The inhibitory action of the compound was assessed by comparing the growth of bacilli in the experimental tubes with that in the controls. Readings were taken at the end of each week up to 4 weeks. The results of experiments carried out in triplicate agreed, and results observed at the end of 4 weeks are presented in Table II.

Acknowledgment. We thank N. Naganathan, National Tuberculosis Institute, Bangalore, India, for supplying drug-resistant strains of *M. tuberculosis*.

Registry No. 1, 70955-02-1; **2**, 104411-19-0; **3**, 121810-69-3; **4**, 121810-70-6; **5**, 100850-37-1; **6**, 121810-71-7; **7**, 121810-72-8; **8**, 121810-73-9; **9**, 121810-74-0; **10**, 89504-69-8; 11, 580-15-4; **12**, 121810-75-1; **15**, 121810-76-2; **16**, 75793-57-6; **16**·NH_3, 121810-86-4; **17**, 121810-77-3; 18, 121810-78-4; **19**, 98797-17-2; **20**, 121810-79-5; **21**, 121810-80-8; **21** (methyl ester), 121810-87-5; **22**, 121810-81-9; **23**, 121810-82-0; **24**, 121810-83-1; **25**, 121810-84-2; **26p**, 121810-85-3; (±)-$CH_3CHBrCOOEt$, 41978-69-2; $ClCH_2COOEt$, 105-39-5; OHCCOOH, 298-12-4; CH_3COCH_2COOEt, 141-97-9; 2-naphthylamine, 91-59-8; *N*-methyl-2-naphthylamine, 2216-67-3; 2-amino-6-methoxynaphthalene, 13101-88-7; pyridine-4-aldehyde, 872-85-5.

Synthesis of Methotrexate–Antibody Conjugates by Regiospecific Coupling and Assessment of Drug and Antitumor Activities

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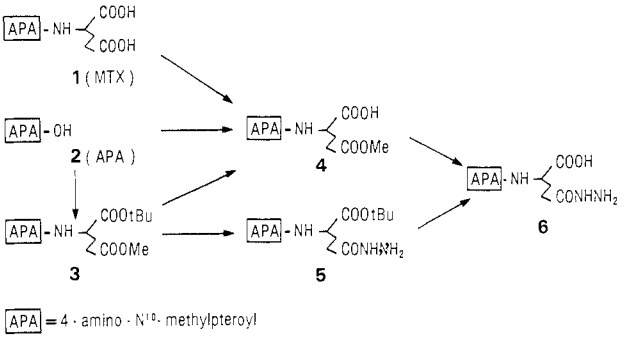
Received November 22, 1988

In order to increase the retention of drug activity, regiospecific coupling has been used to synthesize conjugates of methotrexate (MTX, 1) with normal rabbit IgG (NRG) and a mouse anti-human renal cancer monoclonal IgG (Dal K-20). MTX γ-methyl ester (4) was produced either by selective esterification of MTX or by coupling of 4-amino-4-deoxy-N^{10}-methylpteroic acid (2) with suitable glutamic acid derivatives. The MTX γ-methyl ester (4) was then converted to the corresponding hydrazide 6. An amide-linked conjugate was formed when the MTX γ-hydrazide (6) was converted to reactive acylating species 7 by using *tert*-butyl nitrite or trifluoroacetaldehyde, which were reacted with nucleophilic centers, presumably ε-amino groups, in native IgG. A hydrazone-linked conjugate was formed when MTX γ-hydrazide (6) was reacted directly with IgG that had first been oxidized with periodate to form polyaldehyde IgG. The regiospecifically synthesized conjugates were somewhat more effective inhibitors in vitro of dihydrofolate reductase and of colony formation by human renal cancer (Caki-1) cells than were control nonregiospecific conjugates.

Antibody-mediated drug targeting offers a potentially important approach for increasing the therapeutic efficiency of antineoplastic agents and therefore opens a new dimension in cancer treatment.[1-3] The choice of methotrexate (MTX) as a cytotoxic component of drug–antibody conjugates is based on the fact that it has proved to be a very effective anticancer agent in clinical use.[4] It is stable in an acidic milieu such as the lysosomal compartment where the conjugate may be degraded to release MTX or its low molecular weight derivatives.[5] MTX acts as a potent inhibitor of dihydrofolate reductase (DHFR), an enzyme responsible for recycling 7,8-dihydrofolate to its reduced, physiologically active, 6(*R*)-tetrahydro form, and therefore the cytotoxic effect of MTX has been ascribed to the depletion of the intracellular pool of reduced folates.[6-8]

A key objective in conjugate synthesis is that the linkage incorporating the drug into the conjugate must preserve optimal drug activity either in the intact conjugate per se or in a moiety cleaved by target tumor cells. MTX has been coupled to IgG after activating carboxyl groups by formation of the *N*-hydroxysuccinimide active ester which reacts with nucleophilic centers, presumably amino groups in the protein.[9] Alternatively, the active ester can be converted to the corresponding hydrazide, which reacts with aldehyde groups produced in the IgG by periodate oxidation.[10,11] When conjugated by these methods, bound MTX retains only 10–25% of the DHFR inhibitory effect of equimolar amounts of free MTX. Stereochemical factors play an important role in the interaction between

Scheme I



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0022-2623/89/1832-2426$01.50/0

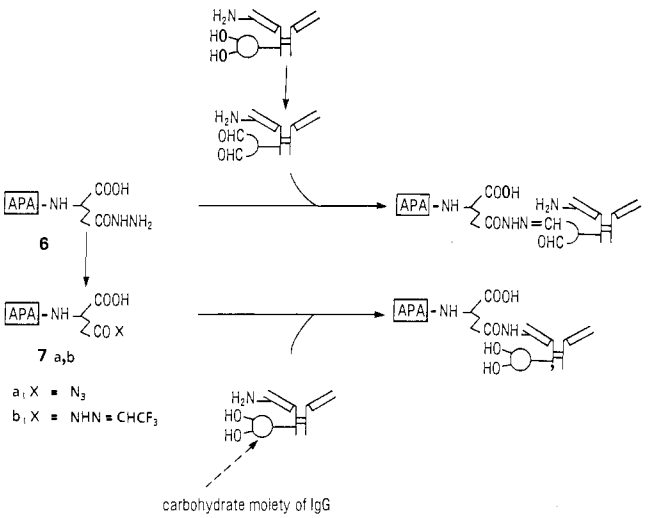
IgG-linked MTX and DHFR and contribute to the decrease in the anti-DHFR activity of conjugated MTX. Another factor[12,13] that is important in the interaction between MTX and DHFR binding relates to the binding of the α-carboxyl group of the glutamic portion of MTX to a highly conserved arginine of DHFR.[14] Therefore, conjugates that preserve the α-carboxyl group should be more potent. The present methods of conjugation based on an activation of carboxyl groups of MTX do not distinguish between them.[9-11]

We have developed a method for regiospecific linkage of MTX to immunoglobulin molecules via the γ-carboxyl group so that the α-carboxyl group remains free. Our approach to regiospecific MTX conjugation is based on the suitable selective activation of the γ-carboxyl group of MTX. A key intermediate in these syntheses was the γ-methyl ester of MTX (**4**). The conjugates produced following this regiospecific method were tested as inhibitors of DHFR as well as of target tumor cells in vitro.

Chemistry. The key intermediate of the coverall reaction scheme is γ-methyl (4-amino-4-deoxy-N^{10}-methylpteroyl)glutamate[12] (**4**, Scheme I), which was synthesized both directly from MTX and also by coupling 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA, **2**) with glutamic acid derivatives. The direct specific esterification of the γ-carboxyl group of MTX is difficult on account of the small difference in the acidity of the two carboxyl groups (pK_a = 3.36 ± 0.2; pK_a = 4.70 ± 0.1),[15] and therefore a substantial amount of MTX diester is also formed. The regiospecific approach, which is commonly used for the preparation of γ-alkyl esters of glutamic acid based on copper complex formation,[16] is of no use in the case of MTX because of the substitution of the amino group of the glutamic acid moiety. However, by use of carefully controlled conditions, the required MTX γ-Me ester (**4**) was prepared in 73% yield by means of absolute methanol in the presence of tetrafluoroboric acid etherate.[17] It is essential that the catalyst is pure and is used in the molar ratio specified. Too little of the catalyst results in unreacted MTX, and too much leads to the dimethyl ester **8** as the major product of the reaction. Our findings are in accord with the experiments of Johns et al.[18] where dry boron trifluoride etherate in ca. 15-fold molar excess over MTX was used to yield MTX dimethyl ester.

An alternate approach to the synthesis of compound **4** involved the direct condensation of APA (**2**) with γ-methyl glutamate using diethyl cyanophosphonate in the presence of a hindered tertiary amine (47% yield) or deblocking the protecting tertiary butyl group of α-*tert*-butyl γ-methyl (4-amino-4-deoxy-N^{10}-methylpteroyl)glutamate (**3**) with boron trifluoride etherate in dry dichloroethane. The α–γ rearrangement was not observed when compound **4** was stored at room temperature.[19] However, TLC revealed slow decomposition to MTX; i.e., after 4-month storage at 23–25 °C less than 2% conversion was observed.

Scheme II



The key intermediate **4** was then converted in excellent yield to MTX γ-hydrazide (**6**) at room temperature with a large molar excess of hydrazine in methanol. This compound served as a derivative capable of being site-specifically conjugated to the oligosaccharide moieties of the immunoglobulin molecules (Scheme II). MTX γ-hydrazide (**6**) also appeared to be a suitable derivative for the amide-based linkage of MTX to IgG since the hydrazide function could be converted to a reactive acylating species (**7a,b**).[20-22] The conversion of hydrazides to azides has often been part of the synthetic scheme in peptide preparation.[23,24] However, this approach has certain limitations in the chemistry of antifolates as they contain sensitive amino groups which are likely to produce unwanted side products. Using *tert*-butyl nitrite hydrochloride,[20,25] we converted hydrazide **6** to the corresponding azide **7a** and used it in situ for the coupling to IgG. The resulting conjugates retained both drug and antibody activity. This indicates that the weakly nucleophilic amino groups of the pteridinyl moiety of MTX were not involved in the process of nitrosation since they are necessary for proper binding to DHFR. A major drawback of this method is its low efficiency in terms of the capability of introducing higher loadings of MTX on the IgG molecule. We did not succeed in achieving loadings greater than 4 mol of MTX/mol of IgG. This could be explained by the lower reactivity of azides compared to succinimidyl active esters and by the formation of MTX amide (which does not react with IgG) during the conversion of MTX γ-hydrazide (**6**) to MTX γ-azide (**7a**). In peptide synthesis using the azide method it was suggested[20] that *N*-nitrosohydrazide formed in the first step is in equilibrium with an oxime form that undergoes dehydration to yield the azide. However, under certain conditions (i.e., low temperature, low acid con-

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centration), a shift in the equilibrium favoring the *N*-nitrosohydrazide can be established, which is followed by an elimination of nitrous oxide to give an MTX amide byproduct.

An alternative method of transformation of **6** to the required synthon was based on reaction with trifluoroacetaldehyde similar to that of Kametani et al.[21] The reaction yielded presumably 2,2,2-trifluoroethylidenehydrazide **7b** which could undergo nucleophilic attack by amino groups of IgG to form the carboxamide MTX–IgG linkage.

The reaction sequence for MTX coupling to IgG which was mediated by an amide linkage is depicted in Scheme II. MTX γ-hydrazide (**6**) was converted to **7b** which, in a hundredfold molar excess, was coupled to IgG in PBS by utilizing the same aproach that has been established for active ester conjugation.[9-11] This approach allowed production of conjugates with higher molar incorporation of MTX (up to 7 mol/mol of IgG) compared to the azide method (up to 4 mol/mol), but the protein recovery was approximately 50% instead of 80–85%.

The hydrazone-mediated synthesis of MTX–IgG conjugates (site specific) consisted of two basic steps as shown in Scheme II. This method involved the generation of aldehyde groups in the carbohydrate portion of the IgG molecule followed by the formation of the hydrazone bond by reaction with **6**. The oxidation of the carbohydrate moieties of IgG by sodium periodate is dependent on a number of variables including the concentration of oxidant, pH, temperature, and time.[26-28] The reaction conditions that were employed here were based on those previously established[10,11] in which IgG was exposed to a 10 mM concentration of sodium periodate at pH 5.5 at 4 °C. The coupling reactions were performed at 4 °C with a 100-fold molar excess of **6**.

The regiospecific conjugation of MTX to immunoglobulins is complicated by the sensitivity of IgG to reaction conditions, and therefore the application of an α-carboxyl protecting group is a serious problem. The protecting group has to be sufficiently stable during coupling and also easily removable under nondenaturing conditions. For instance, the conditions used for the removal of the blocking group in the case of MTX–poly-L-lysine conjugates[29] are too drastic for immunoglobulin conjugates. Removal of an acid-sensitive protecting group (e.g., *tert*-butyl) after the formation of the acylating species and before the conjugation would likely lead to the formation of a cyclic anhydride and therefore to loss of the regiospecific integrity of the conjugates formed in the next step. Cleavage of protecting groups sensitive to alkali or nucleophiles (e.g., methyl, phenacyl) would lead automatically to hydrolysis or a nucleophilic substitution and therefore to an inactivation of the activated γ-carboxyl group (*N*-hydroxysuccinimidyl ester, azide, or 2,2,2-trifluoroethylidenehydrazide).

The methods outlined in this paper for the preparation of the regiospecific conjugates based on transformation of hydrazides have the advantage that the final γ-acylating species of MTX, **7**, can be prepared in three steps from APA (**2**), and its synthesis includes the preparation of the potentially useful compound MTX γ-hydrazide (**6**). The activation of the γ-carboxyl of MTX was achieved without protection of the α-carboxyl group, and therefore, questions about the side reactions involving the α-carboxyl group can arise. The inactivity of this carboxyl function was confirmed when free MTX was treated either with trifluoroacetaldehyde or with *tert*-butyl nitrite under the conditions used for **6**. On the basis of data from literature, the tendency of *N*-benzoylglutamic acid to form oxazolone or *N*-benzoylglutamic acid and glutaric acid to form cyclic anhydrides under the conditions used by us is unlikely.[30-34] An intramolecular cyclization of **6** involving the amino group of the glutamyl moiety of MTX to form a lactam ring,[35] as well as variations of the Dakin–West reaction,[36]

Table I. Inhibition of Beef Liver Dihydrofolate Reductase by Derivatives of MTX (Part A) and MTX–IgG Conjugates (Part B)[a]

Part A

compd	IC_{50}	inhibn index	compd	IC_{50}	inhibn index
MTX	10.8	100	**6**	14.4	75
3			**8**	19.6	55
4	11.9	91	**9**	18.3	59
5	21.6	50			

Part B

MTX–IgG conjugate index	molar incorp	expt 1 IC_{50}	expt 1 inhibn index	expt 2 IC_{50}	expt 2 inhibn index
MTX (free)		12.1	100	9.9	100
MTXA-NRG	4	69	18	58	17
MTXA-NRG	6	63	19	57	17
MTXA-NRG (r)	4	42	29	36	28
MTXA-NRG (r)	6	46	26	40	25
MTXH-NRG	6	72	17	58	17
MTXH-NRG	7	66	18	63	16
MTXH-NRG (r)	5	59	21	50	20
MTXH-NRG (r)	7	62	20	50	20
MTXA-IgG_1 (r)	4	42	29	36	28
MTXA-IgG_1	6	61	20	49	20
MTXA-Dal K-20 (r)	7	45	27	40	25
MTXA-Dal K-20	7	60	20	52	19
MTXH-Dal K-20 (r)	6	45	27	40	25
MTXH-Dal K-20	7	53	23	43	23

[a] Spectrophotometric assay of conversion of dihydrofolate to tetrahydrofolate by DHFR.[48] Results in part A are means of triplicate assays, and results in part B are means of duplicate assays. All determinations were done on the same day, and those for MTX were repeated at the beginning and end of the experiment. The differences between measurements were not more than 5%. Inhibition index = (IC_{50} of MTX/IC_{50}) × 100, where IC_{50} = concentration of inhibitor (MTX derivative or MTX–IgG conjugate) to decrease ΔOD_{340} by 50% and IC_{50} of MTX = concentration of MTX to decrease ΔOD_{340} by 50%. This ratio facilitates comparison between DHFR inhibition experiments where the effect of free MTX on DHFR varies slightly. MTXA-NRG = amide-linked conjugate of MTX and NRG. MTXH-NRG = hydrazone-linked conjugate of MTX and NRG. MTX conjugates with IgG_1 and Dal K-20 are abbreviated similarly. (r) = regiospecific coupling.

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Table II. Colony Formation by Caki-1 Cells. Effect of MTX, Free or Conjugated to Dal K-20[a]

test agent	IC_{50} for inhibn of colony formation, μg/mL	test agent	IC_{50} for inhibn of colony formation, μg/mL
MTX	25	MTXH-Dal K-20	3.4
MTXA-Dal K-20	4.5	MTXH-Dal K-20 (r)	3.0
MTXA-Dal K-20 (r)	3.3		

[a] Results are means of triplicate assays, and the differences between measurements were not more than 5%.

should produce MTX derivatives unable to further react with IgG.

DHFR Inhibition by Conjugates. The retention of drug activity when the drug is bound to IgG depends upon avoiding the introduction of steric hindrance either due to a change in the drug structure produced by introduction of the linkage groups or as a result of interference with the interaction between the bound drug and its target molecules by the adjacent region of the IgG molecule. DHFR inhibition by conjugated MTX has been found to be substantially lower than by the free drug and has varied with different conjugates and DHFR preparations. Typical values range between 15% and 25% of the effectiveness of free MTX[9-11] when comparison is based on the total amount of MTX present, i.e., the concentration of the conjugate multiplied by the incorporation ratio of MTX in moles per mole of IgG. Inhibition index values (i.e., the ratio of the IC_{50} value of MTX to that of a given conjugate × 100) are in Table IB. This method of comparison was necessary because of slight variations in the IC_{50} values of MTX from experiment to experiment. If the values were expressed in terms of the concentration of conjugate (i.e., carrier IgG), almost all conjugates would appear to be equal or superior to the free drug. For example, the inhibition index for MTXA-Dal K-20 (r) would be 189 compared to 100 for free MTX. This suggests that more than one molecule of DHFR can simultaneously bind to individual MTX substituents at distinct sites on the carrier IgG, albeit with somewhat reduced affinity due to steric hindrance. It is not likely that DHFR can be bound simultaneously to all MTX substituents in any of the conjugates, considering that multiple MTX substituents could be sufficiently close together to allow only one DHFR molecule to bind in that region of the conjugate molecule. The differences in the inhibition index values for DHFR among the MTX-IgG conjugates (Table IA) are small but consistent with the inhibition produced by the corresponding γ and disubstituted low molecular weight derivatives in Table IA.

Immunoreactivity of the Conjugates and Tumor Inhibition. Membrane immunofluorescence of 2×10^7 Caki-1 cells with serial dilutions of Dal K-20 (an MAB raised against Caki-1 cells) or its MTX conjugates starting at 1.0 mg/mL yielded titers (i.e., detectable staining of 50% of cells) of 12.5 μg/mL for the monoclonal antibody and its four MTX conjugates. This confirms our previous observations[10] that, at these levels of MTX incorporation (<10 mol of MTX/mol of IgG), there was no detectable change in the immunoreactivity of the antitumor antibodies. MTX inhibited colony formation by Caki-1 cells when conjugated to Dal K-20. Substantial inhibition was observed at concentrations at which free MTX had little effect. The IC_{50} value for both amide-linked and hydrazone-linked regiospecific conjugates was less than that for the corresponding nonregiospecific conjugates (Table II). However, the differences in the IC_{50} values of the four conjugates are quite small and not significant even though

Table III. Effect of MTX Linked to Dal K-20 via Various Linkages on Colony Formation by Melanoma M21 Cells[a]

agents to which cells were exposed	no. of colonies per plate at concn of MTX, μg/mL		
	10	15	20
MTXA-Dal K-20	109 ± 9.10	117 ± 6.12	119 ± 4.10
MTXA-Dal K-20 (r)	115 ± 4.12	118 ± 7.10	112 ± 6.10
MTXH-Dal K-20	112 ± 4.10	109 ± 5.10	108 ± 4.50
MTXH-Dal K-20 (r)	120 ± 10.11	121 ± 7.12	115 ± 8.12

[a] M21 cells alone (untreated); 121 ± 6.10 colonies per plate; cells used = 1000 M21 cells per plate. IC_{50} (the concentration that reduces colony formation by 50%) of MTX for the M21 cells was 10 μg/mL. (r) = regiospecific coupling.

on repeating the colony inhibition assay the order of the inhibitory capacity of the conjugates was the same as in Table II. We have demonstrated that reasons for the increase in cytotoxic potential of MTX when conjugated to targeting antibodies include higher uptake of MTX linked to antibodies against cell surface associated antigens (compared to the uptake of free MTX or MTX linked to a nonspecific IgG), followed by slow sustained catabolism so that there is prolonged maintenance of intracellular active derivatives at a level exceeding that of DHFR.[37-41] We have also demonstrated that exposure of MTX-Dal K-20 conjugates to lysosomal enzymes (pH 4.6, 37 °C) for 24 h releases 50–60% of the bound drug from hydrazone-linked (MTXH) conjugates and 20–30% of the drug from amide-linked (MTXA) conjugates.[11] As free MTX is 4–5 times more potent than its various IgG conjugates in inhibiting DHFR (Table I), the slow sustained intracellular release of free MTX from endocytosed conjugates may add to the potency of MTX-antibody conjugates. Furthermore, the release of free MTX in larger amounts from the hydrazone-linked conjugates (compared to the amide-linked conjugates) may explain its somewhat greater cytotoxic potency (Table II). That the specificity of the inhibitory effect of MTX-Dal K-20 conjugates was associated with the specificity of the carrier antibody is supported by the observations that (i) MTX conjugated to normal mouse IgG (NMG) up to a concentration of 25 μg/mL (the IC_{50} concentration of the free drug) had no effect on colony formation by Caki-1 cells and (ii) none of the four types of MTX-Dal K-–20 conjugate had any effect on the nontarget human M21 melanoma cells up to a concentration of 20 μg/mL conjugated MTX (Table III) even though M21 cells were more sensitive to MTX compared to Caki-1 cells. The IC_{50} concentrations of free MTX for the M21 melanoma and Caki-1 cells were 10 and 25 μg/mL, respectively. In contrast, when MTX was conjugated to MAB 225.28S (a monoclonal antibody raised against M21 melanoma cells), or a polyclonal antihuman melanoma antibody, the conjugates were seen to be more potent inhibitors of M21 cells compared to the free drug or MTX linked to a nonspecific IgG.[42] Dal K-20 alone did not have any significant inhibitory effect on colony formation by Caki-1 cells up to a concentration of 400 μg/mL IgG because the number of colonies formed by Caki-1 cells after exposure to 0, 100, 200, and 400 μg/mL

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Dal K-20 were respectively 185 ± 7.63, 184 ± 8.54, 181 ± 8.54, and 173 ± 7.63. As the amounts of Dal K-20 in conjugates that were potent inhibitors of Caki-1 cells were below 400 μg/mL, the increase in the effectiveness of MTX after linkage to Dal K-20 in suppressing colony formation by Caki-1 cells was not due to the inhibitory effect of Dal K-20 itself on Caki-1 cells.

Experimental Section

The structures of all compounds (Scheme I) were consistent with ^1H or ^{13}C NMR spertra. NMR spectra were measured on a Nicolet NT369NB instrument with chemical shifts reported relative to tetramethylsilane. TLC was performed on fluorescent Merck–Keiselgel 60 silica gel plates using a 5:4:1 $CHCl_3$–EtOH–H_2O solvent system. The spots were visualized under 254-nm ultraviolet light or by special reagents as specified in the individual experimental descriptions. The melting points were measured in Pyrex capillary tubes in a Kofler block and are not corrected. The chemicals used were purchased from Aldrich Chemical Co., Milwaukee, WI, and Sigma Chemical Co., St. Louis, MO.

4-[*N*-[(2,4-Diamino-6-pteridinyl)methyl]-*N*-methylamino]benzoic Acid (2). A modification based on data reported by Rosowsky et al.[13,43] was used. A mixture of dibromotriphenylphosphorane (19 g, 45 mmol) and 2,4-diamino-6-(hydroxymethyl)pteridine (2.88, 15 mmol) in dry DMA (36 mL) was stirred at room temperature for 24 h. The resulting bromide was then coupled with 4-(methylamino)benzoic acid (3.02 g, 20 mmol) in the presence of *N*-ethyldiisopropylamine (7.8 mL, 45 mmol) under stirring at room temperature for 48 h. The reaction mixture was poured into 0.33 M NaOH (250 mL), the precipitate was removed by filtration, and the filtrate was adjusted to pH 5.5 with 10% acetic acid. The product was filtered off, washed with water, and dried at 78 °C in vacuo over phosphorus pentoxide, to give an orange powder (4.64 g, 86%): mp 242 °C; R_f 0.59.

α-*tert*-Butyl γ-Methyl *N*'-[4-[*N*-[(2,4-Diamino-6-pteridinyl)methyl]-*N*-methylamino]benzoyl]-L-glutamate (3).[13] To a suspension of 2 (2.0 g, 5.50 mmol) in dry DMF (200 mL) were added *N*-ethyldiisopropylamine (1.92 mL, 11.0 mmol) and diethyl cyanophosphonate (1.52 mL, 11.0 mmol). The solution was stirred for 4 h, and then α-*tert*-butyl γ-methyl L-glutamate (2.90 g, 11.0 mmol) was added. A second portion of *N*-ethyldiisopropylamine (1.92 mL, 11.0 mmol) was added, and stirring was continued at room temperature for 48 h. The reaction was terminated by adding solid sodium bicarbonate (0.90 g, 11.0 mmol), and the solvent was evaporated under reduced pressure. The residue was chromatographed on a silica gel column by using 5% methanol in chloroform. Pooled TLC homogenous fractions were concentrated to obtain the product as bright yellow fine crystals: mp 142–144 °C; R_f 0.71.

γ-Methyl *N*'-[4-[*N*-[(2,4-Diamino-6-pteridinyl)methyl]-*N*-methylamino]benzoyl]-L-glutamate (4). Method A. To a suspension of **3** (104 mg, 0.2 mmol) in dry dichloroethane (5 mL) was added dropwise boron trifluoride etherate (37.5 μL, 0.3 mmol). The suspension was stirred for 18 h, and then water (1 mL) was added to break up the complex. The organic solvent was evaporated to yield the product as a yellow oil which crystallized upon standing for 24 h. It was then recrystallized from aqueous ethanol (81 mg, 88%): mp 170–174 °C; R_f 0.50.

Method B. A solution of **2** (2 g, 5.50 mmol), *N*-ethyldiisopropylamine (1.92 mL, 11.0 mmol), and diethyl cyanophosphonate (1.52 mL, 11.0 mmol) in dry DMF (200 mL) was stirred for 4 h at room temperature, and then solid γ-methyl glutamate (1.78 g, 11.0 mmol) and a second portion of *N*-ethyldiisopropylamine (1.92 mL, 11.0 mmol) were added. The reaction mixture was stirred at room temperature for 72 h, the solvent was evaporated, and the residue was chromatographed on a silica gel column using chloroform–methanol (95:5). Crystalization from aqueous ethanol gave yellow crystals: mp 172–176 °C; R_f 0.50.

Method C. A mixture of dry MTX (182 mg, 0.40 mmol), anhydrous sodium sulfate (85 mg, 0.60 mmol), and tetrafluoroboric acid etherate (85%, 0.054 mL) in methanol (0.65 mL) was stirred at room temperature for 18 h. The reaction mixture was neutralized by saturated ammonium bicarbonate, and then water (5 mL) was added and the mixture was extracted with chloroform to separate the formed diester 8. Yellow crystals (38 mg, 19%) were obtained: mp 130–134 °C; R_f 0.58. The aqueous phase was lyophilized to yield the monoester 4 (yield 73%) which was either used directly or further purified by column chromatography as indicated above to remove a trace of its α-isomer. The product was isolated in the form of yellow crystals: mp 172–174 °C; R_f 0.50. The products formed by all three methods were indistinguishable from each other as determined by ^1H and ^{13}C NMR spectra.

***tert*-Butyl *N*'-[4-[*N*-[(2,4-Diamino-6-pteridinyl)-methyl]-*N*-methylamino]benzoyl]-L-glutamic Acid γ-Hydrazide (5).** Compound **5** was synthesized as described in the literature;[13] after 72 h of reaction time, the product was separated from the starting material in 77% yield by using preparative TLC. Longer reaction times did not result in greater conversion of the starting diester **3**.

***N*'-[4-[*N*-[(2,4-Diamino-6-pteridinyl)methyl]-*N*-Methylamino]benzoyl]-L-glutamic Acid γ-Hydrazide (6).** To a solution of 4 (194 mg, 0.720 mmol) in methanol (4 mL) was added hydrazine hydrate (100 μL), and the reaction mixture was stirred at room temperature for 48 h. Then, the solvent was removed under vacuo, and the product was obtained as a yellow oil (86 mg, 91%), R_f 0.06.

***N*'-[4-[*N*-[(2,4-Diamino-6-pteridinyl)methyl]-*N*-methylamino]benzoyl]-L-glutamic Acid Dihydrazide (9).** The compound was prepared from 4 under conditions similar to those used by Rosowsky et al.[44] in 92% yield, R_f 0.15; on reaction with acetone, it gave the corresponding hydrazone derivative with R_f 0.62.

Periodate Oxidation of IgG. General Procedure. To a solution of rabbit IgG (10 mg, $6{,}67 \times 10^{-5}$ mmol) in sodium acetate buffer (0.1 M, pH 5.6, 1 mL) was added sodium periodate (0.02 M, 0.1 mL), and the reaction mixture was stirred at 4 °C. The course of the reaction was followed spectrophotometrically,[45] and after 2 h the reaction was stopped by the addition of ethylene glycol (10 μL) and desalted by Bio-Gel P-6 column chromatography.

Conjugation of 6 to Oxidized IgG by a Site-Specific Method. General Procedure. To a stirred solution of oxidized IgG (4.2 mg, 28 nmol) in PBS (1 mL) was dropwise added a solution of **6** (13.5 mg, 2.8 μmol) in DMF (200 μL), and the stirring was continued at 4 °C for 14 h. The reaction mixture was adjusted to pH 8.0 with 0.1 M NaOH and passed through the Bio-Gel P-6 column to remove unreacted material. The isolated conjugates contained 5–7 mol of drug/mol of IgG.

Conjugation of 6 to IgG by a Non-Site-Specific Method. General Procedure. Method A. A solution of **6** (9.7 mg, 0.02 mmol) in dry DMF (0.5 mL) was mixed at −20 °C with 2.4 M HCl (13 μL) and 0.1 M *tert*-butyl nitrite (0.15 mL), both in dry THF, and the mixture was stirred at −20 °C for 1 h. The resulting solution of **7a** was then added dropwise (0.2 mL, 3 μmol) to a stirred solution of IgG (5 mg, 33.3 mmol) in PBS (1 mL) and stirred at 4 °C for 2 h. The reaction mixture was passed through a Bio-Gel P-6 column, and the isolated conjugate was characterized. Molar incorporations were 3–4 mol of MTX/mol of IgG, with protein recoveries ranging from 80% to 85%.

Method B. A solution of **6** (9.7 mg, 0.02 mmol) in dry DMF (0.5 mL) was mixed with a solution of trifluoroacetaldehyde[46] (0.25 M, 0.4 mL) in dry THF, and the reaction mixture was stirred at 25 °C for 18 h. The unreacted aldehyde was removed by a stream of N_2, the reaction mixture containing **7b** (3.3 μmol) was cooled to 4 °C and then added dropwise to a stirred solution of IgG (5 mg, 33.3 nmol) in PBS (1 mL), and the stirring was continued at room temperature for 48 h. The reaction mixture was passed through a Sephadex G-25 (PD-10) desalting column. The purified conjugates contained 4–7 mol of drug/mol of IgG, with protein recoveries ranging from 45% to 65%.

Nonregiospecific Preparation of MTXA–IgG and MTXH–IgG Conjugates. This method of preparation has been described in detail.[11] The molar incorporation and inhibition index

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values of the nonregiospecifically synthesized conjugates prepared for this experiment are shown in Table I.

Characterization of Conjugates: Molar Incorporation of MTX in Conjugates. The amount of MTX incorporated per mole of IgG was determined by measuring the absorbance at 370 nm, using the previously established molar absorbance coefficient of 6500 M^{-1} cm^{-1} for bound MTX.[9] Protein was determined by the method of Lowry,[47] using a protein standard containing 30 mg/mL HSA and 50 mg/mL human IgG.

Determination of Drug Activity by the DHFR Inhibition Assay. The DHFR assay is based on the method of Peterson.[48] To a 3.0-mL cuvette were added reagents and test samples to the required volume of distilled water in the order given: sodium acetate buffer (pH 6.0, 1.0 mL, 1.5 M); KCl (1.0 mL, 1.8 M); NADPH (150 μL, 1.0 mM); 0–100 μL of drug containing sample in 0.01 M PBS (2.2×10^{-5} and 2.2×10^{-6} M with respect to MTX); 0.02 unit of beef liver DHFR. The DHFR was introduced, mixed, and incubated at 22 °C for 2 min. The reaction was initiated by the similar addition of dihydrofolic acid (Sigma) (100 μL, 1 mM), and the change in absorbance per minute at 340 nm was calculated.

Tumor Lines and Antibodies. The Caki-1 cell line derived from a skin metastasis of an RCC from an adult male Caucasian[49] came from the Human Tumor Cell Bank through the American Type Culture Collection (Rockwell, MD). The M21 melanoma cell line was from Dr. Soldano Ferrone, New York Medical College, Valhalla, NY. The monoclonal antibody Dal K-20, a mouse IgG_1, was from a hybridoma constructed in our laboratory by the fusion of mouse myeloma SP-2 with splenic cells from BALB/c mice immunized with Caki-1 cells. This antibody reacted with 5/6 human renal cell carcinomas and the lining epithelium of normal proximal and distal convoluted tubules. The details of the reactivity and specificity of Dal K-20 have been reported.[50] Ascites fluid was harvested 15 days after ip inoculation of 1.5×10^7 clone Dal K-20 cells in Pristane-primed BALB/c mice.

Dal K-20 was isolated by caprylic acid purification[51] with a yield of 2–3 mg of IgG/mL of ascites fluid. Isolation of NRG from rabbit serum was performed in similar manner, and the purity of all preparations was checked by SDS-PAGE.[52]

Colony Inhibition Assay. The procedure was based on that described by Lathan et al.[53] In brief, single-cell suspensions of Caki-1 or M21 cells (>99% impermable to trypan blue) were incubated at 37 °C for 6 h in McCoy's medium supplmented with 15% dialyzed fetal calf serum (1×10^6 cells/mL) and containing various concentrations of the MAB, NRG, MTX, or the conjugates, washed three times, and mixed with 0.3% agar which was poured onto a 0.5% basal layer of agar in Multiwell plates (Becton Dickinson, Lincoln Park, NJ) (10^4 cells/plate) that were incubated at 37 °C in an atmosphere containing 5% CO_2. The plates were monitored periodically with an inverted stage microscope. Colonies were counted visually after 14 days, and only those colonies with more than 30 cells (≤5 cell divisions) were included. An average of three plates were counted for each concentration. For storage, the agar layer was covered with 2.5% glutaraldehyde in PBS and kept at 4 °C.

Registry No. **1**, 59-05-2; **2**, 19741-14-1; **3**, 79648-88-7; **4**, 67022-39-3; **5**, 79640-69-0; **6**, 79640-75-8; **7a**, 121788-83-8; **7b**, 121788-84-9; **8**, 34378-65-9; **9**, 77410-28-7; **9** [bis(acetone hydrazone)derivative], 121788-82-7; DHFR, 9002-03-3; 4-(MeNH)C_6H_4COOH, 619-84-1; H-Glu(OMe)-OBu-*t*, 79640-72-5; H-Glu(OMe)-OH, 1499-55-4; 2,4-diamino-6-(hydroxymethyl)-pteridine, 945-24-4.

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