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Research Paper

The Effect of Molecular Weight, Drug Load, and Charge of Gelatin–MTX Conjugates on Growth Inhibition of HL-60 Leukemia Cells

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Purpose. Gelatin–methotrexate conjugates (G-MTX) with known molecular weight (MW), drug load, and charge were prepared and evaluated for growth inhibition on leukemia cells.
Methods. Gelatin (34 to 171 kDa) was reacted with a carbodiimide to prepare G-MTX with high (G-MTX-H) and low (G-MTX-L) drug loads. Cationic conjugates were prepared by ethylenediamine modification. MTX:gelatin molar ratios were determined spectrophotometricaly. Isoelectric focusing electrophoresis (IEF) and turbidity were used to measure isoelectric points (IEP). Growth inhibition profiles and IC_{50} values were determined on HL-60 cells using a modified MTT assay.
Results. IC_{50} values of anionic G-MTX-L (drug loads 0.5:1 to 2.2:1) increased linearly from 46 to 180 nM with MW. But, IC_{50} values for anionic G-MTX-H (drug loads 7.4:1 to 25:1) showed little, if any, MW dependence and were about two times higher. IC_{50} values for cationic G-MTX-L ranged from 770 to 2,900 nM and the relationship with MW was non-linear.
Conclusions. The growth inhibition ranking was MTX > anionic G-MTX-L > anionic G-MTX-H > cationic G-MTX-L. High drug load may hinder lysosomal enzyme degradation and drug release and contribute to suppression of the MW effect observed with G-MTX-L. A mechanism change is suggested as the cationic conjugates increase to the highest MW.

KEY WORDS: charge; drug load; HL-60 growth inhibition; macromolecular drug conjugates; molecular weight.

INTRODUCTION

Methotrexate (MTX) has been in clinical use for 50 years since the therapeutic effect of MTX against choriocarcinoma and chorioadenoma was reported by Li *et al.* in 1956 (1). It is an antifolate drug used not only for a variety of cancers but also used in the treatment of various autoimmune diseases (2). MTX prevents cell growth by inhibiting the target enzyme, dihydrofolate reductase (DHFR) and subsequently interrupts DNA and RNA synthesis (3). However, MTX also has a substantial nonselective toxicity and a high occurrence of drug resistance (2–4).

Macromolecular conjugates of low molecular weight drugs at molecular weights ranging from a thousand daltons up to several million daltons have been developed for cancer therapy because they offer several potential therapeutic advantages compared to the free drug. A 2006 review notes that 21 anticancer polymer conjugates were either in clinical trials or on the market (5). The potential advantages include higher maximum tolerated doses (6), an extended half-life of the conjugate in circulation (7,8) a passive intra-tumor accumulation by the enhanced permeability and retention (EPR) effect (9–11) as well as the potential to overcome multidrug resistance in cancer treatment (12,13). However, the *in vivo* and *in vitro* fate of macromolecular conjugates are affected by both the physicochemical properties of the conjugates and the anatomical and physiological characteristics of the body or tissues (14–16). The macromolecular carrier dominates the physicochemical properties of the conjugate, such as molecular weight and charge, since it typically constitutes over 80% of the total weight.

The influence of conjugate molecular weight on cellular effect has been explored in the literature but the results are inconclusive. The uptake of two nonionic polymer carriers without drug, *i.e.*, *N*-(2-hydroxypropyl)methacrylamide (HPMA) and poly(vinylpyrrolidone) (PVP), increased in rat yolk sacs as the molecular weight of the carriers decreased but uptake into macrophages and rat intestine cells increased proportionally with carrier molecular weight (17,18). For conjugates, three anticancer conjugates, polyethylene glycol-MTX (PEG-MTX), poly-L-lysine-MTX, and mitomycin C–dextran, have been examined for their molecular weight

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ABBREVIATIONS: DHFR, dihydrofolate reductase; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl; EPR, enhanced permeability and retention; G-MTX-H, gelatin–methotrexate conjugate of high MTX to gelatin molar ratio; G-MTX-L, gelatin–methotrexate conjugate of low MTX to gelatin molar ratio; HL-60, promyelocytic leukemica cells; HPLC-SEC, high performance liquid chromatography-size exclusion column; HPMA, *N*-(2-hydroxypropyl) methacrylamide; LOD, loss on drying; MTX, methotrexate; MW, molecular weight; PEG, polyethylene glycol.

effect on cytotoxicity. Results from PEG-MTX conjugates of 750 to 40,000 Da showed that cytotoxicity was inversely proportional to molecular weight in HL-60 cells even though no apparent molecular weight influences were observed on other cell lines investigated (19). Anionic mitomycin C–dextran conjugates displayed a slight molecular weight effect on cytotoxicity against L1210 leukemia cells while the cationic dextran conjugates showed no molecular weight effect on cytotoxicity (20,21). Cationic poly-L-lysine–MTX conjugates ranging from 3,100 to 130,000 Da also showed comparable cytotoxicity in Chinese Hamster Ovary cells regardless of molecular weights of poly-L-lysine (22). However, the different drug load per conjugate in mitomycin C–dextran and poly-L-lysine–MTX conjugates may obscure the actual molecular weight effect of these macromolecular conjugates. Cationic poly-L-lysine–MTX conjugates have shown a greater cytotoxicity against rat hepatoma cells and L1210 leukemia cells when the MTX load per conjugate was lower (23). An anionic gelatin–MTX conjugate has demonstrated that different drug load affects the *in vitro* growth inhibition of HL-60 cells (24). Therefore, a molecular weight effect on *in vitro* cytotoxicity is not clear and may be affected by the macromolecule, drug load, and cell type.

Cationic macromolecular conjugates usually have better cellular uptake than anionic conjugates due to an interaction with negatively charged cell membranes (21,25–28). The charge effects of two carriers were examined by *in vitro* cytotoxicity studies (21,26). Cationic mitomycin C–dextran conjugates produced a greater growth inhibition than anionic conjugates on L1210 leukemia cells and Ehrlich ascites carcinoma cells (21). However, a neutral poly-L-lysine chlorin e6 conjugate was more cytotoxic than anionic and cationic conjugates in human endothelial hybrid cells and human epidermoid squamous carcinoma cells (26). These studies showed that the charge effect on *in vitro* cytotoxicity can vary depending on the macromolecule and cell type.

The current investigation is a continuation of our studies on the properties of gelatin–MTX conjugates for cellular drug delivery (24). The current investigation is designed to examine the effect of molecular weight, drug load, and charge of gelatin–MTX conjugates on HL-60 leukemia cell growth. The cytotoxicity of these conjugates against HL-60 cells was evaluated by measuring growth inhibition and comparing IC_{50} values.

MATERIALS AND METHODS

Materials

Type A gelatin (250, 175, and 75 Bloom) and Type B gelatin (250 Bloom) were donated from Kind & Knox (Sioux City, IA). The molecular weights for type A gelatins were 100,000, 51,800 and 34,400 Da, respectively. The molecular weight of type B gelatin was 171,000 Da. The average molecular weight of gelatin was determined by Chromaceutical Advanced Technologies (Hopkinton, MA) using a laser light scattering detector coupled to an HPLC-SEC. Moisture contents of gelatins were determined by loss on drying (LOD) at 105°C for 72 h. Citraconic anhydride was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sephadex G-50, MTX (±amethopterin, 95% (*w*/*w*), 12% (*w*/*w*) moisture), 1-ethyl-3-(dimethylaminopropyl) carbodiimide HCl (EDC), ethylenediamine (98.9% (*w*/*v*) pure), RPMI-1640 medium (with L-glutamine and $NaHCO_3$, sterile-filtered), fetal bovine serum, and gentamicin solution (10 mg/ml, sterile-filtered) were purchased from Sigma Chemical Co. (St. Louis, MO). BCA protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL). Spectra/Por molecularporous membrane tubing (molecular weight cut off 6,000–8,000 Da) was purchased from Spectrum Medical Industries, Inc. (Houston, TX). Trichloroacetic acid was purchased from Fisher Chemical (Fair Lawn, NJ). Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Gaithersburg, MD). Water was purified by reverse osmosis. All other chemicals were at least ACS reagent grade.

Methods

Preparation of Anionic Gelatin–MTX Conjugates with Varying Molecular Weight and Different Drug Loads

A total of eight different anionic gelatin–MTX conjugates were prepared from four different molecular weights of gelatins with low and high MTX loadings, *i.e.*, G-MTX-L and G-MTX-H, respectively. These conjugates were essentially synthesized as described previously (29) (Fig. 1A) with the exception of the unsuccessful deblocking step of type A gelatins noted below. Briefly, gelatin (100 mg) was dissolved in 6 ml of 0.1 M $NaHCO_3$ and reacted with citraconic anhydride for 5 h at pH 8.0–9.0 and 25°C with 50 rpm shaking. The blocked gelatin was then separated from excess citraconic anhydride by a preparative Sephadex G-50 column (42×2 cm) and eluted with 0.05 M $NaHCO_3$ at 37°C. A 12 ml blocked gelatin solution mixed with either 15 or 37.5 mg of MTX in 3 ml 0.1 M $NaHCO_3$ for 2 h at pH 7.0 and 25°C with 50 rpm shaking was followed by the addition of either 12.5 or 75 mg EDC at pH 7.0 for 24 h to prepare G-MTX-L or G-MTX-H, respectively. The blocked conjugates were separated from unreacted MTX by a Sephadex G-50 column and lyophilized.

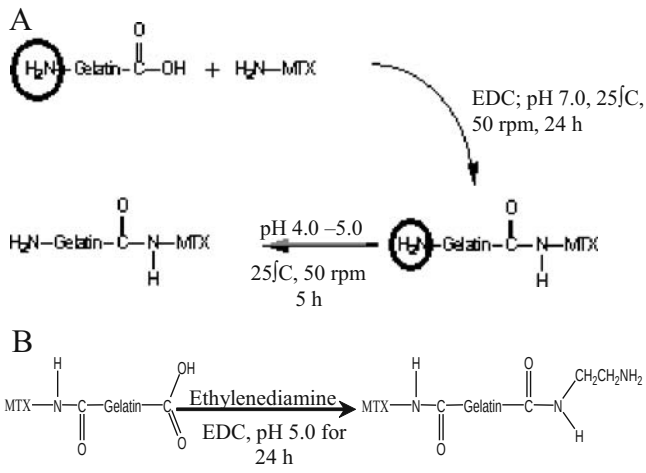

Fig. 1. A Conjugation process of anionic gelatin–MTX conjugates from type B gelatin. *Circled amino groups* indicate these groups are blocked by citraconic anhydride and not available for the EDC conjugation. Product of the conjugation also includes an isourea by-product not shown. **B** Cationization step of gelatin–MTX conjugates.

The blocked conjugates were dissolved in 5 ml of water and then subjected to an amino group deblocking process at pH 4.0–5.0 for 5 h. Finally, all conjugates were purified by SEC, lyophilized and stored in a desiccator at −20°C. The three type A gelatin conjugates unexpectedly retained the citraconic specie with its carboxylic acid group after the deblocking step which converted it to an anionic charge as indicated by their isoelectric points (see below). The highest molecular weight anionic conjugate was prepared from the type B gelatin sample (see Fig. 1A).

Preparation of Cationic Conjugates with Varying Molecular Weight

Anionic gelatin–MTX conjugates were chemically modified with ethylenediamine to acquire amino groups and a resulting cationic charge at physiological pH as described previously (30) (Fig. 1B). Two anionic conjugates from type A gelatin (35,000 and 101,000 Da) were used without the deblocking step while the anionic conjugate from type B gelatin (172,000 Da) was exposed to a deblocking step. A 60 mg sample of anionic gelatin–MTX conjugate in 1 ml of water was mixed with 1.74 ml of ethylenediamine, and the pH of the mixture was adjusted to 5.0. To this mixture, 49.8 mg of EDC in 0.15 ml of water was added, and the pH was re-adjusted to 5.0. The reaction mixture was kept at room temperature for 24 h with constant stirring, followed by SEC purification. The collected fractions were then transferred into a dialysis membrane tube for further purification. The tube was immersed in 2 l of water for 24 h accompanied by three changes of water and constant stirring. After dialysis, the product was lyophilized and stored in a desiccator at −20°C. Three cationic gelatin–MTX conjugates were prepared at the molecular weights of 35,000, 101,000, and 172,000 Da.

Determination of Gelatin and MTX Content in Gelatin–MTX Conjugates

All conjugates were characterized by the methods described previously (29). In summary, gelatin content of the conjugates was analyzed by a BCA protein assay and MTX content was determined at 372 nm by UV spectroscopy. The moisture content of conjugates was determined by mass balance. All determinations were triplicate or more measurements from one sample.

Determination of Conjugate Isoelectric Point

Conjugate isoelectric point (IEP) was determined by isoelectric focusing (IEF) electrophoresis or a turbidity measurement method. For the IEF method, samples (0.9 mg/ml) were loaded into the IEF gels (Bio-Rad, Hercules, CA) with IEF standards (Bio-Rad, Hercules, CA). The gel was run at 100 V for 1 h, 250 V for 1 h, and 500 V for 30 min. The gel was fixed with 20% trichloroacetic acid for 15 h. Then, the gel was stained with IEF gel staining solution (Bio-Rad, Hercules, CA). All IEF gels were scanned and stored electronically. Values were determined from one or two independent experiments. For the turbidity measurement method (31), several conjugate solutions (5 mg/ml) were prepared to cover the pH range of pH 10–12.5. The samples were adjusted to different pH values with either HCl or KOH solutions. At the desired time points, pH values of the sample solutions were measured by an Orion Model 720A pH meter (Orion Research Incorporated, Boston, MA) with a semi-micro combination pH electrode (Thermo Orion, Beverly, MA). For turbidity measurements, 200 μl of each sample solution was transferred into a 96-well plate in triplicate, and the absorbance was measured at 490 nm in a microplate reader (MolecularDevices, Spectramax Plus, Sunnyvale, CA).

Growth Inhibition of Conjugates on HL-60 Cells

The HL-60 promyelocytic leukemia cell line was kindly donated by Dr. Ruy Tchao (Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, University of the Sciences in Philadelphia, Philadelphia, PA). The cells had a 25 h (31) doubling time and were cultured with RPMI-1640 medium (with L-glutamic acid) supplemented with 20% fetal bovine serum and 50 μg/ml gentamicin at 37°C in a humidified surrounding and 5% CO_2 atmosphere. HL-60 cell suspension (200 μl) at 1×10^5 cells/ml was seeded in the wells of a 96-well plate for 24 h. Growth medium, MTX, or conjugates solutions (50 μl) were then introduced to cells in the plate. All concentrations are expressed as MTX equivalent concentrations. At the time of addition, untreated cell growth in the controls was measured at 24 h to establish a baseline for determination of any cytocidal effects on growth. After 72 h incubation, a 30 μl cell suspension was withdrawn from each well and placed into a new well. Each new well was then mixed with 70 μl of growth medium. Viable cells were determined with a CCK-8 assay (modified MTT) in a reproducible linear working cell concentration range of 5,000 to 50,000 cells/ml. UV assay values of viable cells in the sample were expressed as a percentage to that of untreated controls to obtain values of percent growth. However, values of growth in the controls for the first 24 h were subtracted from measurements at each time point and from the controls to remove the contribution of cell growth before drug addition. This calculation is used to allow detection of growth inhibition to a cell concentration below that at the time of drug addition. Such an extent of growth inhibition would be shown as "negative growth" and would represent a cytocidal, rather than a cytostatic drug effect. MTX runs were conducted with different conjugates to serve as a control for each growth inhibition study. Samples were prepared in triplicate.

Determination of IC_{50} Values by Curve Fitting

To determine the individual sensitivity of HL-60 cells toward MTX or different conjugates, the IC_{50} value of each sample replicate was determined after nonlinear regression by Sigma Plot 2001, Version 7.0. The curve was first established by the Hill equation (three parameters) described as follows: $Y = (aX^b)/(c^b + X^b)$, where Y is the percentage of growth inhibition (100% minus percentage of sample growth relative to untreated controls), X is the MTX concentration, and a, b, c are the parameters determined by the software. The IC_{50} value of each sample replicate was determined by algebraic manipulation for the concentration that caused a 50% growth inhibition. Each IC_{50} value was calculated as the average of three replicates.

Table I. Characterization of Gelatin–MTX Conjugates with Low and High MTX Loading

	Molecular weight[a] (Da)	Molar ratio (MTX moles/gelatin mole)	Isoelectric point (IEP)[b]
Anionic[c]	172,000	1.9	4.9
G-MTX-L	101,000	2.2	4.9
	52,000	0.9	4.9
	35,000	0.5	4.9
Anionic[d]	182,000	25	4.9
G-MTX-H	107,000	15	4.9
	56,000	9.3	4.9
	38,000	7.4	4.9
Cationic[c]	172,000	2.0	10.8[e]
G-MTX-L	101,000	1.5	10.6[e]
	35,000	0.6	>9

[a] Molecular weight is calculated as original gelatin value plus incorporated MTX
[b] IEP determination by isoelectric focusing electrophoresis
[c] Low drug molar ratio
[d] High drug molar ratio
[e] IEP determination by turbidity measurement method

RESULTS

Characterization of Gelatin–MTX Conjugates

Characterizations of the gelatin–MTX conjugates are summarized in Table I. The conjugates have a molecular weight range from 35,000 to 182,000 Da. Anionic G-MTX-L and cationic G-MTX-L conjugates have a MTX molar ratio ranging from 0.5 to 2.2. Anionic G-MTX-H conjugates have a molar ratio ranging from 7.4 to 25. Anionic G-MTX-L and G-MTX-H have the same IEP value of pH 4.9 and are negatively charged at physiological pH. Cationic G-MTX-L conjugates, however, are positively charged at physiological pH because of their high IEP values of 9 to 10.8.

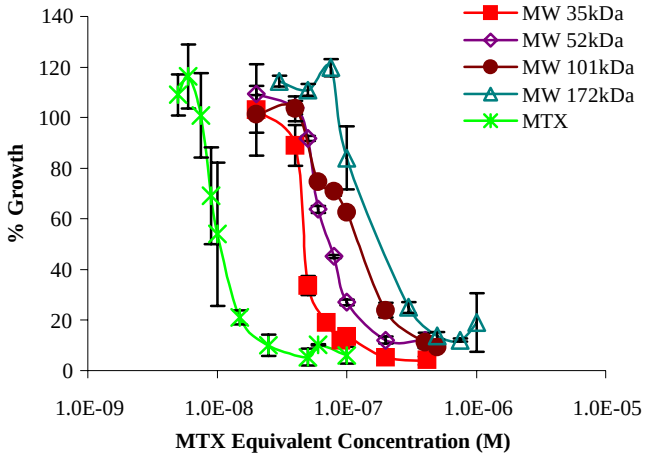

Fig. 2. Growth inhibition curves of HL-60 cells after 72 h incubation with anionic gelatin–MTX conjugates (anionic G-MTX-L) with a low MTX load ranging from molar ratios of 0.5 to 2.2. Values are the mean of nine replicates in three experiments for MTX and mean of three replicates in one experiment for the conjugates; *error bars* are ±SD.

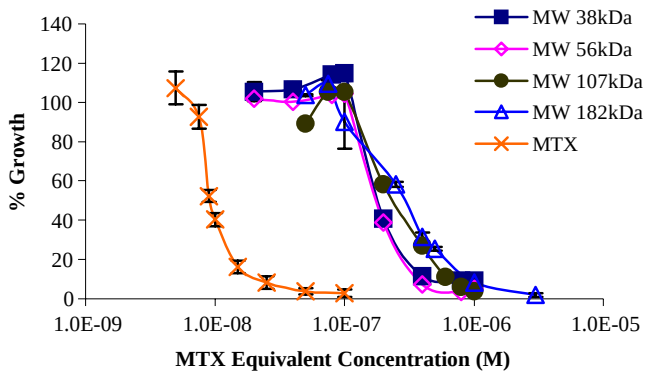

Fig. 3. Growth inhibition curves of HL-60 cells after 72 h incubation with anionic gelatin–MTX conjugates (anionic G-MTX-H) with a high MTX load ranging from molar ratios of 7.4 to 25. Values are the mean of nine replicates in three experiments for MTX and mean of three replicates in one experiment for the conjugates; *error bars* are ±SD. Error bars not shown for the 56 kDa and 107 kDa conjugates.

Growth Inhibition by Gelatin–MTX Conjugates

The percentage growth of HL-60 cells incubated with anionic G-MTX-L, anionic G-MTX-H, and cationic G-MTX-L, with MTX controls is shown in Figs. 2, 3 and 4. The MTX growth curve is located at lower concentrations than for the conjugates and demonstrates that MTX has a stronger growth inhibition than the conjugates. The growth curves of anionic G-MTX-L (Fig. 2) shift to lower concentration as the molecular weight of conjugate decreases. However, little or no growth curve shift for anionic G-MTX-H is noted in Fig. 3 as the molecular weight of conjugates changes. When the cells are incubated with cationic G-MTX-L, the growth inhibition curves of the two lowest molecular weights are similar but shift to higher concentration for the highest molecular weight in Fig. 4. The highest molecular weight cationic conjugates produced a "negative" growth at 2×10^{5} M equivalent MTX concentration. A repeat of this experiment produced the same results.

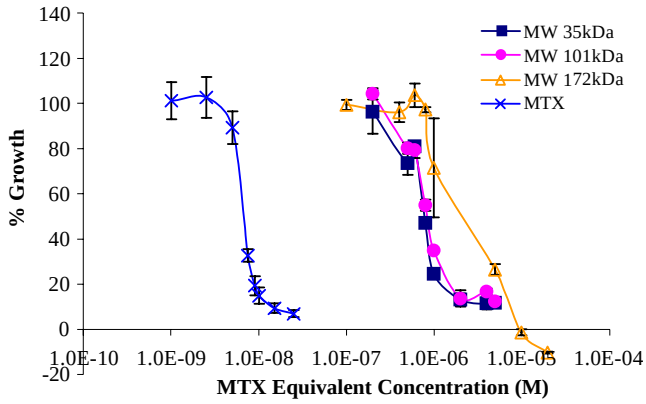

Fig. 4. Growth inhibition curves of HL-60 cells after 72 h incubation with cationic gelatin–MTX conjugates (cationic G-MTX-L) with a low MTX load ranging from molar ratios of 0.6 to 2.0. Values are the mean of nine replicates in three experiments for MTX and mean of three replicates in one experiment for the conjugates; *error bars* are ±SD.

Table II. IC_{50} Values of Gelatin–MTX Conjugates and MTX in HL-60 Cells

	Molecular weight (Da)	IC_{50} values[a] (nM)
Anionic[b] G-MTX-L	172,000	180±70
	101,000	120±2
	52,000	75±0
	35,000	46±1
Anionic[c] G-MTX-H	182,000	290±10
	107,000	250±10
	56,000	190±5
	38,000	200±2
Cationic[b] G-MTX-L	172,000	2,900±560
	101,000	840±9
	35,000	770±3
MTX	454	10±5[d]

[a] Mean±SD of three replicates in one experiment
[b] Low drug molar ratio (see Table I)
[c] High drug molar ratio (see Table I)
[d] Mean±SD of three replicates in nine experiments (n=27)

IC_{50} values of conjugates and MTX were determined and summarized in Table II. The average IC_{50} value of free MTX was 10 nM. Anionic G-MTX-L had IC_{50} values ranging from 46 to180 nM. Anionic G-MTX-H had an IC_{50} values ranging from 190 to 290 nM. Cationic G-MTX-L had IC_{50} values ranging from 770 to 2,900 nM. The results illustrate the growth inhibition trend of MTX > anionic G-MTX-L > anionic G-MTX-H > cationic G-MTX-L. The IC_{50} values for anionic G-MTX-L are directly proportional to molecular weight while anionic G-MTX-H has little or no molecular weight effect on growth inhibition. The IC_{50} value of anionic G-MTX-L is decreased almost 4 fold when conjugate molecular weight decreases from 172 kDa to 35 kDa. When the MTX load of anionic conjugates increases by 7 to 15 fold, the IC_{50} value decreases by 1.6 to 4.3 fold at all molecular weights. Cationic conjugates are less effective than anionic conjugates by 7 to 17 fold. Figure 5 shows an inverse linear relationship between IC_{50} values and molecular weight for anionic G-MTX-L. However, while the IC_{50} values of the two lower molecular weight cationic conjugates are very close, the IC_{50} value of highest molecular weight cationic conjugate increased more than three fold to reflect a substantial loss of effect.

DISCUSSION

The effect of molecular weight, drug load, and charge of gelatin–MTX conjugates on growth inhibition against HL-60 leukemia cells has been examined in this investigation. This is a continuation of our previous work with these conjugates for cellular drug delivery (24). Growth inhibition was measured in the current investigation by a modified MTT assay based on cellular metabolism while growth measurement in the previous study was measured by the trypan blue dye exclusion method based on membrane integrity. The growth measurements for free MTX and comparable conjugates in the two investigations were virtually identical which indicates comparisons between the two investigations are appropriate.

Effect of Molecular Weight

The molecular weight effect of anionic G-MTX-L (35 kDa to 172 kDa) with a relatively constant low molar drug load (shown in Fig. 2) was inversely proportional to growth inhibition. This trend is similar to that reported for PEG-MTX conjugates ranging from 750 to 40 kDa also against HL-60 cells. These results indicate a potential therapeutic advantage for G-MTX compared to the free drug. The high molecular weight biodegradable gelatin carrier could have greater tumor accumulation compared to the free drug because of the EPR effect which could enhance anti-tumor effects as well as reduce toxicity to non cancerous organs and tissues. Indeed, the high molecular weight gelatin carrier may produce greater tumor accumulation than reported with synthetic polymer carriers of lower molecular weight because the biodegradable gelatin may be able to safely exceed the molecular weight limitation of glomorular filtration in the kidney. Another potential advantage of this conjugate is that after tumor accumulation, tumor enzymes such as cathepsin B could breakdown the gelatin carrier to smaller fragments which would be more effective against the tumor cells than higher molecular weight species.

In earlier non-drug macromolecular carrier studies, cell uptake was attributed to an endocytosis process of fluid-phase pinocytosis (17,18). Our previous work demonstrated that gelatin (145 kDa) uptake in HL-60 cells occurs with accumulation in intracellular compartments (24). It is therefore likely that anionic G-MTX-L, and perhaps G-MTX-H, enter cells by fluid-phase pinocytosis into an endosome. The endosome becomes a lysosome in which cleaved MTX and/or MTX containing fragments of the conjugate diffuse out of the lysosome into the cytoplasm to exert an effect.

The inverse correlation between conjugate molecular weight and IC_{50} value is notable for its high linearity (r^2= 0.989) as shown in Fig. 5. Such a degree of linearity in this

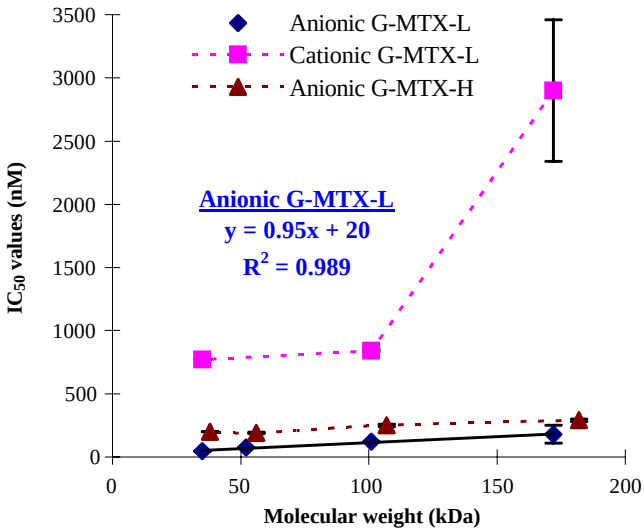

Fig. 5. Molecular weight relationship with IC_{50} values of gelatin–MTX conjugates with low and high MTX loading (G-MTX-L and G-MTX-H) for growth inhibition studies. Values are the mean of three replicates from one experiment; *error bars* are ±SD.

correlation has apparently not been reported in the pharmaceutical literature. This linearity suggests the same rate controlling mechanism in the process of growth inhibition by these low drug load conjugates, but it is unclear at this time which specific mechanism is involved. This process includes at least uptake, endosome transitions, and lysosomal processing. It is also notable that the molecular weight effect correlation is substantially suppressed, if not absent, when the drug load increases to molar ratios >7.4 (see Fig. 3). The explanation for this suppressed effect is unknown at this time. One possibility is that uptake may change to a receptor-mediated endocytosis which is considered to be independent of molecular weight. Another possibility involves a drug load hindrance on lysosomal degradation that is discussed below.

The molecular weight effects of the cationic conjugates were only examined for low drug loads since this effect was suppressed at higher drug loads in the anionic conjugates. The lowest (35 kDa) and intermediate (101 kDa) molecular weight cationic conjugates had virtually the same IC_{50} values which indicates no molecular weight effect by these two conjugates. The highest molecular weight cationic conjugate (172 kDa), however, had a substantially reduced effect which resulted in an almost four-fold increase in its IC_{50} value (see Figs. 4 and 5). This non-linear molecular weight effect suggests that this conjugate (172 kDa) has a different rate controlling mechanism than the two lower molecular weight cationic conjugates. A mechanism involving adsorption is discussed below.

The Effect of Drug Load

The lower drug load conjugates in the current investigation have a stronger effect against cells than the high drug load conjugates (see Figs. 2 and 3). Rosowsky *et al.* reported a similar effect and suggested that a high drug load lowered the net positive charge of the carrier and caused a decreased cell uptake (23). The explanation, however, does not apply for this conjugate because both G-MTX-L and G-MTX-H have the same IEP (see Table I). We previously reported a decreased *in vitro* lysosomal enzymatic degradation of gelatin–MTX conjugates as the MTX molar drug load increased and hypothesized a non-specific hindrance on cathepsin B degradation from the higher drug load (24). A reduced conjugate degradation would be expected to reduce the availability and interaction of MTX with DHFR and reduce the drug effectiveness. The current results support this hypothesis. A similar drug-load hindered degradation of a dextran–methotrexate conjugate for reduced cell growth inhibition also has been suggested (32). A hindered conjugate degradation within the lysosome could also contribute to suppression of the molecular weight effect by the high drug load conjugates.

The Effect of Charge

Results of the current investigation demonstrated that these cationic G-MTX conjugates produced the least effect on HL-60 leukemia cells (see Figs. 4 and 5 and Table II). In other reports, fluorescence images of poly-L lysine chlorin e6 conjugate uptake in EA.hy926 and A431 cells showed that this cationic conjugate was bound to the plasma membrane while the anionic and neutral charged conjugates were internalized in organelles and membranes (26). Subcellular distribution of cationic mitomycin C–dextran conjugates also demonstrated that 90% or more of adsorbed conjugate was associated with the plasma membrane of EAC cells (21). In a similar manner, the positively charged G-MTX conjugate could be bound to the inside plasma membrane of the endosome following adsorptive-mediated endocytosis leading to a hindered lysosomal enzymatic degradation and subsequent hindered release of drug. It is unclear at this time, however, what the mechanism change noted above represents for the highest molecular weight cationic conjugate (see Fig. 5). One possibility is a disproportionate enzyme degradation protection from both charge and high molecular weight contributing to membrane adsorption. Another possibility is a lysosomalytic effect from the high number of positively charged amino groups on the conjugate.

A minimum cell growth of 1.9% to 13% was observed for MTX and most conjugates except the highest molecular weight cationic conjugate. This indicates the MTX and these gelatin–MTX conjugates have a growth inhibition, or cytostatic effect. However, the highest molecular weight cationic conjugate at the two highest concentrations reduced cell growth after 72 h of drug incubation to below that at the time of drug addition which indicates a cytocidal effect. By comparison, preliminary results with the equivalent free MTX concentrations on these cells did not produce this cytocidal effect (33) which suggests a different conjugate mechanism compared to the free drug and a possible conjugate advantage. The flattened growth curves of the lower molecular weight cationic conjugates also suggest that this cytocidal effect would not be observed by these lower molecular weight conjugates at the higher concentrations. The high concentration of the large positively charged conjugate may react strongly with the negatively charged cell membrane leading to membrane disruption. Cell membrane disruption has been observed with other polycations (25,34,35).

CONCLUSIONS

This investigation showed that molecular weight, drug load, and charge of an anticancer macromolecular conjugate can affect growth inhibition of a leukemia cell line. Anionic conjugates with low drug load enhanced growth inhibition in a linear manner as their molecular weight decreased. The linearity suggests the same rate controlling mechanism in cell effect by these conjugates. However, when the drug load of these anionic conjugates was increased 7 to 15 fold, little or no molecular weight effect on growth inhibition was observed. It was also found that low drug load conjugates inhibited growth more than high drug load conjugates which corroborates our earlier hypothesis of non-specific lysosomal enzyme hindrance by the higher drug load (24). The high drug load protection may also contribute to the suppression of the molecular weight effect observed with low drug load anionic conjugates. The growth inhibition of all cationic conjugates was less effective than that of comparable anionic conjugates. The reduced effect may be due to a charge induced adsorption to the inner lysosome membrane and a resulting hindered drug release. In addition, the cationic conjugates did not follow a linear relationship between IC_{50} and molecular weight as was observed in the comparable anionic conjugates. This non-linearity is attributed to a mechanism change, possibly a lysosomalytic effect by the

highest molecular weight cationic conjugate. IC_{50} values of gelatin–MTX conjugates decreased by 63-fold (thereby enhancing growth inhibition) when molecular weight, drug load, and charge were varied under these experimental conditions. In general, the effects of conjugate molecular weight, drug load, and charge observed in this study may occur in cellular uptake and/or lysosomal processing with subsequent release of drug.

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