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Antitumor efficacy of a novel polymer–peptide–drug conjugate in human tumor xenograft models

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We have designed a new dextran–peptide–methotrexate conjugate to achieve tumor-targeted delivery of chemotherapeutics. The dextran carrier was selected to allow passive targeting and enhanced permeation and retention (EPR). The peptide linker has also been optimized to allow drug release in the presence of matrix-metalloproteinase-2 (MMP-2) and matrix-metalloproteinase-9 (MMP-9), 2 important tumor-associated enzymes. The new conjugate was assessed for its *in vivo* antitumor efficacy and systemic side effects. It was compared with free methotrexate (MTX) and a similar conjugate, differing by an MMP-insensitive linker, at equivalent intraperitoneal dosages. The MMP-sensitive conjugate demonstrated tolerable *in vivo* side effects and effective inhibition of *in vivo* tumor growth by 83% in each of the 2 separate tumor models that overexpress MMP (HT-1080 and U-87). The antiproliferative effect of the drug contributed to the inhibition of tumor growth. In contrast, free MTX resulted in no significant tumor reduction in the same models. Neither free MTX nor the conjugate caused any tumor inhibition in the mice bearing RT-112, a slower growing model that does not overexpress MMP. MMP-insensitive conjugates, though able to inhibit tumor growth, caused toxicity in the small intestine and bone marrow.

Key words: methotrexate; dextran; matrix metalloproteinase; gelatinase; tumor; enhanced permeation and retention (EPR); tumor-associated enzymes; polymer-drug conjugate; cancer targeting

Polymer–drug conjugates have shown promise as drug-delivery vehicles for targeting low molecular weight drugs to tumor tissues.[1] By tailoring the polymeric carriers, the pharmacokinetics and tumor target ratio of the attached drug molecules can be favorably altered. The key to the success of this application is that covalent attachment to the polymer enables passive targeting of the drug molecules. The high molecular weight of the polymer increases the size of the conjugate and slows the drug clearance by the kidneys. The drug concentration in the plasma is thereby maintained above the therapeutic level for a prolonged period of time. Additionally, many solid tumors display unique pathophysiology features, including highly permeable vasculature and impaired lymphatic drainage, that are absent in normal tissues. High molecular weight polymer–drug conjugates extravasate into the tumor tissues but not the normal tissues with less permeable vessels. Once inside the tumor tissues, the polymer–drug conjugates do not readily return to the general circulation because of the poor lymphatics. This phenomenon, termed enhanced permeation and retention (EPR), was first discovered by Maeda.[2]

To take advantage of passive targeting and EPR, we have considered the necessary criteria in our design of a new polymer–peptide–drug conjugate. The polymer backbone needs to be biocompatible and biodegradable. It should be hydrophilic, not highly charged, and should have a size above the renal threshold limit to increase its circulation time. Chemically, it should provide functional sites to allow covalent attachment to create the polymer–drug conjugate. To this end, dextran (70,000 Da) was used because of its biocompatibility[3] and biodegradability.[4] Its hydrophilic property and its high molecular weight should also allow for passive targeting.[5] The hydroxyl groups on the dextran backbone provide the necessary sites for covalent modification.

We selected methotrexate (MTX) as the model drug in our new dextran–peptide–drug conjugate. It is a folic acid analog that inhibits dihydrofolate reductase and thus hinders the synthesis of RNA and DNA. This molecule has been used for cancer treatment for many years and is effective against a number of tumors, including lymphoblastic leukemia in children, choriocarcinoma and related trophoblastic tumors in women, osteosarcoma and carcinomas of the breast, head, neck, ovary and bladder. However, MTX has a short circulation half-life and undesirable systemic side effects. Like most chemotherapeutics, it is nonspecific and is toxic to all rapidly dividing cells, with major clinical side effects in the gastrointestinal tract and the bone marrow.[6] These problems can potentially be reduced by altering the pharmacokinetics and biodistribution using the approach of polymer–drug conjugates. Recently, there has been renewed interest in MTX conjugation because of the promising clinical results of albumin–MTX conjugates.[7] Besides albumin, studies of MTX conjugates with synthetic polymers such as poly(ethylene glycol)[8] and poly *N*-(2-hydroxypropyl)methacrylamide[9] have been carried out by different research groups. Along with the above properties, MTX also has 2 carboxyl groups available for covalent linkage, making it an attractive drug to use in our system.

For efficient targeting, MTX should remain attached to the carrier in the circulation and be released when it reaches the tumor site. For albumin–MTX, such release presumably follows the endocytosis of the conjugate. Inside the cells, albumin is digested to amino acid residues and MTX is freed to inhibit dihydrofolate reductase.[10] For synthetic polymers, the release usually depends on 2 types of linkers: (1) an acid labile linker, which is cleaved at low pH in the endosomal–lysosomal pathway[11] or (2) a peptide linker, which is degradable intracellularly by ubiquitous lysosomal enzymes. Kopecek's group has established the usefulness of Gly-Phe-Leu-Gly as a biodegradable linker in the second category.[5] In our conjugate, we intend to incorporate peptide linkers that are cleavable by 2 tumor-associated matrix-metalloproteinases (MMP), MMP-2 and MMP-9, that are secreted in elevated levels by many types of human cancers[12–17] These enzymes help the tumor cells to survive and grow by breaking down the extracellular matrix, releasing growth factors to stimulate cell proliferation and releasing angiogenesis factors to promote blood vessel formation.[18] The optimal oligopeptide substrates for the MMPs have been identified by Turk and coworkers using a combinatorial library;[19] our linker, Pro-Val-Gly-Leu-Ile-Gly (PVGLIG), is based on their findings.[20]

We have successfully synthesized the new dextran–peptide–MTX conjugate, with structure shown in Figure 1. We demonstrated in a previous report that the conjugate MTX–PVGLIG–dextran was capable of releasing peptide–MTX in the presence of MMP-2 and MMP-9. A similar conjugate with a scrambled peptide linker, MTX–GIVGPL–dextran (GIVGPL: glycine-isoleu-

Abbreviations: eq., equivalent; GIVGPL, glycine-isoleucine-valine-glycine-proline-leucine; H&E, hematoxylin and eosin; i.p., intraperitoneal; MMP, matrix metalloproteinase; MTX, methotrexate; PVGLIG, proline-valine-glycine-leucine-isoleucine-glycine.

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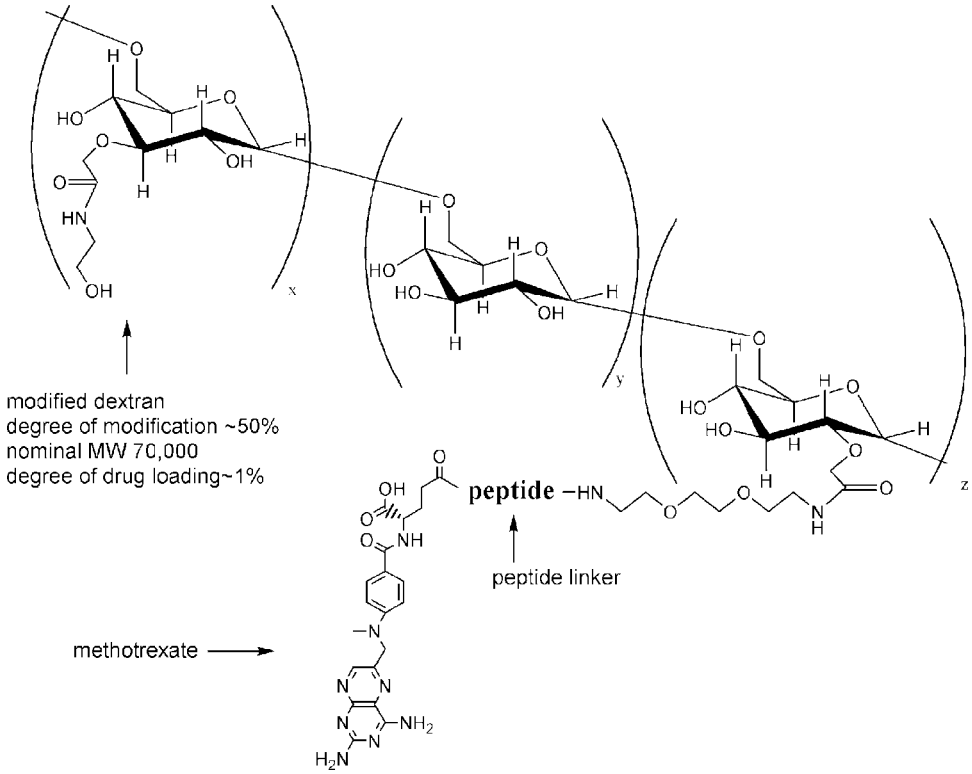

FIGURE 1 – Chemical structure of the new dextran–peptide–MTX conjugate in the current study.

cine-valine-glycine-proline-leucine), remained uncleaved in the same conditions.[20] The goal of the current study was to evaluate the antitumor efficacy and assess the drug-related toxicity of our novel conjugates. These results were compared with the free drug and the conjugate with a scrambled peptide linker, to shed some light on the targeting mechanism and the effect of using MMP-labile linkers.

Material and methods

Materials

Methotrexate (MTX) was purchased from Sigma-Aldrich. The 2 conjugates, MTX–PVGLIG–dextran and MTX–GIVGPL–dextran, were synthesized and purified as described.[20] The 4 MTX–peptide analogs, MTX-G, MTX-GI, MTX-GIV and MTX-PVG, were prepared using the reported methods with slight modifications: (1) different amino acid sequences were synthesized on the solid-phase resins and (2) the HPLC purification of these MTX–peptide analogs was performed on a smaller 20 ml column. Human tumor cell lines HT-1080 and U-87 were grown from seed vials purchased from American Type Cell Culture. RT-112 was a gift from Dr. Marsha Moses at the Children's Hospital (Boston, MA). The cell lines were maintained as described[20] and tested negative for murine pathogens and mycoplasma prior to being used in animal study.

Animal model

Six-week old female SCID mice were obtained from Charles River Laboratory. Animal studies were conducted in accordance with an approved protocol by the Department of Comparative Medicine at the Massachusetts Institute of Technology. One to two million tumor cells were injected subcutaneously at the mid-dorsal level. Treatment was initiated after the tumor was allowed to grow to about 100 mm^3on the back of the mouse. This experimental protocol is intended to mimic the clinical situation when treatment begins after a tumor has already been established in a patient. Free MTX, MTX–PVGLIG–dextran and MTX–GIVGPL–dextran were injected intraperitoneally (i.p.) once a week. The dosage was normalized according to the body weight of the mouse. In the control group, each mouse was injected with 0.5 ml of phosphate buffered saline. Up to 3 injections were performed for HT-1080 and RT-112 and 2 injections for the fastest growing U-87. For all the 3 models, the groups receiving MTX–GIVGPL–dextran were sacrificed after the first injection because of severe weight loss. Weight and tumor size were monitored 3 times a week. Tumor size was estimated as an ellipsoid volume according to the formula[21]: size = $\text{width}^2 \times \text{length} \times \pi/6$.

Histology and immunochemistry

Tissues were fixed in 10% buffered formalin overnight, and were processed for paraffin embedding and sectioning using standard histological procedures. For bone marrow sections, femurs were decalcified after fixation. Tumor sections were stained with hematoxylin and eosin (H&E) for general morphologic evaluation, with MMP-2 and MMP-9 antibodies for the expressions of the 2 enzymes, and with Ki67 for proliferating cells.

Preparation of tumor extract and measurement of MMP concentrations

The procedure of preparing tumor extract was adopted from the Moses Laboratory at the Children's Hospital (Boston, MA).[22] Samples of dialyzed tumor extract were analyzed for their MMP-2 and MMP-9 concentrations using ELISA kits (Calbiochem) according to the manufacturer's instructions.

Results

Design of animal study

We used 3 tumor models to assess the efficacy of the new polymer–peptide–drug conjugates and compared the results with the free drug: HT-1080 human fibrosarcoma, U-87 human glioblastoma and RT-112 human bladder carcinoma. HT-1080 and U-87

are known to overexpress MMP-2 and MMP-9.[23–26] RT-112 was selected to contrast the other 2 cell lines by its absence of MMP overexpression to assess whether the treatment efficacy of the new conjugate would depend on the expression of MMP in the tumor model. The enzyme expression of the tumor lines were assessed in cell-conditioned media using gelatin zymography (data not shown). Both proenzymes and active enzymes of MMP-2 and MMP-9 were observed for HT-1080 and U-87. In comparison, MMPs were present at much lower concentrations and only the proenzymes were found. The enzyme concentrations from *in vivo* tumor extracts were measured by ELISA (Table I). This method did not distinguish between the proenzyme from the active enzyme but gave a more quantitative estimate of the amount of enzyme. The concentration of MMP-2 was 10-fold higher and the concentration of MMP-9 was 50-fold higher in HT-1080 and U-87 tumors than in the RT-112 tumor. Immunohistochemical staining of tumor sections harvested from the mice also indicated that HT-1080 and U-87 were MMP-2 positive and RT-112 was MMP-2 negative (Fig. 2). MMP-9 staining was positive for all cell lines but the staining was significantly weaker in RT-112 (Fig. 3). The peritumor tissue for the different tumor model, when it was present, consisted of skeletal muscle, adipose tissue and fibrous connective tissue, and was negative for MMP-2 and MMP-9 staining (data not shown).

Pilot studies were performed in HT-1080 bearing mice to assess the maximum safe dose of the free drug and the conjugate (MTX–PVGLIG–dextran), which was 50 mg/kg/week; the results are summarized in Table II. Three weekly dosages were injected by the end of the pilot study when the tumor burden reached the maximally bearable size in the control group. The mice in the conjugate group had slightly more weight loss compared to the saline-treated control group but generally remained healthy and active. The conjugate did not cause any toxic death at this dosage, whereas free MTX caused an average of about 20% death rate for 2 pilot studies. There was more variability in the death rate in the free-MTX treated group, which could not be attributed to drug-related toxicity with full certainty because symptoms such as severe hypoactivity and sharp weight loss were absent. In addition to the weekly injection study, a twice-weekly dosing study was performed with 50 mg/kg free MTX or MTX–PVGLIG–dextran. Doubling the injection frequency resulted in severe and acute toxicity in both treatment groups, so the subsequent studies were carried out with weekly injections of 50 mg MTX eq. (equivalent)/mouse body weight, and a maximum of 3 injections were administered.

TABLE I – ELISA MEASUREMENT OF MMP-2 AND MMP-9 IN EXTRACTS FROM TUMORS HARVESTED FROM THE THREE MICE TUMOR MODELS

	HT-1080 Concentration (ng/ml)	U-87 Concentration (ng/ml)	RT-112 Concentration (ng/ml)
MMP-2	17.0 ± 6.3	16.1 ± 12.4	1.5 ± 1.1
MMP-9	5.2 ± 1.7	5.0 ± 1.0	0.1 ± 0.01

Measurement of antitumor efficacy

The antitumor efficacy in the full-scale study was measured by monitoring the tumor volume after treatment and by histological examination of the tumor tissues. Figure 4*a* shows the results for HT-1080 bearing mice. Three weekly i.p. injections of free MTX at 50 mg/kg caused only a slight reduction in tumor size relative to tumor size in the control group. The difference in the tumor size was not significant ($p = 0.194$, two-tailed t-test) when compared to the control group treated with phosphate-buffered saline. A week after the third injection, the study was terminated because the tumor size in the control group exceeded 10% of the mouse body weight and the mice needed to be euthanized as per the guidelines of the animal care committee at our institute. At this point, the tumor size of the group treated with MTX–PVGLIG–dextran at an equivalent dosage (3 × 50 mg of MTX eq./kg of

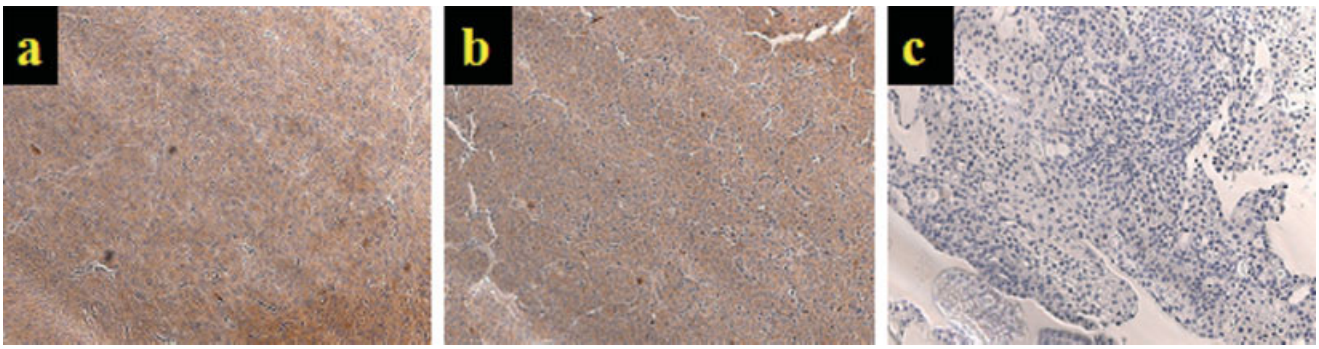

FIGURE 2 – Tumor sections harvested from tumor bearing mice stained against MMP-2 antibody at ×20 magnification. (*a*) HT-1080, (*b*) U-87, (*c*) RT-112. Brown coloration indicates the presence of MMP-2.

FIGURE 3 – Tumor sections harvested from tumor bearing mice stained against MMP-9 antibody at ×20 magnification. (*a*) HT-1080, (*b*) U-87, (*c*) RT-112. Brown coloration indicates the presence of MMP-9.

TABLE II – TOLERANCE OF HT-1080 TUMOR BEARING MICE ON DIFFERENT DOSAGE REGIMES OF FREE METHOTREXATE AND DEXTRAN–PEPTIDE–METHOTREXATE IN PILOT STUDIES[1]

Dosage form	Weekly i.p. dosage (mg methotrexate eq./kg of mouse body weight)[2]	%weight drop at the end of the study	Number of deaths due to drug-related toxicity
Free methotrexate	12.5	8%	0/3
	25	20%	0/3
	50	16%, 15%	2/5[3], 0/5
	100	24%	0/4
MTX–PVGLIG–dextran conjugate	12.5	1%	0/3
	25	12%	0/3
	50	13%, 10%	0/3, 0/4
	100[4]	19%	3/4
Saline control	0	3%, 11%, 8%, 9%	0/3, 0/3, 0/3, 0/3

[1]Multple numbers are reported if more than one pilot experiment was performed using the same conditions.–[2]Three weekly dosages were administered unless further noted.–[3]Deaths due to unknown causes; drug-related toxicity was suspected.–[4]Only 2 weekly injections were administered because of severe toxic response and serious weight loss.

body weight) averaged only (16.8 ± 3.6)% of that of the control group. The inhibition on tumor growth was statistically significant ($p = 1.86 \times 10^{-4}$, one-tailed *t*-test). The study with MTX–GIVGPL–dextran was terminated prematurely, after the first injection on day 6, because of severe toxicity. Nevertheless, this conjugate was effective in suppressing the tumor growth with tumor size averaging (37.5 ± 9.0)% of that of the control group on day 6 ($p = 0.00056$, two-tailed *t*-test).

The results were similar in U-87 bearing mice (Fig. 4*b*), whose tumor cells have a higher growth rate than that of the HT-1080. The animal study was terminated a week after the second i.p. injection so as to avoid excessive tumor burden in the control group. Treatment using free MTX at 50 mg/kg did not have any effect on the tumor size. ($p = 0.48$ compared to the tumor size of the control group using a two-tailed *t*-test). In contrast, the tumor growth was significantly inhibited in the MTX–PVGLIG–dextran group, with the tumor size averaging (17.2 ±3.7)% of the size of the control group ($p = 1.52 \times 10^{-4}$, two-tailed *t*-test). Similar to the case in HT-1080, the study with MTX–GIVGPL–dextran was terminated prematurely on day 6, because of severe toxicity. The conjugate appeared effective in suppressing the tumor growth with

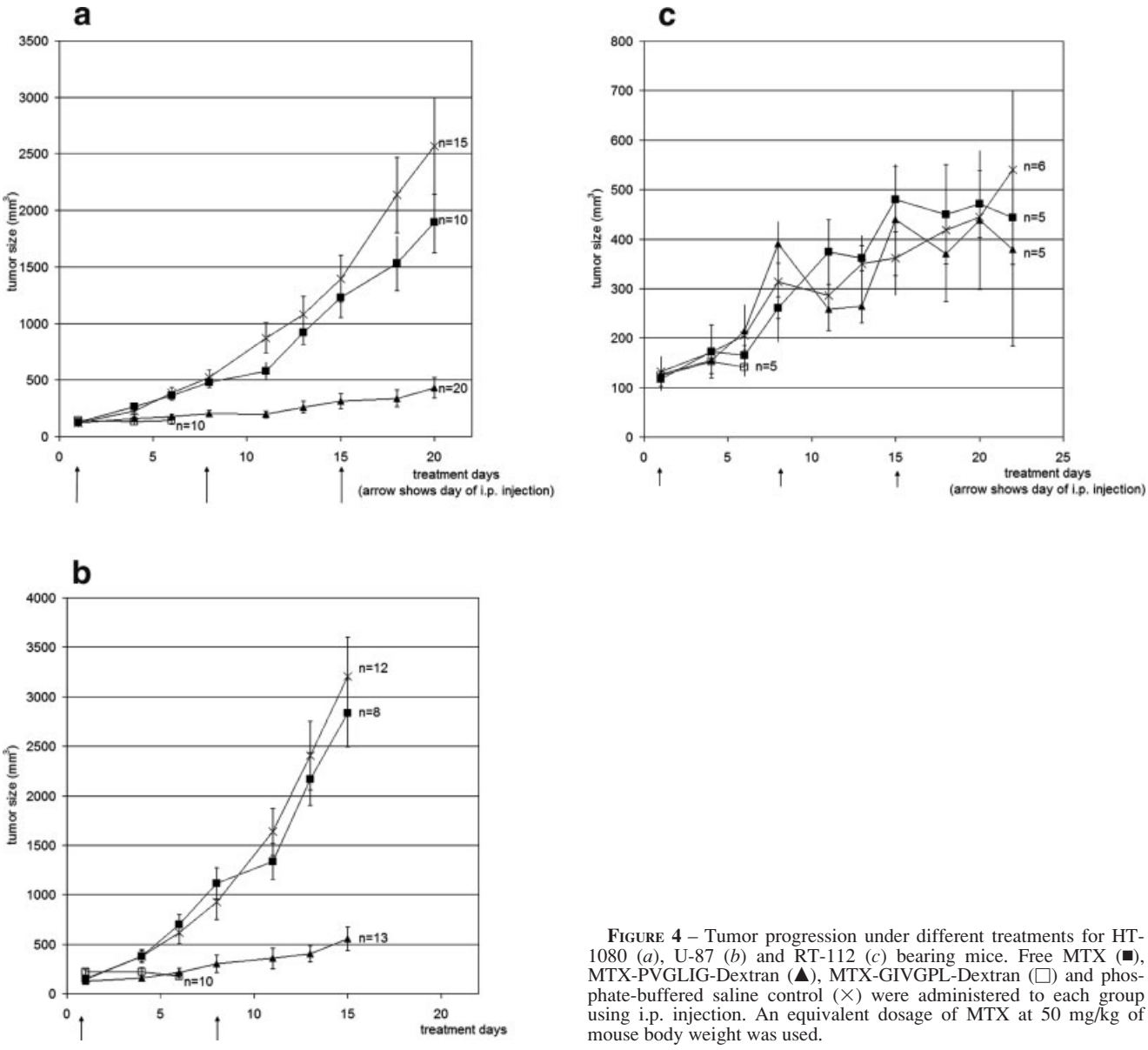

FIGURE 4 – Tumor progression under different treatments for HT-1080 (*a*), U-87 (*b*) and RT-112 (*c*) bearing mice. Free MTX (■), MTX-PVGLIG-Dextran (▲), MTX-GIVGPL-Dextran (□) and phosphate-buffered saline control (×) were administered to each group using i.p. injection. An equivalent dosage of MTX at 50 mg/kg of mouse body weight was used.

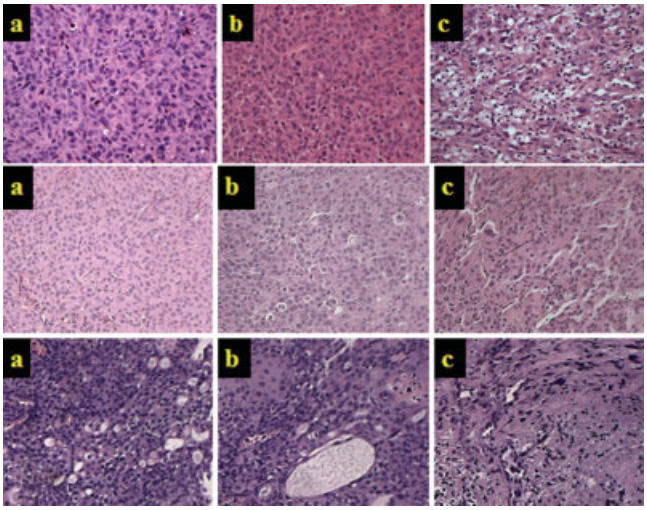

FIGURE 5 – Histological examination of H&E stained tumor sections from HT-1080 (upper panel), U-87 (middle panel) and RT-112 (lower panel) bearing mice at ×20 magnification. Tumors were harvested a week after the last injection from mice treated with (*a*) saline (control), (*b*) free MTX and (*c*) conjugate MTX-PVGLIG-dextran.

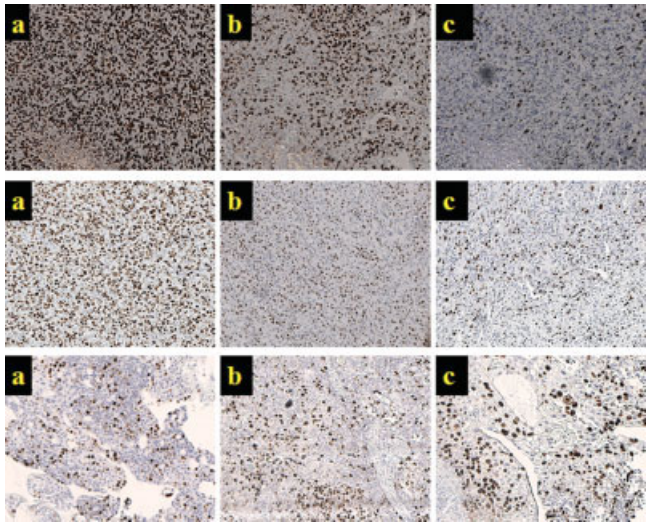

FIGURE 6 – Histological examination of Ki67 stained tumor sections from HT-1080 (upper panel), U-87 (middle panel) and RT-112 (lower panel) bearing mice, at ×20 magnification. Tumors were harvested a week after the last injection from mice treated with (*a*) saline (control), (*b*) free MTX and (*c*) conjugate MTX-PVGLIG-dextran. Dark brown spots indicate the presence of proliferative cells.

tumor size averaging (28.9 ± 5.6)% of that of the control group on day 6 ($p = 0.0026$, two-tailed *t*-test).

In RT-112 bearing mice, neither MTX nor the conjugate MTX–PVGLIG–dextran had any significant effect in suppressing tumor growth, as shown in Figure 4*c*. The two-tailed *t*-tests between the treatment groups and the control groups gave $p = 0.48$ and 0.30, respectively. RT-112 tumor grew much slower than HT-1080 and U-87. At 1 week after the 3rd injection, the tumor size of the control group averaged 541 ± 161 cm^3 in RT-112 bearing mice whereas the tumor size averaged 2,569 ± 427 cm^3 in the control group of HT-1080 bearing mice. Treatment with MTX–GIVGPL–dextran did not result in a significant antitumor effect ($p = 0.079$, two-tailed *t*-test compared with the control) but severe toxicity was again observed after the 1st injection. The study with this MMP-insensitive conjugate was aborted on day 6 for the same reasons as for the aforementioned studies.

The HT-1080 fibrosarcoma in the control and free MTX groups consisted of hypercellular sheets of a monomorphic population of oval to spindle shaped cells with coarse chromatin, prominent nucleoli and a moderate amount of eosinophilic cytoplasm. There were numerous mitotic figures (Figs. 5*a* and 5*b* in the upper panel). In contrast, the tumor in the group treated with MTX–PVGLIG–dextran was much less cellular, had fewer mitotic figures and contained an infiltrate of foamy histiocytes, most likely as a response to the treatment (Fig. 5*c* in the upper panel).

The U-87 glioblastoma in the control and free MTX groups consisted of hypercellular sheets of large pleomorphic tumor cells with marked nuclear atypia, prominent nucleoli and abundant eosinophilic cytoplasm. There were numerous mitotic figures. In contrast, the tumors in the group treated with MTX–PVGLIG–dextran were much less cellular, had fewer mitotic figures and contained an infiltrate of foamy histiocytes (Fig. 5 middle panel). The observation was similar to that for the HT-1080 treatment group.

The RT-112 bladder tumor in all the treatment groups consisted of an epithelial tumor with predominantly transitional features, but with focal glandular formation. There was no significant difference between the control group and the various treatment groups from the standpoint of the histological evaluation (Fig. 5 lower panel).

The antitumor effect from the conjugate MTX–PVGLIG–dextran for HT-1080 and U-87 bearing mice was also evident by the measurement of the proliferative index using Ki67 stain, a marker for dividing cells. The tumor sections in the saline control and free-MTX treated group had a much higher proliferative index than the conjugate treated group (Fig. 6 upper and middle panels). For RT-112 bearing mice, Ki67 staining showed that the proliferative index was similar among the control, the free-MTX treated and the conjugate treated groups (Fig. 6 lower panel). Compared to HT-1080 and U-87, the control tumor of RT-112 had a lower proliferative index.

Study of systemic side effects

The body weight of the mice was monitored as an indicator of systemic toxicity. To ensure that the modified dextran carrier of the conjugates did not induce toxicity, mice were injected with carboxymethyl dextran that was charge neutralized with ethanolamine during the pilot study (data not shown). A week after 3 injections, the body weight increased slightly by 7.8% ± 4.8%. The lack of any toxic response supported that the modified dextran was biocompatible. One week after multiple injections with the MMP-sensitive conjugate MTX–PVGLIG–dextran, we observed a small decrease in body weight in the HT-1080 (−11% ± 1.8%) and U-87 (−1.3% ± 1.6%) bearing mice. In RT-112 bearing mice, the body weight increased at the end of the study (8.6% ± 2.5%). These weight changes were not significantly different from the groups receiving phosphate-buffered saline as a control or with free-MTX injections. The most drastic weight loss was observed in the groups treated with MTX–GIVGPL–dextran, the conjugates with the scrambled peptide linkers insensitive to MMP. In all the 3 tumor models, the mice receiving only single injection of MTX–GIVGPL–dextran suffered 15–25% weight loss (Table III). The mice were severely hypoactive and their hair was ruffled, significantly worse than the control or the free-MTX group. As a result, they were sacrificed on day 6 posttreatment because of the drug-related toxicity.

The side effects were further evaluated by histological examinations of the major tissues, including small intestine, bone marrow, liver, kidney, spleen and skin. Regardless of the type of tumor

TABLE III – SUMMARY OF SIDE EFFECTS FROM TREATMENT WITH FREE METHOTREXATE, MTX-PVGLIG-DEXTRAN AND MTX-GIVGPL-DEXTRAN[1]

		%Body weight change[2]	Drug-related toxic response in small intestine	Drug-related toxic response in bone marrow[3]
Phosphate-buffered saline (control)	H	−4.5% (± 2.6%)	None	None
	U	−1.5% (± 3.0%)	None	
	R	+10% (± 3.7%)	None	None
Free methotrexate	H	−16% (± 2.3%)	None-to-minimal	None
	U	+4.0%(± 2.8%)	None-to-minimal	
	R	+10% (± 3.7%)	None	None
MTX–PVGLIG–dextran conjugate	H	−11% (± 1.8%)	None-to-minimal	None
	U	−1.3% (± 1.6%)	None-to-minimal	
	R	+8.6%(± 2.5%)	None	None
MTX–GIVGPL–dextran conjugate	H	−18% (± 3.0%)	Moderate	Moderate-to-severe
	U	−23% (± 1.9%)	Moderate	
	R	−21%(± 1.6%)	Moderate	Moderate-to-severe

[1]H = HT-1080, U = U-87, R = RT-112.–[2]The body weight at the last day of the study (a week after the last injection) was compared with the initial body weight prior to treatment.–[3]Bone marrow tissues were not sampled from U-87 bearing mice.

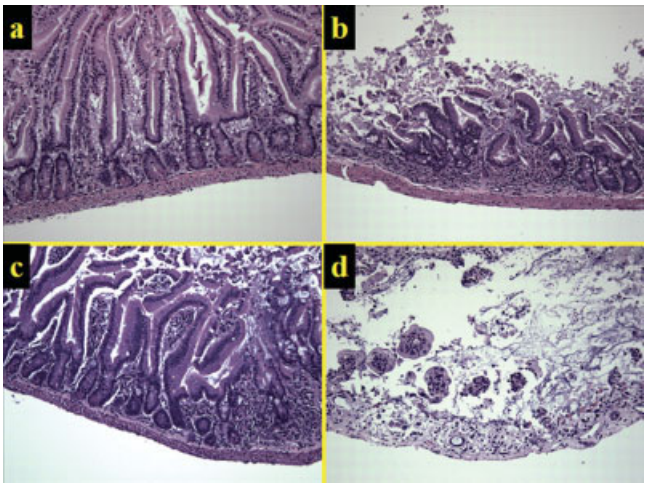

FIGURE 7 – Histological examination of the small intestine sections from HT-1080 bearing mice, at ×10 magnification. Tissues were harvested from mice treated with (*a*) 3 injections of saline (control), (*b*) 3 injections of free MTX, (*c*) 3 injections of conjugate MTX-PVGLIG-dextran and (*d*) 1 injection of conjugate MTX-GIVGPL-dextran.

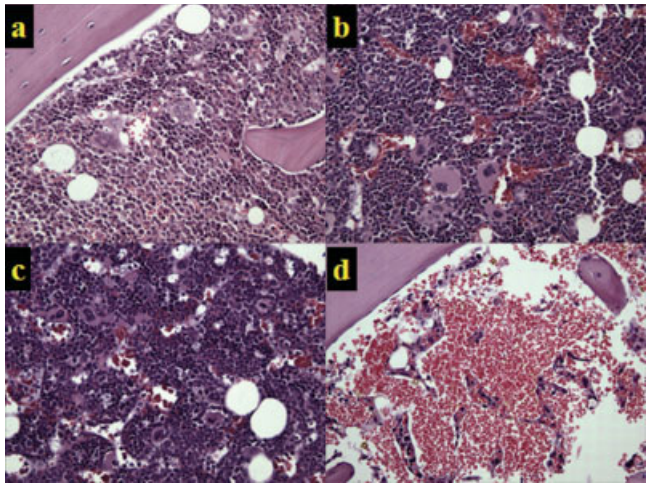

FIGURE 8 – Histological examination of the bone marrow sections from HT-1080 bearing mice at ×20 magnification. Tissues were harvested from mice treated with (*a*) 3 injections of saline (control), (*b*) 3 injections of free MTX (*c*) 3 injections of conjugate MTX-PVGLIG-dextran, and (*d*) 1 injection of conjugate MTX-GIVGPL-dextran.

model, there was moderate-to-severe toxicity in the small intestines and the bone marrows in all the mice treated with the MMP-insensitive conjugate MTX–GIVGPL–dextran (Table III). The drastic weight loss and these tissue damages showed that MTX–GIVGPL–dextran caused toxicity, independent of the MMP expression level of the tumor model.

The histologic changes in the small intestine of MTX–GIVGPL–dextran treated mice consisted of epithelial necrosis with focal regenerative changes, and occasional degeneration of the muscularis without evidence of frank perforation. There were only a limited number of cases with minimal small intestinal toxicity in the mice treated with the MTX–PVGLIG–dextran or with free MTX, consisting of regenerative changes in the epithelium and increased chronic inflammation in the lamina propria. In most cases, the small intestines appeared healthy and similar to the control mice treated with saline (Fig. 7).

In the bone marrow, there was necrosis and hemorrhage with a loss of all hematopoetic lineages in the MTX–GIVGPL–dextran treated mice. These signs of tissue damage were absent in the mice treated with saline, free MTX or MTX–PVGLIG–dextran (Fig. 8).

Liver, kidney, spleen and skin appeared healthy in all treatment groups (data not shown). There was occasional nonspecific, mild extramedullary hematopoiesis in the liver and spleen.

Discussion

The novel conjugate, MTX–PVGLIG–dextran, was designed to achieve targeted delivery of chemotherapeutics to the tumor tissue. The pharmacokinetics and biodistribution of the drug (MTX in this case) can be changed through covalent attachment to a polymeric carrier. In our conjugate, dextran has the properties suitable to passively localize the therapeutic to the tumor tissue. The increase in drug concentration at the tumor tissue for a prolonged period of time should increase its antitumor efficacy. Preferential localization of the therapeutics to the target site should also result in a reduction in systemic side effects. The primary goal was to establish antitumor efficacy of the new polymer–peptide–drug conjugate in an *in vivo* model system.

For the conjugate to be effective, MTX needs to be released from the polymer at the appropriate anatomic location. In our conjugate, it is linked to the dextran carrier *via* peptide linkers cleavable by MMP-2 and MMP-9. A second goal is to determine whether the main mechanism of drug release *in vivo* is MMP dependent. Hence, we evaluated whether anti tumor efficacy and drug related toxicity were dependent on the MMP expression levels in the tumor models, or on the MMP sensitivity of the peptide linkers.

On the basis of the pilot experiments, the dose of 50 mg MTX eq./kg of body weight administrated weekly up to 3 times was selected. This dosage equals half the maximum tolerated dose

found by Burger using free MTX in mice.[10] At this dosage, we did not detect any significant antitumor effect for free MTX using the animal models. On the other hand, the conjugate MTX-PVGLIG-dextran demonstrated enhanced efficacy without significant side effects. In the tumor models overexpressing MMPs, the conjugate resulted in significant inhibition of tumor growth (by 86%). This antitumor effect is comparable to albumin–MTX at its optimal dosage in the mouse models.[10] Since the potency of the released MTX–peptide analog was 2 orders of magnitude lower than that of the free MTX,[20] the promising result suggested that the conjugate had a significantly higher tumor targeting ratio.

The superior antitumor efficacy could possibly be due to the prolonged circulation of MTX due to passive targeting. In its free form, MTX is cleared rapidly from the body by the kidney.[6] The dextran carrier we selected for conjugation with the drug has a nominal molecular weight of 70,000 Da. Reports from Kopecek's group and others have shown that the renal excretion limit is about 40,000 Da[5] and our conjugate is intentionally sized above this threshold. The tumor targeting ratio could also be potentially enhanced by the mediation of MMPs. These tumor-associated enzymes could release more active peptide–MTX from the conjugate at the tumor tissue.

We attempted to shed some light on the targeting mechanism by comparing the *in vivo* observations among tumor models with different levels of MMP expression. Out of the 3 tumor models, only RT-112 did not show any treatment effect by the conjugate MTX-PVGLIG-dextran. It was also the cell line with the lowest level of MMP expression. As an antimetabolite, MTX must be released from the carrier to exert its effect on cell growth by inhibiting DNA synthesis. The release can result from the cleavage of the peptide linker by MMP in the extracellular vicinity of tumor tissue. This route of localized drug release from MTX-PVGLIG-dextran is not as probable in RT-112 as in HT-1080 or U-87, because of their differential MMP expression levels.

However, RT-112 also differed from HT-1080 and U-87 in *in vivo* growth kinetics, so the lack of treatment effect may not be solely due to the low expression of MMP-2 and MMP-9 in RT-112. The treatment effectiveness of the conjugate was accompanied by a reduction in the proliferative index. This is in agreement with the pharmacological action of MTX, which hinders proliferation by inhibiting DNA synthesis through its binding of dihydrofolate reductase. As the main mechanism of tumor growth suppression is to inhibit tumor cell proliferation,[6] if the tumor model is not highly proliferative, as in the case of RT-112, the therapeutic effect of free MTX or the conjugate will be limited.

Drug uptake by tissues can also happen nonspecifically *via* cellular endocytosis. After conjugates are internalized by cells, they presumably go though the endosomal–lysosomal pathway, where drug release can take place *via* the enzymatic cleavage of lysosomal enzymes that are present inside all cells. Although the peptide sequences of the conjugates are not designed for lysosomal enzymes, the large number of proteases present and their broad specificities can degrade the peptide linkers. This nonspecific drug release is possible for both MTX-PVGLIG-dextran and MTX-GIVGPL-dextran. It potentially decreases the efficiency of targeting of MTX-PVGLIG-dextran and causes the toxic response we observed for the twice per week dosing. It may also explain the tumor-inhibiting ability of the conjugate MTX-GIVGPL-dextran despite its lack of sensitivity towards MMPs.

The new conjugate MTX-PVGLIG-dextran was well tolerated by the mice with the current treatment regime (once per week dosing). The weight drop was minor, and histological examination of the major organs and tissues did not find any significant toxicity. In clinical uses, free MTX is known to cause side effects in the gastrointestinal tract and in the bone marrow.[6] In our study, the mice treated by free MTX at the designated dosage did not show any severe drug-related toxicity. However, some HT-1080 and RT-112 bearing mice died from unknown reasons prior to the completion of the free-MTX treatment. These accounted for ~13% of the total number in the MTX group and we did not observe any similar case in the saline control group.

The severe and acute drug-related toxicity caused by the MMP-insensitive conjugate MTX-GIVGPL-dextran was surprising. This conjugate resulted in more than 20% weight drop, causing us to sacrifice the mice after the first injection itself. Severe necrosis within the small bowel and bone marrow were present. This form differs from the well-tolerated MTX-PVGLIG-dextran only in the sequence of the amino acids in the peptide linker. Differences in the systemic side effects suggest that drug release from MTX-GIVGPL-dextran at normal tissues is more likely than from MTX-PVGLIG-dextran. Furthermore, this undesirable drug release is not due to MMPs.

From the current results, it was evident that MTX-PVGLIG-dextran is a promising vehicle to achieve tumor-targeted delivery of MTX in MMP-overexpressing models. Further studies in assessing the biodistribution of the conjugate and the release profile should be useful in understanding the targeting mechanism of the new conjugate.

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