Targeted Delivery of Antineoplastic Agent to Bone: Biodistribution Studies of Technetium-99m-Labeled Gem-Bisphosphonate Conjugate of Methotrexate

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A methotrexate-bisphosphonate conjugate containing a peptide bond has been found to possess over five times greater antineoplastic activity against osteosarcoma in experimental animal models compared with methotrexate alone. **Methods:** The conjugate was labeled with ^{99m}Tc in the presence of stannous ions to determine biologic distribution, with special reference to osseous tissue. Biodistribution studies were carried out in mice after intravenous administration of the labeled conjugate. Radionuclide imaging of rabbits was also performed. **Results:** The labeled conjugate behaved like a bone-seeking agent. **Conclusion:** The present study indicates that the concept of treating osteosarcoma or metastatic tumors of bone with this class of agents has a firm basis.

Key Words: methotrexate; bisphosphonate; technetium-99m-diphosphonate; bone tumors

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Specific delivery of antineoplastic agents to bone tumor sites has remained a major problem. Aside from instances in which an artery supplying the tumor can be cannulated, there are few site-specific alternatives. Bone-seeking radionuclides, such as ^{89}Sr, have been used to offer some relief from pain due to bone metastases but are not curative (*1*). Bisphosphonates are bone-seeking agents (*2*). Most have two phosphate groups bound to the same carbon atom (*geminal*) and are known as gem-bisphosphonates. Two of them, methylenediphosphonate and hydroxymethylenediphosphonate, labeled with ^{99m}Tc are routinely used in skeletal scintigraphy for delineation of bone metastases (*3*). Uptake of these phosphonates in a bone tumor is usually several times higher than that of corresponding normal bone. Conjugates of cytotoxic agents, such as methotrexate bound to a bisphosphonate, represent a new class of chemotherapeutic drugs in which the bisphosphonate moiety seems to carry the linked drug to osseous tissue (*4*). Methotrexate is extensively used as an antineoplastic agent, and in higher doses it can produce a therapeutic response in some patients with bone metastases (*5*). Several analogs of methotrexate-bisphosphonate (MTX-BP) conjugates have been synthesized by Sturtz et al. (*4,6*), and some have been evaluated for efficacy of tumor inhibition in experimental animal models. One of these has shown five to six times greater anticancer activity compared with methotrexate alone in animal models of transplanted osteosarcoma. The conjugate also appeared to be three to four times more toxic than methotrexate, possibly due to a myelosuppressive effect from increased delivery of methotrexate to skeletal bone. We explored the possibility of labeling this conjugate with ^{99m}Tc to determine its biodistribution with special reference to bone.

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MATERIALS AND METHODS

Synthesis of a gem-bisphosphonate conjugate of methotrexate (MTX-BP) with increased anticancer activity has been reported previously (*4*), as well as an improved synthesis for a higher yield (*6*). The MTX-BP conjugate is linked with a peptide bond, and the molecular weight of the sodium salt is 809, as shown in Figure 1.

The sodium salt of the compound was found to be soluble in isotonic saline at concentrations of 1–5 mg/ml; the pH was observed to be around 10. It was anticipated that the conjugate could be labeled with ^{99m}Tc due to the presence of phosphonate moiety. A method using stannous chloride, similar to those used previously for labeling several bone-seeking agents (*7,8*), was adopted for this purpose. The MTX-BP conjugate was dissolved in isotonic saline at a concentration of 1–2 mg/ml. The pH was then adjusted between 6 and 6.5, using dilute HCl. A stannous chloride solution was prepared in 0.1 *N* HCl (1 mg/ml) and added to the conjugate (the conjugate-to-tin solution ratio was 20:1). The final pH was around 5. The product was passed through a 0.22-μm filter and stored in rubber-capped glass vials in amounts of 1 ml. Some of them were placed in a freezer, after flushing with nitrogen gas, for future use as a kit. Technetium-99m-pertechnetate was added to a vial (or a kit after thawing) in amounts of 0.2–0.5 ml containing 20–100 MBq of activity. Products with lower activities were used in mice and those with higher activities in rabbits.

Chromatographic Studies

Chromatographic studies were carried out using two solvent systems, as recommended for quality control of ^{99m}Tc-labeled radiopharmaceuticals (*9*). Whatman 3M paper strips (8 cm × 2 cm) were used for ascending chromatography using (a) acetone and (b) 0.9% saline to determine the presence of free pertechnetate and colloidal materials, respectively. Strips were cut into 0.5-cm sections and assayed for radioactivity, and the percent binding was determined for the labeled conjugate.

Biologic Studies

Biologic studies were carried out by approaches used in developing ^{99m}Tc-labeled bone-imaging agents (*7,8*). Specifically, biodistribution studies were performed using male white mice weighing between 32 and 39 g (CD-1, Charles River, MA). These studies were conducted using several preparations of the labeled conjugate. The ^{99m}Tc-labeled compound was diluted to 1:4 in isotonic saline for studies in mice. Mice were injected with dosages of 0.1 ml (~600 kBq) into the tail vein and killed at different time intervals between 0.25 and 4 hr. A blood sample was obtained, and various organs were excised, including samples of skin, muscle and a femur. These samples were weighed. The urinary bladder was removed carefully to avoid radioactive contamination from urine.

A 1% reference sample was prepared with a solution that was made by diluting a dosage (0.1 ml) to 100 ml in isotonic saline. This sample was used to compute uptake of radioactivity in various organs. Samples were counted in a well-type scintillation counter.

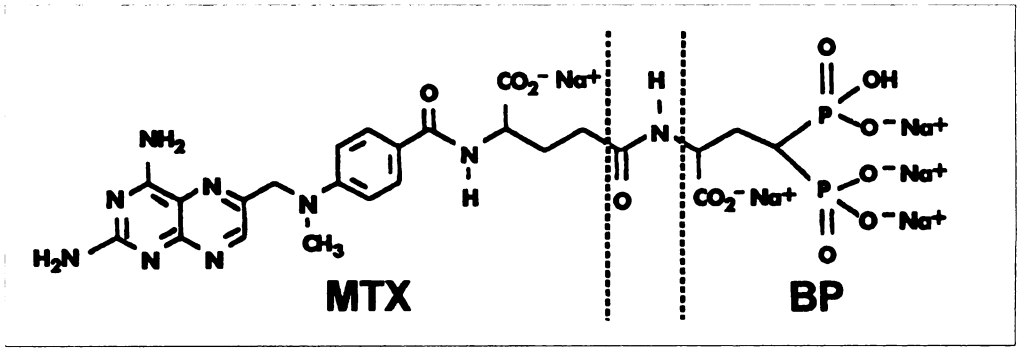

FIGURE 1. Structure of methotrexate-bisphosphonate conjugate. Methotrexate (MTX) and bisphosphonate (BP) moieties are linked with a peptide bond (shown within dotted lines).

Results were expressed as percent of injected dosage per organ. Blood, skin, muscle and bone were assumed to represent 7%, 13%, 40% and 10% of the body weight, respectively. Carcasses were also counted using a scintillation probe and compared with a 5% reference sample under a similar geometric condition. Radioactive excretion, essentially in the urine, was estimated from the difference between 100% and the sum of percent activities in all samples, including the carcass.

Mean values and standard deviations were obtained for each time point with three to five mice. Only animals in whom the radioactive content of the tail was less than 10% of the injected dosages were included in these calculations.

Radionuclide imaging was done in male New Zealand rabbits after intravenous injection of 0.3 ml (20–30 MBq) of the undiluted radiopharmaceutical into the ear vein. A gamma camera, with parallel-hole collimator, was used for this purpose. Posterior views were obtained between 1.5 and 2 hr after injection.

RESULTS

Radiopharmaceutical preparations showed negligible amounts of free pertechnetate (<1%, as observed in chromatograms with acetone). Presence of colloidal materials could not be estimated from the chromatograms with saline because the bulky molecule of MTX-BP did not move far from the origin. It is likely, however, to be less than 5%, as evident from the low liver uptake values in mice.

Biodistribution pattern of the ^{99m}Tc-labeled MTX-BP conjugate appeared similar to that of previously studied ^{99m}Tc–labeled bone-seeking agents (*7,8*), with slightly higher retention of radioactivity in blood and soft tissues, such as skin, muscle and kidneys, and slightly decreased uptake in skeletal bone. After intravenous injection, radioactivity appeared to clear from the blood rapidly, with concomitant uptake in bone and excretion in urine. In mice, by 1 hr, about 55% was excreted in the urine and 20% distributed in the skeleton. Activity in the skin, muscle, liver, kidneys, gastrointestinal tract (with content) and the tail ranged between 2% and 7% in each. By 1 hr, radioactivity in brain, eyes, thyroid, heart, lungs, spleen and testes was found to be very low (0.01–0.11% of the injected dosage). Bone uptake in mice remained essentially the same between 0.5 and 2 hr. Table 1 summarizes the biodistribution results obtained in mice. Figure 2 shows posterior images obtained in a rabbit 1.5 hr after intravenous administration of ^{99m}Tc-labeled conjugate. Skeletal bones were well delineated, and activity in the urinary bladder was evident.

TABLE 1
Biodistribution of Technetium-99m-Labeled MTX-BP Conjugate in Mice

	%ID/organ at different times (mean ± s.d.)				
Organ	15 min (n = 4)	30 min (n = 3)	1 hr (n = 5)	2 hr (n = 3)	4 hr (n = 3)
Blood	5.80 ± 0.761	2.60 ± 0.364	1.72 ± 0.259	1.19 ± 0.173	0.60 ± 0.046
Skin	10.77 ± 0.931	8.78 ± 1.312	7.00 ± 1.896	5.25 ± 1.244	3.50 ± 0.490
Muscle	9.94 ± 1.310	5.48 ± 0.780	4.28 ± 0.376	4.58 ± 1.521	2.89 ± 0.368
Skeleton	12.65 ± 0.912	17.77 ± 1.697	19.58 ± 1.545	18.68 ± 0.646	15.30 ± 0.800
Brain	0.04 ± 0.005	0.02 ± 0.004	0.02 ± 0.003	0.01 ± 0.001	0.01 ± 0.005
Eyes	0.03 ± 0.003	0.02 ± 0.004	0.01 ± 0.002	0.01 ± 0.001	0.01 ± 0.001
Thyroid	0.09 ± 0.033	0.03 ± 0.008	0.04 ± 0.013	0.04 ± 0.005	0.03 ± 0.008
Heart	0.23 ± 0.017	0.11 ± 0.018	0.09 ± 0.008	0.07 ± 0.010	0.06 ± 0.015
Lungs	0.26 ± 0.016	0.18 ± 0.050	0.11 ± 0.010	0.07 ± 0.009	0.06 ± 0.015
Liver	5.09 ± 1.475	4.78 ± 0.953	3.60 ± 1.228	3.04 ± 0.690	3.36 ± 1.725
Spleen	0.19 ± 0.059	0.18 ± 0.061	0.08 ± 0.025	0.11 ± 0.026	0.06 ± 0.024
Kidneys	6.29 ± 0.678	5.15 ± 0.886	4.57 ± 0.713	6.10 ± 0.919	5.76 ± 0.945
Testes	0.10 ± 0.028	0.08 ± 0.007	0.06 ± 0.014	0.07 ± 0.016	0.04 ± 0.013
GI+cont	3.55 ± 0.416	2.50 ± 0.260	2.26 ± 0.731	1.82 ± 0.078	1.67 ± 0.460
Tail	8.81 ± 1.114	7.89 ± 1.340	6.57 ± 2.689	4.97 ± 2.407	4.15 ± 0.927
Excretion	44.59 ± 1.776	51.29 ± 3.880	54.75 ± 1.648	55.29 ± 2.949	61.15 ± 3.678

GI+cont = gastrointestinal tract and contents.

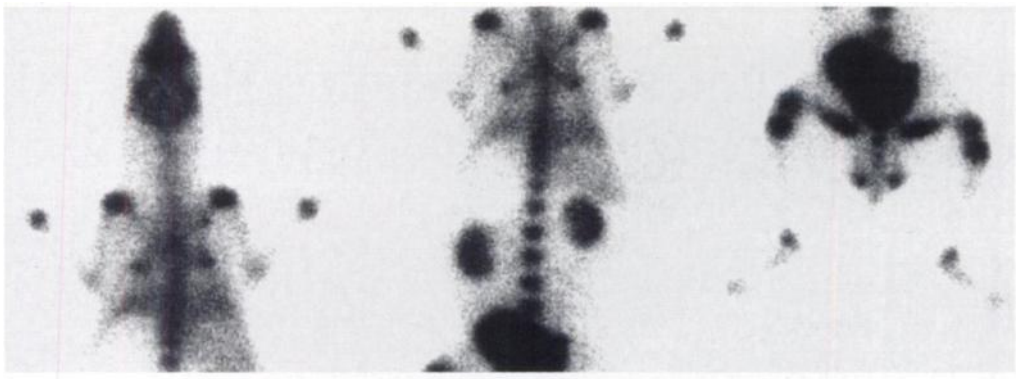

FIGURE 2. Posterior images obtained in a rabbit 1.5 hr after intravenous injection of ^{99m}Tc-labeled MTX-BP conjugate. Bone and joints are well delineated in all three segments of the view.

DISCUSSION

Rapid blood clearance, high urinary excretion and low liver uptake indicated ^{99m}Tc binding to MTX-BP conjugate with a purity of about 95%. Further, these studies in mice and rabbits showed that the ^{99m}Tc-labeled conjugate behaved similarly to bone-seeking agents, such as ^{99m}Tc-labeled diphosphonates (*7,8*).

The MTX-BP conjugate has a peptide bond that is likely to resist rapid cleavage of the compound in the bloodstream. It was anticipated that the bisphosphonate moiety would carry methotrexate along with it to bone. This was based on the observation that equivalent tumor inhibition in experimental models was observed with doses of the MTX-BP conjugate several times lower than doses with methotrexate alone (*6*). Bone tumors would most likely accumulate several-fold higher amounts of conjugated methotrexate than would have been taken up if it had not been conjugated with bisphosphonate.

We do not recommend ^{99m}Tc-labeled MTX-BP conjugate for routine bone imaging. It might, however, be a valuable adjunct when a patient is treated with such a conjugate for bone metastases to know the spatial and temporal variation of distribution of the drug. It could be used along with administration of a therapeutic dose of MTX-BP conjugate. In the present biodistribution studies in mice, the amount of ^{99m}Tc-labeled conjugate was approximately 1 mg/kg. The therapy dose is likely to be less than 5 mg/kg (*6*).

Wingen et al. (*10*) initiated studies with bisphosphonate-linked cytotoxic agents in the hope of treating osteosarcoma. Sturtz et al. (*4,6*) were able to obtain a conjugate that appeared to fulfill this idea.

CONCLUSION

The present study indicates the potential of targeted delivery of antineoplastic agents to bone for the treatment of osteosarcoma or bone metastases. Analogous conjugates with reduced toxicity and dose fractionations are likely to offer greater therapeutic efficacy. Further, the MTX-BP conjugate might be labeled with other radionuclides, such as ^{186}Re, to seek additional therapeutic advantages. Rhenium-186-hydroxyethylidine diphosphonate alone has been found to be effective in palliation therapy for bone metastases (*11*). In addition, it might be possible to obtain a bisphosphonate coupled with an iodo-derivative of methotrexate (*12*). Thus, incorporation of a beta-emitting radionuclide to a MTX-BP conjugate might offer a synergistic effect in the treatment of bone metastases.

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