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In vitro Drug Release Characteristics of Methotrexate-Human Serum Albumin and 5-Fluorouracil-Acetic Acid-Human Serum Albumin Conjugates

Chong-Kook Kim[§], Myung Gull Lee, Man Ki Park, Heejoo Lee* and Hae Jin Kang

*College of Pharmacy, Seoul National University, Seoul 151-742, and *College of Pharmacy, Ducksung Women's University, Seoul 132-714 Korea*

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Abstract The release rates of methotrexate (MTX) from MTX-human serum albumin (HSA) conjugate, and 5-fluorouracil (5-FU) from 5-FU acetic acid (AA)-HSA conjugate were determined after incubation of the conjugates in various conditions. The concentrations of 5-FU released from the conjugate increased monoexponentially, however those of MTX increased biexponentially in all studies. It indicated that there are two distinct types of MTX-HSA linkage, weakly and tightly bound linkages. The release rates of 5-FU were lower than those of MTX in all studies indicating that the bond of 5-FU-AA-HSA conjugate is very stable, which is supported by the higher value of activation energy (39.9 vs 10.7 Kcal/mole) using Arrhenius equation. The release rates of MTX and 5-FU from the conjugates increased with incubation temperatures. Proteolytic enzyme and liver homogenates accelerated significantly the release rates of MTX and 5-FU. Approximately 1.30 and 22.0% of MTX were released after 12 hours of incubation in the absence and presence of protease, respectively. The corresponding values for 5-FU were 1.0 and 17.0%. Approximately 10.3 and 11.9% of 5-FU and MTX, respectively were released after 12 hours of incubation with rat liver homogenates which were diluted 6 times with phosphate buffer of pH 6.0. The MTX-HSA and 5-FU-AA-HSA conjugates were very stable in rat plasma.

Keywords Drug release, methotrexate, albumin, 5-fluorouracil, acetic acid, drug conjugate.

One of the major limitations of systemic cancer therapy is the incidence of toxic side effects related to drug action. Nephrotoxicity and gastrointestinal tract disturbances, such as mucositis, bloody diarrhea, nausea and vomiting are the main side effects of methotrexate (MTX). Myelosuppression and gastrointestinal mucositis are the most significant toxic effects of 5-fluorouracil (5-FU).[1]

The ideal dosage form in cancer therapy is the one that provides a specific delivery of anticancer drug to the tumor site(s) in sufficient amounts, for a long period of time with no interaction with the normal tissues.[2] For this purpose, anticancer drug-macromolecule (such as human serum albumin, HSA, or polypeptides) conjugates were synthesized and their *in vitro* anticancer activities were reported.[3-6]

The purpose of this paper is to report our preliminary results on *in vitro* release of MTX from MTX-HSA conjugate, and 5-FU from 5-FU-acetic acid (AA)-HSA conjugate at various conditions.

[§]To whom inquires should be directed

MATERIALS AND METHODS

Materials

MTX and 5-FU were the gifts of Choong-Wae Pharm. Co. (Seoul, Korea). HSA in fraction V, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Sephadex G-50 and G-25 were purchased from Sigma Chemical Co. (St. Luois, Mo.). Alphachloroacetic acid and N,N-dimethylformamide were products of Aldrich Chemie (Steinheim, West-Germany). The 1,3-Dimethylhexylcarbodiimide (DDC) and N-hydroxysuccinamide (NHS) were obtained from Junsei Chemical Co. (Tokyo, Japan). Protease (activity of 31,400 units/g at pH 8.0) was kindly supplied by Dong-A Pharm. Co. (Seoul, Korea). All other chemicals

were commercial reagent grade and used without further purification. Synthesis and identification of MTX-HSA and 5-FU-AA-HSA conjugates, and the contents of MTX and 5-FU in the conjugates were also reported.[9,10]

Release of drug from the conjugates at various pHs

A weight of the conjugates equivalent to 2 mg of MTX or 5-FU was dissolved in 50 m*l* of various buffer solutions with different pHs of 3.0, 5.0, 7.0 and 9.0. These mixtures were incubated in a water bath shaker at 37 °C and 68 oscillations per min (opm). Two 100 μl samples were collected with appropriate time intervals and stored in the freezer prior to HPLC analysis for MTX[7] and 5-FU.[8]

Release of drug from the conjugates at various temperatures

The same amount of the conjugates was dissolved in 50 m*l* of pH 7.4 buffer solution and the mixture was incubated in a water bath shaker at various temperatures (25, 37 and 45 °C) and 68 opm.

Release of drug from he conjugates in the presence of proteolytic enzyme

The same amount of the conjugates was dissolved in 50 m*l* of pH 7.4 buffer solution having 200 mg of protease, and the mixture was incubated in a water bath shaker at 37 °C and 68 opm.

Release of drug from the conjugates in liver homogenate

The liver homogenate was prepared by following method described by Graham[11]. One male Sprague-Dowley rat was anesthetized with ether. The liver was excised, rinsed in ice-cold normal saline solution, dried, blotted with paper towel, and weighed. All subsequent procedures were carried out at 0-4 °C. The liver was minced into small pieces with scissors and then homogenized in 5 volumes of phosphate buffer of pH 6.0 in a glass homogenizer. The homogenate was then centrifuged (Han-Il Centrifuge, Seoul, Korea) at 9000 g for 20 min. After discarding the floating fat layer, the supernatant fraction was collected for experiment. The same amount of the conjugates was dissolved in 50 m*l* of the above homogenates. The mixtures were then incubated in a water bath shaker at 37 °C and 68 opm.

Release of drug from the conjugates in plasma

The same amount of the conjugates was dissolved in 50 m*l* of rat plasma and the mixture was incubated in a water bath shaker at 37 °C and 68 opm.

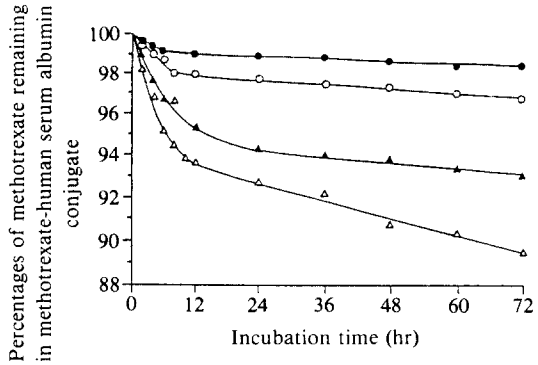

Fig. 1. Percentages of methotrexate (MTX) remaining in MTX-human serum albumin conjugate after incubation of the conjugate in water bath shaker at 37 °C and 68 oscillations per min with pHs of 3.0(●), 5.0( ), 7.0(▲) and 9.0(△) buffer solutions, respectively.

RESULTS AND DISCUSSION

Fig. 1 shows percentages of MTX remaining in MTX-HSA conjugate after incubation with pHs of 3.0, 5.0, 7.0, and 9.0 buffer solutions, respectively, for 72 hours at 37 °C and 68 opm. The concentrations of MTX remaining in MTX-HSA conjugate decayed biexponentially. The MTX which is weakly bound to the conjugate could be released faster in "the earlier period" (up to 6 hours) of incubation and then MTX which is tight covalently bound to the conjugate could be released in "the latter period" (after 6 hours) of incubation. The release rates of MTX increased with increasing pHs. The percentages of MTX released from the conjugate were approximately 0.09, 1.89, 3.40 and 4.90% for pHs of 3.0, 5.0, 7.0 and 9.0 buffer solutions, respectively in 6 hours of incubation. The corresponding values incubated for up to 72 hours were 1.31, 3.20, 6.90 and 10.4%. The release rate constants were 0.297, 0.587, 1.16 and 1.70 day for pHs of 3.0, 5.0, 7.0 and 9.0 buffer solutions, respectively for "the earlier period" of incubation and the corresponding values were 0.010, 0.042, 0.077 and 0.1150 day for "the latter period" of incubation.

It is to be noted that the release rates of 5-FU from 5-FU-AA-HSA conjugate did not increase with increasing pHs. (Fig. 2). The percentages of 5-FU released from the conjugates were 0.20, 2.20, 3.50 and 1.00% for pHs of 3.0, 5.0, 7.0 and 9.0 buffer solutions, respectively in 36 hours of incubation (Fig. 2). The corresponding values of release rate constants were 0.006, 0.179, 0.256 and 0.091

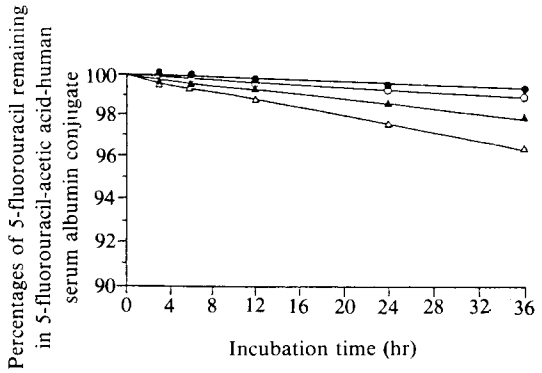

Fig. 2. Percentages of 5-fluorouracil (5-FU) remaining in 5-FU-acetic acid-human serum albumin conjugate after incubation of the conjugate in water bath shaker at 37°C and 68 oscillations per min with pHs of 3.0(●), 5.0(▲), 7.0(△) and 9.0(○) buffer solutions, respectively.

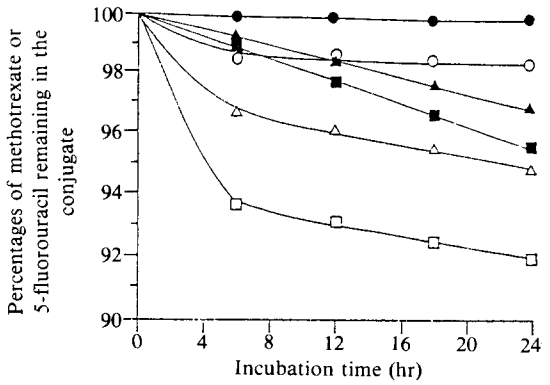

Fig. 3. Percentages of methotrexate (MTX, open) and 5-fluorouracil (5-FU, closed) remaining in MTX-human serum albumin (HSA) conjugate and 5-FU-acetic acid-HSA conjugate, respectively after incubation of the conjugates in water bath shaker at 25°C(○), 37°C(△), or 45°C(□) and 68 oscillations per min with phosphate buffer, pH 7.4.

day^{-1}.

Biphasic release pattern was also obtained when MTX-HSA conjugate was incubated with phosphate buffer of pH 7.4 at different temperatures (Fig. 3). As expected, the release rates increased with increasing temperatures. The percentages of MTX released from the conjugate were approximately 1.30, 3.50 and 6.30% for 25, 37 and 45 °C, respectively in 6 hours of incubation and the corresponding values in 24 hours of incubation were 1.70, 5.20 and 8.90% (Fig. 3). The release rate constants were 0.682, 1.375 and 2.462 day at 25 °C, 37 °C and 45 °C respectively for "the earlier period" of incubation and the corresponding values for "the latter period" of incubation were 0.035, 0.174 and 0.194 day^{-1}.

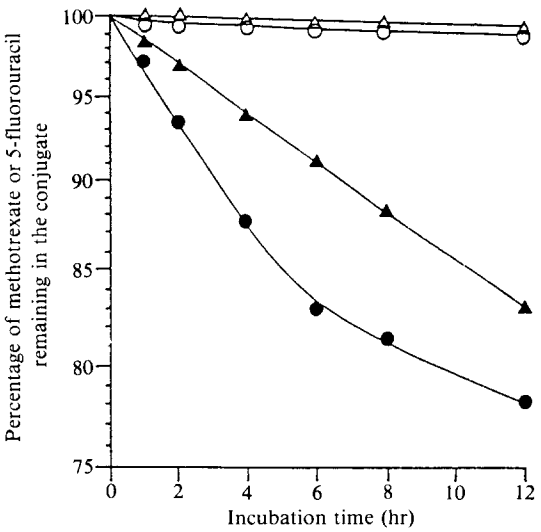

Fig. 4. Percentages of methotrexate (MTX, ○) and 5-fluorouracil (5-FU, △) remaining in MTX-human serum albumin (HSA) conjugate and 5-FU-acetic acid-HSA conjugate, respectively after incubation of the conjugates in water bath shaker at 37°C and 68 oscillations per min with phosphate buffer pH 6.0 in the presence (closed) or absence (open) of proteolytic enzyme.

The release of 5-FU from 5-FU-AA-HSA conjugate was increased with increasing temperature (Fig. 3). The percentages of 5-FU released from the conjugate were approximately 0.10, 3.20 and 4.30% for 25, 37 and 45 °C, respectively for 24 hrs of incubation. The corresponding values of release rate constants were 0.035, 0.174 and 0.194 day^{-1}.

The release of MTX from MTX-HSA conjugate was significantly increased in the presence of proteolytic enzyme (Fig. 4). Approximately 17.0% of MTX was released from the conjugate in 6 hours of incubation in the presence of the enzyme, whereas the value was approximately 0.50% in the absence of the enzyme. The corresponding values were 22.0 and 1.30% in 12 hours of incubation. Similar results were also obtained in 5-FU. Approximately 17.0% of 5-FU was released in 12 hours of incuba-

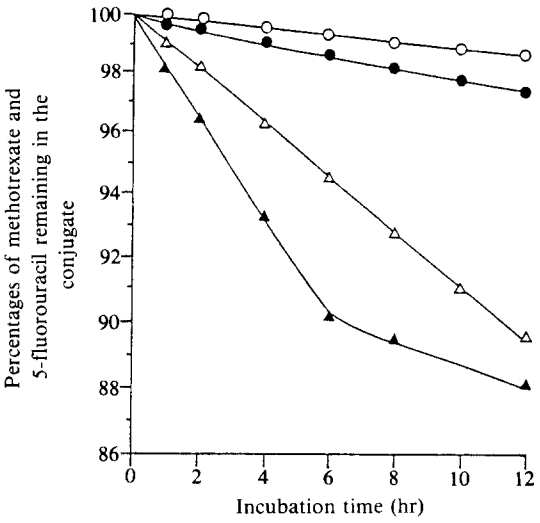

Fig. 5. Percentages of methotrexate (MTX, closed) and 5-fluorouracil (5-FU, open) remaining in MTX-human serum albumin (HSA) conjugate and 5-FU-acetic acid-HSA conjugate, respectively after incubation of the conjugates in water bath shaker at 37°C and 68 oscillations per min with plasma (△) or rat liver homogenate (○).

tion in the presence of the enzyme, however, the value was 1.00% in the absence of the enzyme (Fig. 4).

Liver homogenates accelerated significantly the release rates of MTX and 5-FU from the conjugates (Fig. 5). The percentages of MTX and 5-FU released from the conjugates were approximately 11.1 and 10.3%, respectively in 12 hours of incubation. When one considers the 6 times dilution of liver homogenates in the present study, the release rates of MTX and 5-FU from the conjugates in liver could increase in *in vivo*.

The MTX-HSA and 5-FU-AA-HSA conjugates seemed stable when they were incubated with plasma (Fig. 5). The percentages of MTX and 5-FU released from the conjugates were approximately 2.70 and 1.20%, respectively in 12 hours of incubation with plasma. It might be due to the interaction between the conjugates and albumin or other substances in plasma.

The release rates of 5-FU from 5-FU-AA-HSA conjugate were lower than those of MTX from MTX-HSA conjugate in all studies suggesting that the bond of 5-FU-AA-HSA conjugate is stable. The stable bond of 5-FU-AA-HSA was supported by the higher activation energy (using Arrhenius equation) of 39.9 Kcal/mole when compared to the value of 10.7 Kcal/mole for "the earlier period" of incubation and 24.6 Kcal/mole for "the latter period" of incubation for MTX-HSA conjugate.

Based on the above results, one could expect that MTX-HSA conjugate and 5-FU-AA-HSA conjugate could be used as a slow release dosage form in *in vivo* study.

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