Shenyang Pharmaceutical University, Department of Pharmaceutics[1], Shenyang City, Changli Traditional Chinese Medical Hospital[2], Qinhuangdao, P.R. China

Synthesis and characteristics of the fluorouracil-dextran conjugates

Ai Jun Hao[1], Ying Jie Deng[1], Xu Bin Suo[1], Yan Hong Cao[2]

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Ai Jun Hao, Ying Jie Deng, Xu Bin Suo P.O. Box 52, Shenyang Pharmaceutical University, Shenyang City, Liaoning Province, 110016, Yan Hong Cao, Changli Traditional Chinese Medical Hospital, Qinhuangdao, Hebei Province, 066600 P.R. China
Haoaj2004@yahoo.com.cn

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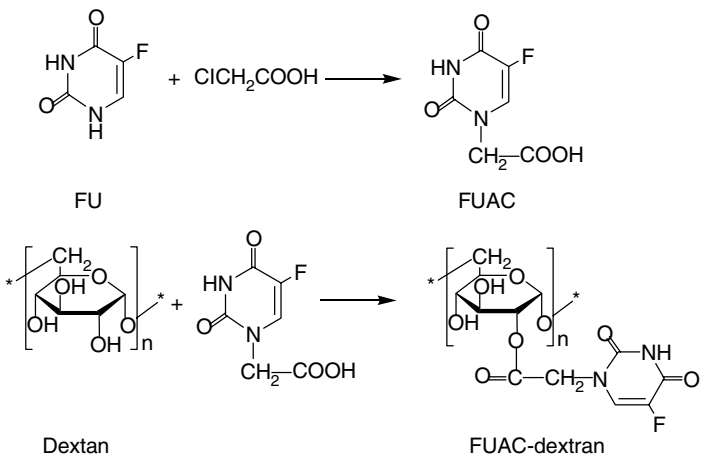
Fluorouracil acetic acid (FUAC) was first chemically bound to dextran in a solvent mixture (FA/DMF/DCM = 10 : 9 : 1, v/v/v) catalyzed by DMAP and DCC. The conjugate obtained was confirmed by analysis of UV, IR and NMR spectra. The degree of substitution (DS) of the conjugates was dependent on the proportions of FUAC and dextran used in the reaction mixture. Investigation of the chemical stability revealed that the conjugate was stable in an acidic buffer solution. The conjugate will find application in drug targeting and controlled release.

5-Fluorouracil (FU) is widely used in the treatment of various kinds of cancer including colon cancer. But due to its serious adverse effects, many efforts have been made to decrease its side effects and increase its therapeutic index. To achieve good therapeutic effects, 5-FU needs to be kept at a low concentration and to act over a long time (Longley et al. 2003). Considering the mechanism of FU, Chung et al. (1991) synthesized fluorouracil acetic acid-human serum albumin conjugates and investigated the pharmacokinetics of FU after intravenous infusion of the conjugates. Their results demonstrated that FU rather than FUAC was released from the conjugates *in vivo*, which indicated that FUAC and polymer conjugates can release FU *in vivo*.

Dextran has been investigated as a most promising carrier for the delivery of drugs, proteins, enzymes and imaging agents (Mehvar 2000). Most dextran conjugates act as polymeric prodrugs, prolonging the release of the active drug *in vitro* and *in vivo*. In view of the mechanism of action of FU and the advantages of polymeric prodrugs, dextran was selected as the carrier for synthesis of a novel macromolecular prodrug of FU. We anticipated that the macromolecular prodrug would be stable *in vitro* and would slowly release FU *in vivo* with passive targeting ability.

Dextran has numerous hydroxyl groups in the glucose unit. Theoretically dextran can be directly esterified with bioactive compounds containing carboxyl groups. Because FU does not have an appropriate functional group to conjugate with the hydroxyl group of dextran, FU was reacted with chloroacetic acid in the presence of potassium hydroxide to obtain FUAC and a carboxyl group was thus introduced to the FU molecule. Then FUAC was esterified with dextran as shown in the Scheme. The reaction was performed under conditions similar to the method (Bamford et al. 1986; Nichifor et al. 2004). The DS of the conjugates depended on the molar ratio of FUAC and glucose units. We tried various solvents such as DMSO, MF and DMF as the reaction medium but the DS of FUAC was much lower than that of the conjugate prepared in the mixed solvent. The solvent mixture MF/DMF/DCM was the best reaction medium among DMSO, MF and DMF, which was consistent with the results obtained from the reaction of dextran with other bioactive molecules containing a carboxylic group (Bamford et al. 1986; Nichifor et al. 2004). The reasons for the increased efficacy of the esterification reaction in the mixture (MF/DMF/DCM) are not clear (Nichifor et al. 2004). However, the FUAC-dextran conjugates were successfully prepared in two steps as shown in the Scheme.

Scheme Synthesis of FUAC-Dextran conjugates



The purity of the conjugates was determined by gel filtration chromatography. The conjugate was found in the elute from 25 ml to 45 ml and FUAC was in the elute from 50 ml to 100 ml. The mixture of FUAC and conjugate was completely separated on this gel column. The elution profiles of the conjugates revealed that there is almost no free FUAC in the conjugate. The absence of free drug in the conjugate was additionally proved by determination of free FUAC in the conjugate by a RP HPLC method which showed that 97.4% (wt) of FUAC was chemically conjugated to dextran and not merely dispersed or incorporated into the polymer. There was good agreement with the analysis by gel filtration chromatography.

The conjugate obtained was confirmed by its UV, IR and NMR spectra. The maximum UV absorbance of free FUAC was observed at 271 nm in 0.1 N HCI solution, but a maximum for the conjugate was found at 268 nm under the same conditions. The IR spectrum of the conjugate was recorded in a KBr tablet and showed a peak at from 1748 to 1680 cm^{-1} originating from the ester bond and lactam of the ring of FU. The ^1H NMR spectrum showed that FUAC was chemically conjugated with dextran through an ester bond, ^1H NMR (DMSO) δ (ppm): 3.2–3.75 and 4.54–4.94 (CH−OH, and $-CH_2-O$, glucose unit), 3.35 ($N-CH_2-COO-$), 5.19 (CH−OCO), 7.98 (C_6-H, FU), 11.6 (N_3-H, FU).

The DS of the conjugate was expressed as the percentage of FUAC in the conjugate. When the molar ratio of FUAC per anhydroglucose unit was 0.5 : 1.0 and 1.0 : 1.0, the DS of the conjugates was 15.1% and 30.1% (wt) respectively which was equivalent to 15.3 and 30.5 molecules of FUAC per 100 glucose units respectively. The DS of the conjugate depended on the relative molecular proportion of the starting materials of the reaction. This result was consistent with that for other bioactive molecules reacted with dextran (Bamford et al. 1986; Nichifor et al. 2004).

In hydrolysis experiments it was noteworthy that no detectable FU was produced throughout the hydrolysis experiments. The rate constant of hydrolysis of the conjugate was therefore calculated from the concentration of FUAC at different times and is shown in the Table. The stability investigation of the conjugates revealed that the conjugate was more stable at pH 2.96 and more sensitive to hydrolysis when the pH value changed from 2.96 to 8.75. This result was consistent with the hydrolysis of glucocorticoid-dextran esters (McLeod et al. 1993). The results demonstrated that the conjugate will remain chemically bonded to the polymer in acid conditions such as those in the stomach and will tend to decompose and release free FUAC under neutral or slightly alkaline conditions such as those in the colon. Therefore this conjugate would be suitable for colon targeting as a polymeric prodrug of FU. As a polymeric prodrug, after intravenous administration the conjugate would slowly release FU and passively target the solid tumor through an enhanced permeability and retention (EPR) effect (Takakura and Hashida 1995).

Table: Hydrolysis rate constant at 60 °C in buffer solutions and physiological saline (DS = 15.1%)

Solution	pH value of buffer solutions					Physiological saline
	1.24	2.96	4.95	6.80	8.75	
k_{ester} (day^{-1})	0.228	0.0336	0.362	10.2	100.1	3.31

In conclusion, FUAC-dextran conjugates with different DS were successfully synthesized in the mixed solvent. The conjugate was more stable at a pH around 2.96 and was labile under neutral and alkaline conditions. The physicochemical properties and pharmacokinetics of the conjugates are being further investigated.

Experimental

1. Materials

A commercial dextran (T-70, Mw = 70,000) and Sephadex G-25 were purchased from Pharmacia-Amersham. FU was purchased from Nantong Jinghua Pharmaceutical Co. Ltd, Jiangsu Province, China. FUAC was synthesized according of Tada (1975). DMF and DCM were stored over 4 Å molecular sieves until use. All other solvents and chemicals were obtained from commercial sources and used without further purification. Freeze drying was performed with a FD-1 Lyophilizer manufactured by Boyikang Co. Ltd., Beijing, China. IR spectra were recorded on a Bruker IR-27G spectrometer. NMR spectrum was recorded on an ARX-300.

2. General synthesis of FUAC-dextran conjugate

1 g Dextran (6.17 mmol anhydrous glucose units) was dissolved in a mixed solvent composed of 10 ml of FA, 9.0 ml DMF and 1.0 ml DCM at 30 °C under magnetic stirring. 0.58 g (3.09 mmol) FUAC was added to the solution of dextran followed by the addition of 38 mg DMAP. After these were totally dissolved, 0.700 g DCC was added to the solution. The reaction mixture was stirred for 24 h at 30 °C and filtered to remove dicyclohexylurea generated by DCC. The filtrate was poured into 200 ml of a mixed solution of methyl alcohol and diethyl ether (3 : 1, v/v) to precipitate the FUAC-dextran conjugate. The crude conjugate obtained was collected by filtration and washed with 50 ml diethyl ether and 50 ml acetone. The conjugate was chromatographed by Sephadex G-25 column. The solution containing the conjugate was collected and was freeze dried in a FD-1 lyophilizer. The final conjugate was a white powder and was stored in a desiccator in the presence of phosphorus pentoxide.

3. Chemical stability in buffer solution at elevated temperature

The stock solution of the conjugates was added to different preheated (60 ± 0.2 °C) buffers to give a final concentration of 50 μM (based on FUAC). The concentration of all buffers was maintained at 0.1 M and was adjusted to an ionic strength of 0.5 M by the addition of NaCl. The buffer solution used was as follows: hydrochloric acid (pH 1.24), citrate (pH 2.96), acetate (pH 4.85), phosphate (pH 6.80) and borate (pH 8.75). The buffer solutions were incubated for a given time. 0.2 ml portions of the reaction mixture were removed at appropriate time intervals and 20 μl was injected into HPLC analysis.

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