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Characterization of in-vitro drug release and biological activity of methotrexate-bovine serum albumin conjugates

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Two series of methotrexate (MTX)-bovine serum albumin (BSA) conjugates have been prepared containing either 96 ± 16 mg (mean ± s.d.) or 32 ± 13 mg of MTX per gram of conjugate. The conjugates released MTX in-vitro in a biphasic manner, the release rate being dependent on the quantity of MTX in the conjugate and on the pH of the release medium. An initial rapid release over 6 h appears to be due to physically adsorbed MTX with the slower secondary release due to covalently bound drug. The conjugates retain a degree of antineoplastic activity in-vitro, but this might be related to the small fraction of MTX that is tightly physically bound.

One of the major limitations of systemic cancer chemotherapy is the incidence of toxic side-effects related to drug action on normal replicating cells such as those of the bone marrow or gastrointestinal tract. One of the methods used in an attempt to reduce this toxicity has been to attach the antineoplastic agent to a carrier molecule which may increase its activity against tumour cells if uptake of the carrier into these cells is preferential. This was first attempted experimentally in 1958 (Mathe et al 1958) when methotrexate (MTX), coupled to gamma globulins through an azo-linkage, produced a greater increase in survival time in mice bearing L1210 leukaemia cells than either free MTX or globulin, or a physical combination of both. More recently this approach has been extended by coupling MTX (and other antineoplastic agents) to a variety of both natural and synthetic carrier molecules.

The attachment of MTX to immunoglobulins has met with varying degrees of success. Coupling to immunoglobulins either by a carbodiimide cross-linking method or by the synthesis of an active ester intermediate of MTX, yielded conjugates that produced a 70% survival rate in tumour-bearing mice compared with a 10% survival rate for MTX and immunoglobulins administered separately (Kulkarni et al 1981). Similar results were obtained with MTX coupled via a mixed anhydride procedure to antibodies raised against a murine ovarian sarcoma (Burstein & Knapp 1977).

Carboxymethylcellulose (mol. wt 30 000) and polyethylenimine (mol. wt 2000) have also been conjugated with MTX (Fung et al 1979). The carboxymethylcellulose conjugate was approximately one hundred times less active in-vitro than free MTX in terms of the inhibition of tetrahydrofolate dehydrogenase and L5178Y cell growth, whereas the polyethylenimine conjugate exhibited only a slight reduction of activity in both systems, an effect perhaps due to the differences in the molecular weight of the molecules.

Water soluble conjugates for instillation into tumour sites have also been investigated (Harding 1971) by linking MTX to either bovine serum albumin (BSA) or dextrans of varying molecular weight. These derivatives when injected intraperitoneally into mice bearing the ascitic form of L1210 increased the half-life of the attached MTX in the peritoneal cavity and, depending upon the dose administered, increased the survival time of the mice (Jacobs et al 1971). The MTX-BSA conjugates were also tested intravenously and proved more effective than free MTX against the development of lung metastases from a subcutaneously transplanted Lewis lung carcinoma (Chu & Whiteley 1979).

The results obtained with the albumin conjugates coupled with the fact that serum albumin has been demonstrated to accumulate at tumour sites (Cerrottini & Isliker 1967) led us to study the properties of MTX-BSA conjugates to assess their suitability as drug targeting agents. In this paper we present results on the in-vitro release of drug from the conjugates, a basic parameter that has received only scant attention in the literature since very few studies fully characterize the drug conjugates used. The biological activity of the conjugates against L1210

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murine leukaemia in-vitro has also been assessed and related to drug release profiles.

MATERIALS AND METHODS

Materials

All buffer salts and other reagents were of Analar grade and purchased from BDH, Poole, UK. Methotrexate was the gift of Lederle Laboratories, Gosport, UK. Bovine serum albumin (Fraction V, 96–99% albumin) was obtained from Sigma Chemical Co., Poole, UK and used without further treatment. Buffers: release buffer pH 7·4 (mM) NaCl 75·3, Na_2HPO_4 53·4, NaH_2PO_4 15·3, pH 5·0 NaCl 90, Na_2HPO_4 0·7, NaH_2PO_4 75. Phosphate buffered saline, (mM) NaCl 137, KCl 3, Na_2HPO_4 8, KH_2PO_4 1·5, pH 7·4.

Synthesis of bovine serum albumin methotrexate high strength conjugates

To BSA (100 mg) dissolved in $NaHCO_3$ buffer (2 mL 0·05 M pH 7·6), was added MTX (40 mg, 0·09 mM) in 4 mL of buffer and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide HCl (25 mg, 0·13 mM) in 1 mL of buffer, and the mixture made to a final volume of 12 mL, then incubated at room temperature in the dark for 4 h. The mixture was then applied to a BioGel P100 gel exclusion chromatography column (2·2 × 35 cm) and eluted with 0·1 M potassium tetraborate (pH 8·2) containing 1 M urea. Collection of 4 mL fractions afforded the conjugate in fractions 6–10 and unreacted MTX in fractions 16–24. The first conjugate fraction was discarded, the remainder pooled and dialysed (Visking tubing 18/32) against distilled water, in the dark at 4 °C, the solution filtered (0·22 μm) then lyophilized (Chu & Whiteley 1977). Low strength conjugates were produced by the same method but half the quantities of MTX and carbodiimide were used.

Measurement of MTX content of the conjugates

The MTX content of the conjugates was calculated from the absorbance of a solution in 0·1 M NaOH at 376 nm, with reference to the absorbance of MTX standards, MTX A1%, 1 cm at 376 nm in 0·1 M NaOH = 163·1. BSA absorbance under these conditions is negligible.

Determination of the water content of the conjugates

The water content of the conjugates was determined by thermogravimetry using a Stanton Redcroft TG 750 thermobalance as follows: balance setting, range 3 ratio 3·0–10 mg; heating rate 10 °C min^{-1} from 20–130 °C with the temperature held at 130 °C until equilibrium weight was achieved; furnace, cooled with water 10 L h^{-1} and purged throughout run with dry N_2 5–10 mL min^{-1}. Results are expressed as the percentage weight loss (average of three determinations on each conjugate) which is taken to be equal to the water content of the conjugates. No visible degradation of the conjugates occurred during this procedure.

Release of MTX from MTX-BSA conjugates

A weight of conjugate equivalent to 1 mg of MTX was dissolved in the appropriate buffer and made up to 10 mL, the solution transferred to 10 cm Visking tubing (18/32) which was placed in 100 mL of the same buffer, magnetically stirred at 37 °C. The MTX content of the external buffer was measured by UV analysis at 303 nm and by HPLC on 1 mL samples of the buffer. Control experiments of physical mixtures of BSA and MTX were also performed.

Determination of rate constant of MTX release

The data from the release experiments were treated as if release was pseudo first order. The rate constant was derived by plotting log $a/(a - x)$ vs t, where a is the initial concentration of MTX inside the dialysis bag and $(a - x)$ is the concentration after time t h. This yields a straight line (determined statistically) which has its slope $k/2{\cdot}303$ where k is the rate constant of release. Two rate constants could be calculated from each set of data using the points between 1–6 h (inclusive) to provide the initial rate constant and the points between 6–72 h to calculate the second rate constant.

Release of MTX from the MTX-BSA conjugates on precipitation by trichloroacetic acid

Conjugate was precipitated from aqueous solution immediately after formation by the addition of an equal volume of trichloroacetic acid (10% w/v), the precipitated protein was removed by centrifugation (4000 rev min^{-1}, 30 min) and the quantity of MTX in the supernatant measured by HPLC.

HPLC analysis for MTX (Lawson & Dixon 1981)

Column, Spherisorb ODS 5 μm (0·5 × 25 cm); pre-column identical packing material (0·5 × 5 cm); buffer, 0·1 M Tris: 0·1 M Na_2HPO_4, (50 : 50) pH 6·8; mobile phase, buffer : methanol (80 : 20); flow rate, 1 mL min^{-1} at 2000 psi (LDC III Constametric pump); detector, 303 nm (LDC Spectromonitor III); range 0·01 AUFS; injection loop, 20 μL. Samples were injected directly without any pretreatment. The retention time for MTX was 9 min. A plot of MTX

concentration against peak height produced a straight line (r = 0·9980, n = 4).

Growth inhibition of L1210 cells

L1210 cells were purchased from Flow Laboratories, Irvine, UK and maintained in RPMI 1640 medium supplemented with 10% v/v foetal calf serum and containing penicillin 100 iu mL^{-1}, streptomycin 100 μg mL^{-1} and 20 mM HEPES. Cells were normally grown in Nunclon flasks and experiments performed in Linbro (24 well) multiwell plates, 5 (× 2 mL) wells being used at each concentration. Experiments were carried out by harvesting cells in the exponential growth phase, adjusting to the starting cell concentration (2·5 × 10^5 cells mL^{-1}) adding the material under test (in 0·1–0·2 mL PBS) and plating out into the dish.

Cell counting

Two wells were sampled from each experiment every 24 h and the cells counted after dilution in PBS with a Coulter Counter (Model Z_B, 100 μm counting tube), four counts being taken on each well and the result of one experiment expressed as the average of the eight counts.

RESULTS AND DISCUSSION

The results presented in Table 1 show that the high strength conjugates contain approximately 10% by weight of MTX whilst the low strength conjugates have just over 3%. The attachment values for the high strength conjugates are comparable with the results of 78–86 mg MTX g^{-1} conjugate reported by Chu & Whiteley (1977). This latter result relates solely to the synthesis of high strength conjugates and provides only basic information so that precise comparisons of the results are not possible. The water content of both conjugates is similar at around 10%, and using these values it is possible to calculate the number of molecules of MTX attached per molecule of BSA (Table 1). This was found to be 15 and 5 for the high and low strength conjugates, respectively, and amounts to an increase in molecular weight of around 9 and 3%. The coupling levels achieved are, however, similar to those reported for the attachment of MTX to antibodies (Kulkarni et al 1981) and to polylysine (Ryser & Shen 1980) by the use of carbodiimide in solution.

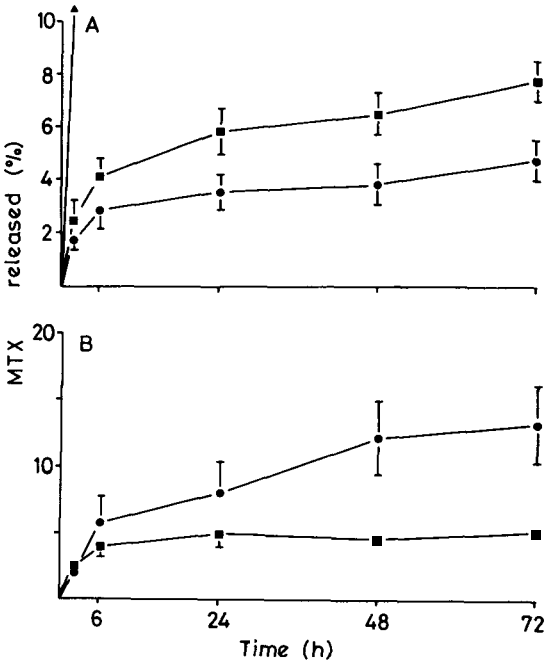

FIG. 1. Release of methotrexate from MTX-BSA conjugates. ■ High strength conjugates, ● Low strength conjugates, mean ± s.d., n = 7. ▲ Physical mixture MTX and BSA equivalent to high strength conjugate, mean n = 3. A, pH 7·4, B, pH 5·0.

Fig. 1 shows the results relating to the release of MTX from the conjugates. At pH 7·4 (Fig. 1A), both conjugates display an initial fast release period up to 6 h. In this interval approximately 4 or 2% of the MTX is released from either type of conjugate, and

Table 1. Properties of MTX-BSA conjugates.

	mg MTX g^{-1} conjugate mean ± s.d.	Range	mol MTX/ molBSA[a] mean ± s.d.	%H_2O content mean ± s.d.	% MTX released on TCA precipitation mean ± s.d.
High strength	96·4 ± 15·6 n = 15	141–76	15·3 ± 2·0 n = 11	10·7 ± 1·5 n = 11	5·1 ± 1·4 n = 8
Low strength	32·1 ± 13·0 n = 15	63–19	4·7 ± 1·6 n = 6	10·8 ± 1·4 n = 6	2·9 ± 0·9 n = 8

[a] Calculated allowing for determined water content and using a molecular weight of 66 500 for BSA.

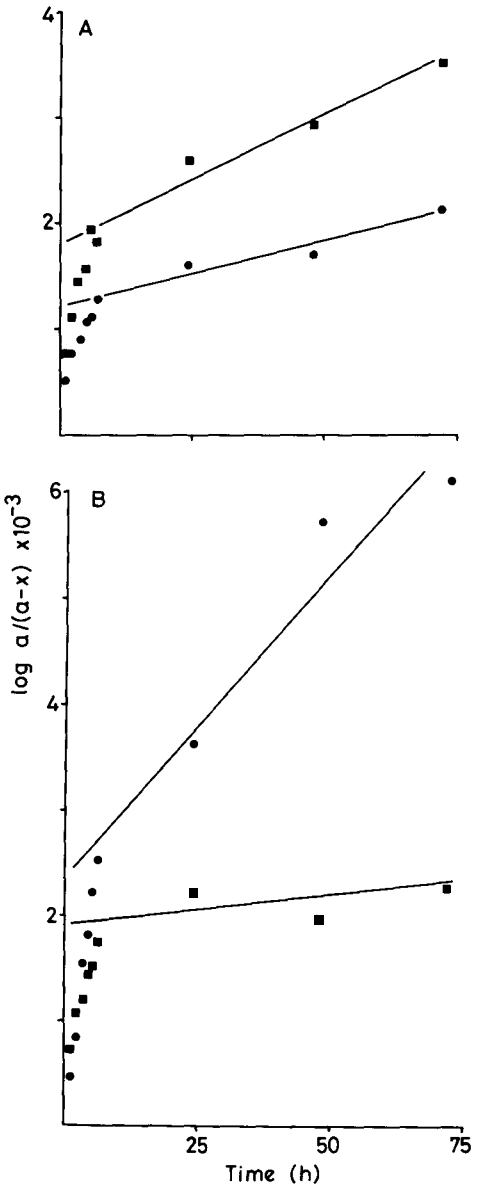

FIG. 2. Plot of data used to calculate rate constants in Table 2. ■ High strength conjugates, ● Low strength conjugates. Mean n = 7. A, pH 7·4, B, pH 5·0.

thereafter the release rate is very much slower with less than 10% released in 72 h. Similar results are obtained at pH 5·0 (Fig. 1B), with a fast release period up to 6 h. After this interval the high strength conjugates precipitated thus hindering further release whilst the low strength conjugates released up to 16% of the attached MTX. The rate constants determined from the release experiments are reported in Table 2 and the data presented graphically in Fig. 2. At pH 7·4 the initial rate constants are generally ten times greater than the second rate constant and this is also true of the low strength conjugates at pH 5·0, but not the high strength due to precipitation of the conjugates.

The data presented in Figs 1 and 2 demonstrate that release of MTX from the conjugates is a biphasic process, a result which suggests that the MTX is attached to the BSA by two distinct types of linkage. It is possible to calculate from the intercept of the second release rate regression line on the log a/(a − x) axis an approximation to the quantity of MTX released by the faster initial release process. These calculated values (Table 2) agree closely with

Table 2. Release Rate Constants of MTX from MTX-BSA conjugates.

Conjugate	pH	Rate Constant	$k \times 10^{-3} h^{-1}$	Intercept as % release
Low strength	7·4	Initial	3·47	—
	7·4	Second	0·27	2·8
High strength	7·4	Initial	5·20	—
	7·4	Second	0·56	4·1
Low strength	5·0	Initial	9·63	—
	5·0	Second	1·32(a)	5·2
High strength	5·0	Initial	4·55	—
	5·0	Second	0·13(b)	4·2

All calculated regression lines had correlation coefficients statistically significant at the 1% level except (a) significant 2·5% level, (b) no significant correlation.

the quantity of MTX released when the conjugates are precipitated from solution (Table 1), with the exception of the low strength conjugates at pH 5. The discrepancy of this latter result could be related to the increased rate of secondary release at the lower pH. These results imply that a small percentage of the attached MTX is tightly physically bound. It would appear therefore that the fast release process is due to the loss of physically bound MTX whilst the slow release can be ascribed to the hydrolysis of covalently attached MTX.

Methotrexate is known to bind physically with albumin to the extent of about 46% at normal clinical drug levels (Paxton 1981). The magnitude of the physical binding in this case is such that the bound drug is not removed during chromatography or dialysis, although traces of MTX were detected in some of the dialysates. Chu & Whiteley (1980) suspected that the conjugates contained a small

percentage of non-covalently bound drug but were unable to substantiate this. Our results suggest a general difficulty that may occur on covalently binding drugs to macromolecules which is that of ensuring that all of the physically bound drug is removed during the processing of the conjugate. This is especially significant if, as in this case, the macromolecule has a high physical affinity for the drug.

Although the coupling of anti-cancer agents to macromolecules by carbodiimides has been extensively used (Harding 1971; Ryser & Shen 1978; Chu & Whiteley 1979; Kulkarni et al 1981) little data exist on the stability or release of the anti-cancer agent after coupling. One study does report on the in-vitro release of mitomycin C from mitomycin C-dextran T70 conjugate at pH 7·4, with the finding that after three days 80% of the drug was released (Kojima et al 1980). The high release is due to the fact that the mitomycin C was not attached directly to the dextran backbone but via a caproic acid spacer group. In our study the MTX is directly attached to the protein backbone, a feature which will act to hinder drug release.

The release at pH 5·0 was also examined since it has been reported that albumin has been found inside tumour cells (Cerrottini & Isliker 1967), probably inside phagolysosomes which have a pH of around 5 (DeDuve et al 1974). Under these conditions the overall release was doubled for the low strength conjugates but was still very low. The increased release rate is probably due to a faster hydrolysis of the covalent bonding, catalysed by the lower pH. The high strength conjugates at pH 5 precipitated after 6 h; further work would be required to assess this property fully but it may be linked to the modification of protein structure occurring during the coupling reaction.

Overall the release of MTX from the conjugates is low with half-lives of release at pH 7·4 (based on second rate constants) of 50 and 110 days (approximately) for high and low strength conjugates. As the conjugates have been reported to have a half-life of only 11 h in the peritoneal cavity (Jacobs et al 1971), they would be cleared from a body cavity before the release of substantial quantities of drug, and this would preclude their use as depot preparations for instillation into tumour sites. The use of the conjugates for drug targeting would also appear to be limited by the poor rates of drug release and a method of drug coupling that facilitates a controlled release would be desirable.

Tissue culture results (Fig. 3) demonstrate that free MTX at a concentration of 10^{-7} M completely inhibits cell growth whereas at 10^{-8} M it has no discernible effect. The conjugates are ineffective at 10^{-7} M (equivalent MTX concentration) but do show growth inhibitory properties at 10^{-6} M. However only when the concentration of the conjugates is increased to 10^{-5} M is the measured growth inhibition similar to that of free MTX at 10^{-7} M. In all the concentrations tested the low strength conjugates were slightly less active than the high strength. The data presented in Fig. 3 are represented diagrammatically in Fig. 4 in which the percentage inhibition of growth at 48 h is plotted with respect to the log MTX concentration.

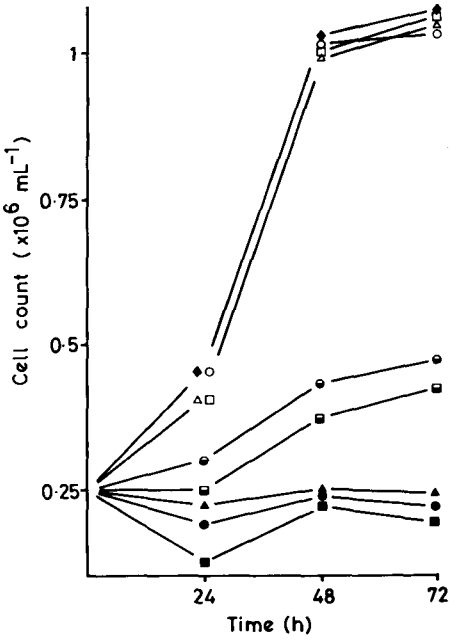

FIG. 3. Inhibition of L1210 cell growth by MTX-BSA conjugates. ◆ Control, ▲ MTX 10^{-7} M, △ MTX 10^{-8} M, ■ high strength conjugate 10^{-5} M, ▣ high strength conjugate 10^{-6} M, □ high strength conjugate 10^{-7} M, ● low strength conjugate 10^{-5} M, ◐ low strength conjugate 10^{-6} M, ○ low strength conjugate 10^{-7} M. N.B. Concentrations of conjugates refer to the equivalent concentration of MTX. Mean $n = 4$.

The concentration of the conjugates required to inhibit L1210 cell growth in-vitro by 50% at 48 h were 14 and 18 times greater than the free MTX concentration, a result similar to that obtained by Chu & Whiteley (1980). This reduction in activity is in broad agreement with the quantity of MTX that is released within 48 h at pH 7·4, since free MTX only

takes 2 h to exert its full inhibitory activity on L1210 cells (Sirontak & Donsbach 1974). This indicates that the in-vitro cytotoxic effect of the conjugates is to a certain extent dependent on the small quantity of physically bound MTX. The reduced activity of the low strength conjugates in comparison with the high strength may therefore be related to the lower percentage of MTX that is physically bound to the BSA in this series. The tissue culture results also indicate that a fraction of the activity cannot be ascribed to simple release processes and must therefore be due to possible uptake and degradation of the conjugate by the cells.

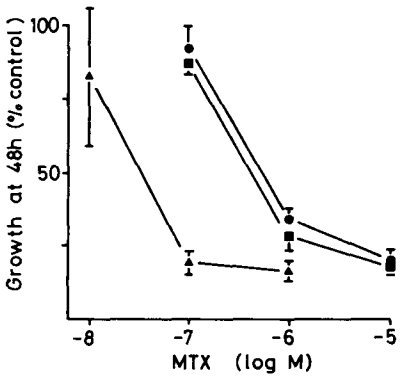

FIG. 4. Percentage inhibition of L1210 growth at 48 h. ▲ MTX, ■ high strength conjugates, ● low strength conjugates, mean ± s.d., n = 4.

These results indicate that a greater degree of physicochemical investigation of the properties of anticancer macromolecular drug conjugates is necessary to comprehend fully their mode of action.

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