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Short Communication

Activation of Methotrexate Prodrugs by Enzyme/Monoclonal Antibody Conjugates

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One of the major goals in cancer chemotherapy is the development of procedures for the *selective* delivery of drugs to tumor cells. This strategy, when perfected, will allow drugs to be used at the high concentrations necessary for virtually complete (i.e., > 99%) eradication of the tumor cells without concomitant destruction of normal cells. Monoclonal antibodies targeted to tumor antigens offer considerable promise for effecting the desired selectivity. To date, however, only limited success has been achieved in clinical trials with monoclonals as vehicles for carrying drugs. Two limitations are evident in the use of drug-monoclonal conjugates: (a) Relatively few drug molecules can be linked to each monoclonal without impairing binding activity; and (b) A mechanism is needed to release covalently-bound drug from the antibody. In an effort to overcome these problems, attention has focused recently on the attachment of enzymes rather than drugs to monoclonals, thereby taking advantage of the catalytic power of the enzyme to generate large amounts of the active drugs from inert prodrugs at the tumor site [reviewed by Senter (1)].

Methotrexate (MTX), employed extensively in cancer chemotherapy, is well-suited to the prodrug/enzyme-monoclonal conjugate strategy. α-Peptides of the drug (i.e., derivatives in which the α-COOH has been linked to an amino acid) are relatively non-toxic, but these derivatives can be hydrolyzed by carboxypeptidases to yield the active parent drug. Alanine, phenylalanine, aspartate and arginine derivatives of MTX (Fig. 1) have been synthesized in good yield by conventional pathways (2, 3). It should be noted, however, that direct coupling of the amino acid to MTX results in considerable racemization of the L-glutamate moiety in the drug which, in turn, limits the carboxypeptidase-mediated cleavage of the MTX-amino acid linkage. This can be avoided by coupling the Glu-amino acid dipeptide to 4-amino-4-deoxy-10-methylpteroic acid. Hydrolysis of the MTX α-peptides by various carboxypeptidases, and particularly MTX-Ala by carboxypeptidase-A (CP-A), has been examined in detail (2). These reactions, although slow relative to the rates with standard substrates for the enzymes (e.g., hippurylphenylalanine for CP-A), are still sufficient to generate toxic quantities of the drug (2).

Regional selectivity of the MTX-Ala/CP-A combination was demonstrated in a model system in which immobilized CP-A was embedded in a semi-solid medium containing the prodrug and L1210 cells (4). The crucial question, however, was whether cytotoxic combinations of MTX could be produced by the amount of CP-A that could be attached to target cells via a monoclonal antibody. Accordingly, CP-A derivatized with succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate and a monoclonal antibody (KS1/4, directed toward a surface antigen on carcinoma cells) derivatized with N-succinimidyl 3-(2-pyridyldithio)propionate were reacted to produce a thioether-linked, enzyme-antibody conjugate preparation (5). The latter, purified by sequential HPLC size-exclusion and DEAE chromatography, contained

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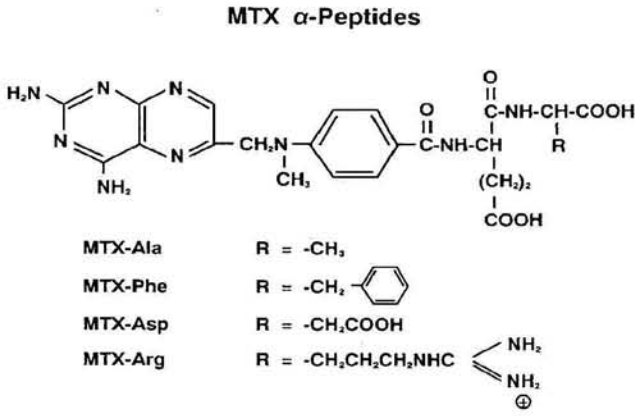

Figure 1. Structures of Methotrexate α-peptides.

Table 1. Cytotoxicity of Methotrexate α-Phenylalanine (MTX-Phe) for UCLA-P3 Cells Treated with Carboxypeptidase-A (CP-A)/Monoclonal Antibody KS1/4 Conjugate

Drug or Prodrug	Enzyme-Monoclonal Conjugate	% Inhibition
MTX	–	100
MTX-Phe	CP-A/KS1/4	94
MTX-Phe	–	23
–	CP-A/KS1/4	3

In vitro cytotoxicity against UCLA-P3 human lung adenocarcinoma cells (10^4 cells/well) was measured in 96-well plates as described previously (5). In samples treated with enzyme-antibody conjugate, excess of the latter was removed by extensive washing. Growth was stopped after 72 h. Inhibition is expressed relative to that observed with MTX (designated as 100%). Concentrations of MTX and MTX-Phe, 1×10^{-6} M.

approximately equal amounts of 1 : 1 and 2 : 1 (enzyme : antibody) conjugates. Binding activity of the conjugate (1.8×10^5 molecules/cell) was similar to that of unreacted antibody.

In vitro cytotoxicity studies using UCLA-P3 human lung adenocarcinoma cells (2×10^4 cells/well) in 96-well plates demonstrated that the amount of *free* CP-A required to produce ID_{50} values for MTX prodrugs equal to that of MTX (ca. 5×10^{-8} M) was 10 mU/well for MTX-Ala, and 1 mU for MTX-Phe. However, calculations based upon the number of antigen binding sites per cell and the CP-A activity of the KS1/4 conjugate indicated that only about 0.03 mU of enzyme would be present on cells saturated with conjugate. It was a pleasant surprise to find that, when the lung tumor cells were treated with the conjugate, and excess unbound conjugate was removed by extensive washing prior to addition of the prodrug, the ID_{50} for MTX-Ala improved from 8.9×10^{-6} M (no CP-A or conjugate) to 1.5×10^{-6} M for cells containing bound conjugate. With MTX-Phe as a prodrug, the results were even better: ID_{50} in the presence of conjugate approached that of MTX (5.2×10^{-8} M). The conjugate-mediated toxicity of MTX-Phe is illustrated by an experiment (Table 1) in which identical concentrations of the drug and prodrug are compared for their ability to inhibit cell replication. Increased potentiation of prodrug cytotoxicity by antibody-bound CP-A over that afforded by free enzyme is attributed to enhanced effectiveness of MTX generated adjacent to folate transporters at the cell surface. These results clearly demonstrate the *in vitro* chemotherapeutic potential of carboxypeptidase-monoclonal antibody conjugates used in conjunction with MTX peptide prodrugs.

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References

1. Senter, P. D. (1990) FASEB Journal *4*, 188–193.
2. Kuefner, U., Lohrmann, U., Montejano, Y. D., Vitols, K. S. & Huennekens, F. M. (1989) Biochemistry *28*, 2288–2297.
3. Kuefner, U., Esswein, A., Lohrmann, U., Montejano, Y., Vitols, K. S. & Huennekens, F. M. (1990) Biochemistry *29*, 10540–10545.
4. Vitols, K. S., Montejano, Y. D., Kuefner, U. & Huennekens, F. M. (1989) Pteridines *1*, 65–70.
5. Haenseler, E., Esswein, A., Vitols, K. S., Montejano, Y., Mueller, B. M., Reisfeld, R. A. & Huennekens, F. M. (1992) Biochemistry, *31*, 891–897.