Methotrexate (MTX)–cIBR Conjugate for Targeting MTX to Leukocytes: Conjugate Stability and *In Vivo* Efficacy in Suppressing Rheumatoid Arthritis

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ABSTRACT: Methotrexate (MTX) has been used to treat rheumatoid arthritis at low doses and leukemia at high doses; however, this drug can produce severe side effects. Our hypothesis is that MTX side effects can be attenuated by directing the drug to the target cells (i.e., leukocytes) using (cyclo(1,12)PenPRGGSVLVTGC) peptide (cIBR). To test this hypothesis, MTX was conjugated to the N-terminus of cIBR peptide to give MTX–cIBR conjugate. MTX–cIBR (5.0 mg/kg) suppressed joint arthritis in adjuvant arthritis rats and prevented periarticular inflammation and bone resorption of the limb joints. *In vitro*, the toxicity of MTX–cIBR peptide against Molt-3 T cells was inhibited by anti-lymphocyte function-associated antigen-1 (LFA-1) antibody and cIBR peptide in a concentration-dependent manner, suggesting that the uptake of MTX–cIBR was partially mediated by LFA-1. Chemical stability studies indicated that MTX–cIBR was most stable at pH 6.0. The MTX portion of MTX–cIBR was unstable under acidic conditions, whereas the cIBR portion was unstable under basic conditions. In biological media, MTX–cIBR had short half lives in rat plasma (44 min) and homogenized rat heart tissue (38 min). This low plasma stability may contribute to the low *in vivo* efficacy of MTX–cIBR; therefore, there is a need to design a more stable conjugate to improve the *in vivo* efficacy. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:3275–3291, 2012

Keywords: targeted drug delivery; peptide degradation; peptides; drug conjugates; active transport; MTX–cIBR; ICAM-1; LFA-1; methotrexate; *in vitro*; *in vivo*; chemical stability; enzymatic stability; rheumatoid arthritis

INTRODUCTION

Intercellular adhesion molecule-1 (ICAM-1)–lymphocyte function-associated antigen-1 (LFA-1) interactions play an important role in the process of T-cell activation and vascular extravasation of leukocytes through vascular endothelium for recruitment to sites of infection and inflammation.[1,2] Inhibition of ICAM-1–LFA-1 interactions by antibodies and peptides has been shown to suppress autoimmune diseases as well as allograft rejections.[1–3] cIBR peptide (cyclo(1,12)PenPRGGSVLVTGC) derived from the domain-1 (D1) of ICAM-1 binds to the I-domain of LFA-1 and inhibits homotypic and heterotypic T-cell adhesion.[1,4–9] Uptake of cIBR peptide by T cells occurs via receptor-mediated endocytosis[4]; thus, it is an attractive molecule for selective delivery of cytotoxic drugs to leukocytes. The uptake of cIBR was determined using fluorescein isothiocyanate (FITC) conjugate with cIBR (FITC–cIBR), and FITC–cIBR was internalized by Molt-3 and HL-60 cells via receptor-mediated endocytosis.[4,10,11] As a negative control, FITC–cIBR was not internalized by human umbilical vein endothelial cells, which do not express LFA-1 receptors.

Abbreviations used: ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; FITC, fluorescein isothiocyanate

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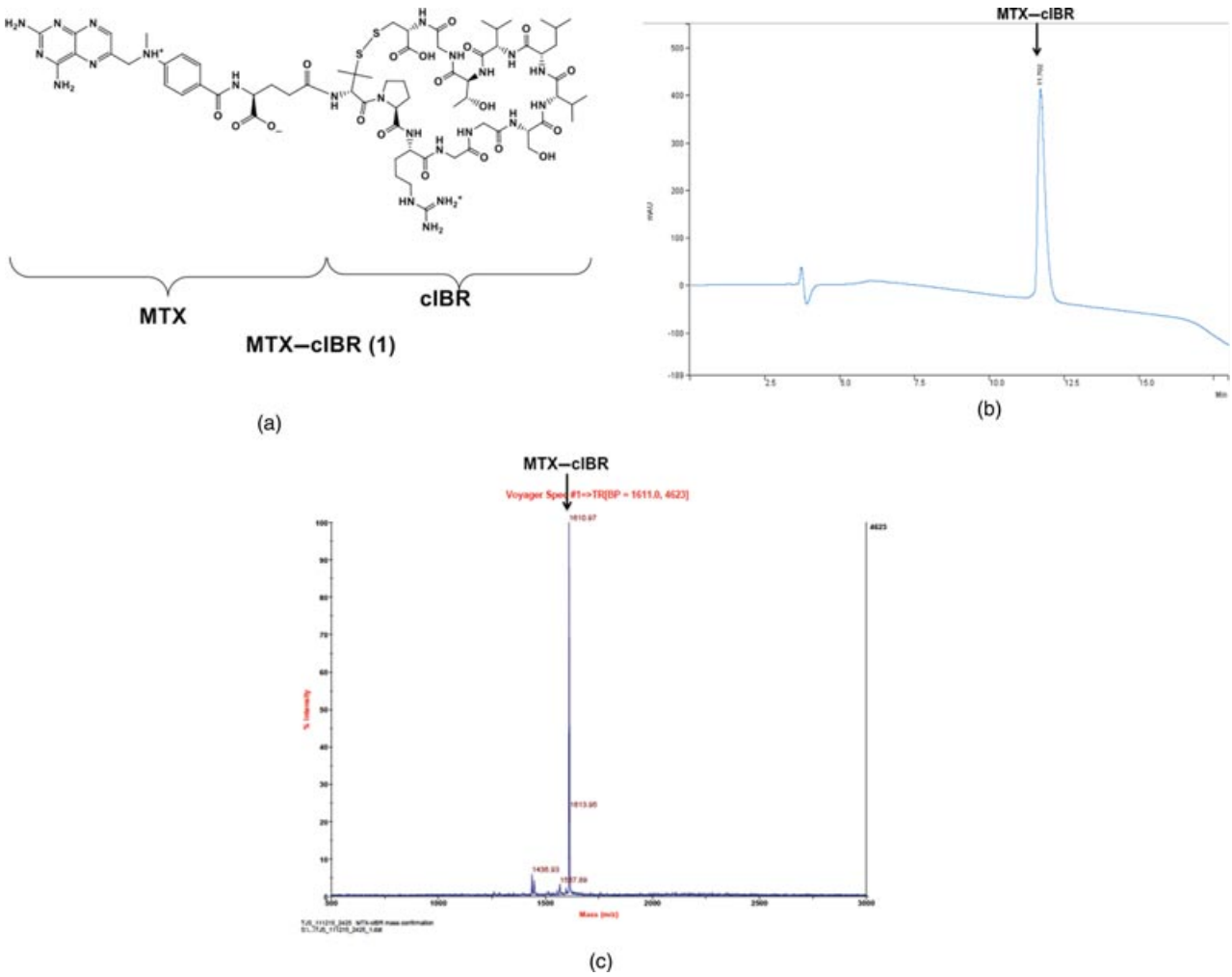

Figure 1. Structure and characterization of MTX–cIBR: (a) chemical structure of MTX–cIBR, (b) analytical reversed-phase HPLC chromatogram of pure MTX–cIBR, and (c) mass spectrometry spectrum of pure MTX–cIBR.

Methotrexate (MTX) is used to treat leukemia at high doses[12,13] and autoimmune diseases such as rheumatoid arthritis (RA) at low doses.[14,15] The normal cellular uptake of MTX is mediated by reduced folate carriers (RFCs) and membrane folate-binding proteins (mFBPs); thus, these cellular uptake processes contribute to nonselectivity for different cells, which causes side effects.[16] MTX may also generate drug resistance due to (a) changes in RFC expression level and altered transport kinetics and (b) increased dihydrofolate reductase (DHFR) expression with continued use of the drug.[12,17–19] Several other reasons for MTX resistance have also been proposed in the literature.[18,20] MTX has been effectively delivered to cells using its conjugates with different carrier molecules, including peptides,[20] proteins,[21,22] and polymers.[23,24] Using target receptors other than RFC for cellular entry, the conjugate may possibly avoid cellular drug resistance. Normally, MTX is conjugated to the carrier peptides or proteins via the γ-carboxylic acid of MTX to the amino group in the carrier because the α-carboxylic acid group of MTX is necessary for binding to DHFR.[4]

In this work, MTX was conjugated to the cIBR peptide to produce MTX–cIBR (Fig. 1a) for selective delivery of MTX to leukocytes for potential treatment of RA and leukemia. The hypothesis is that MTX–cIBR is directed toward LFA-1-expressing leukocytes over cells that do not express LFA-1, so the conjugate would have lower side effects than MTX alone. *In vivo* activity of the conjugate was determined in the rat adjuvant arthritic model. To study the *in vitro* selectivity, the activity of the conjugate to kill human Molt-3 T cells was assessed in the presence and absence of cIBR peptide and anti-LFA-1 antibodies. Chemical stability study of MTX–cIBR was conducted under accelerated conditions at different pH values. Finally, the enzymatic stability determination was carried out in plasma and liver homogenates to determine the dosing regimen during the *in vivo* study and for future