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Mutational 'hot-spots' in mammalian, bacterial and protozoal dihydrofolate reductases associated with antifolate resistance: Sequence and structural comparison

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ABSTRACT

Human dihydrofolate reductase (DHFR) is a primary target for antifolate drugs in cancer treatment, while DHFRs from *Plasmodium falciparum*, *Plasmodium vivax* and various bacterial species are primary targets in the treatment of malaria and bacterial infections. Mutations in each of these DHFRs can result in resistance towards clinically relevant antifolates. We review the structural and functional impact of active-site mutations with respect to enzyme activity and antifolate resistance of DHFRs from mammals, protozoa and bacteria. The high structural homology between DHFRs results in a number of cross-species, active-site 'hot-spots' for broad-based antifolate resistance. In addition, we identify mutations that confer species-specific resistance, or antifolate-specific resistance. This comparative review of antifolate binding in diverse species provides new insights into the relationship between antifolate design and the development of mutational resistance. It also presents avenues for designing antifolate-resistant mammalian DHFRs as chemoprotective agents.

1. Introduction

Antifolates constitute a large family of structurally diverse compounds used in the treatment of a broad range of diseases, the most important of which are various types of cancers as well as protozoal and bacterial infections (Lynch et al., 1982; Mennes et al., 2005; Daw et al., 2006; Bell et al., 2008; Zhanel et al., 2008) (Fig. 1). The principal target of antifolates is dihydrofolate reductase (DHFR; E.C.C. 1.5.1.3), an essential enzyme found in all living organisms. DHFR catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF), using NADPH as a coenzyme. THF is required for the *de novo* synthesis of purines and thymidylate. Inhibition of DHFR leads to the arrest of cell proliferation and, eventually, to cell death.

Antifolates bind to the folate-binding site of DHFR, acting as competitive inhibitors. As a result of high structural homology across species, many antifolates exhibit cross-species inhibition of DHFR. Nonetheless, species-specific inhibition has also been achieved, as illustrated by trimethoprim (TMP; Fig. 1), used in the treatment of bacterial infections. Bacterial DHFR exhibits 2500-fold tighter binding for TMP (K_i = 0.08 nM) than the human homolog (K_i = 200 nM), making TMP an effective antibiotic with few or no side effects (Margosiak et al., 1993). Similarly, pyrimethamine (PYR) and cycloguanil (CYC) (Fig. 1) are effective antifolates for the treatment of malaria (Ferone et al., 1969; Hitchings, 1969). The high structural homology shared by mammalian, parasitic and bacterial chromosomal DHFRs suggests that species-specific inhibition results from slight structural differences within their active site cavities.

Widespread clinical application of antifolates has led to resistance development, greatly impairing their efficacy. A number of different resistance mechanisms have been reported, including DHFR gene amplification, decreased cellular permeability to antifolates and even acquisition of evolutionarily distinct, intrinsically antifolate-resistant DHFR variants. This review focuses on resistance stemming from mutations of the *DHFR* gene. In protozoa and bacteria, the resulting DHFR variants no longer bind the antifolates effectively, yet maintain sufficient catalytic activity to ensure cellular proliferation. Due to the slower rate of mutation in human cells relative to bacteria and protozoa, this resistance mechanism

Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; TMP, trimethoprim; CYC, cycloguanil; PYR, pyrimethamine; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); DHF, 7,8-dihydrofolate; THF, 5,6,7,8-tetrahydrofolate; PMTX, pemetrexed; TMTX, trimetrexate; *p*-ABA, *p*-aminobenzoic acid; HSC, hematopoietic stem cells; RFC, reduced folate carrier; FPGS, folyl polyglutamate synthase; TS, thymidylate synthase; WT, wild-type; NBMPR-P, nitrobenzylmercaptopurine ribose phosphate.

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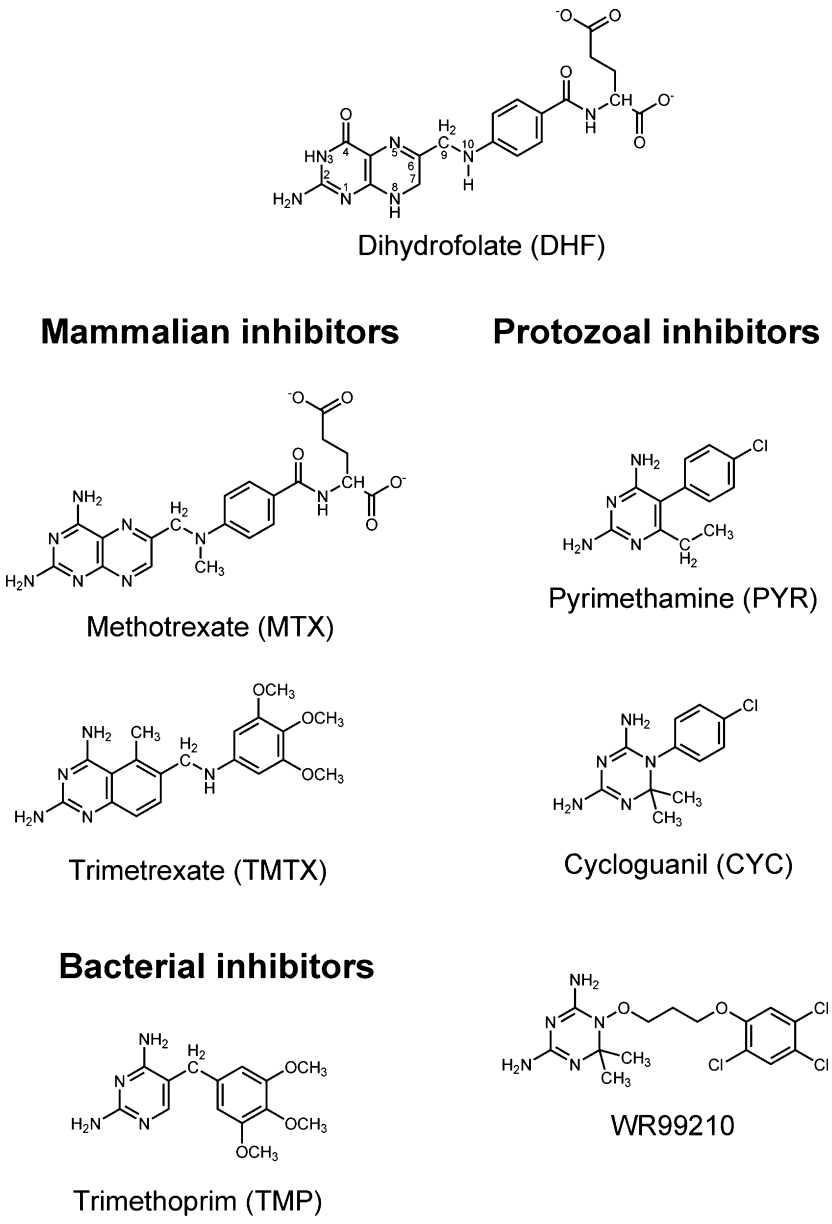

Fig. 1. Chemical structures of dihydrofolate and antifolates used to inhibit DHFR activity in different species. All antifolates shown are currently used for species-specific treatment. Pterin ring atom numbering is shown on DHF.

is not commonly found during the treatment of human proliferative diseases such as cancer (Spencer et al., 1996b). Nonetheless, antifolate-resistant variants of human DHFR offer important applications, such as the potential of protecting healthy bone marrow cells from the cytotoxic effects of antifolates used in cancer therapy (Banerjee and Bertino, 2002). Hence, the identification of mutational 'hot-spots' associated with antifolate resistance may lead to engineering antifolate-resistant DHFRs for myeloprotection.

We attempt to map the antifolate-binding requirements at the DHFR active site utilizing cross-species structural homology, extensive mutational data and inhibition data from a variety of antifolate-resistant DHFRs. Cross-species and cross-drug similarities and differences are highlighted. The aim is to increase our understanding of the balance between substrate binding and binding of specific antifolates in clinically relevant native or mutated DHFR variants.

1.1. Antifolate resistance mechanisms in mammalian cells

Among the antifolates designed to treat proliferative diseases in humans, no drug has been more clinically useful than methotrexate (MTX). First synthesized in the 1940s (Farber et al., 1948), MTX has been used to treat various types of cancers and other proliferative diseases (Chu and Allegra, 1996). MTX is a slow, tight-binding, competitive inhibitor of DHFRs from almost all species, binding stoichiometrically to DHFR. Due to its lack of selectivity, MTX is applied exclusively to the treatment of human diseases (Jones et al., 2000; Mennes et al., 2005; Strojan et al., 2005; Daw et al., 2006; Hashkes and Laxer, 2006; Slamon et al., 2006).

MTX resistance has been observed in mammalian cells exposed to MTX *ex vivo*. At low doses, mutations in the reduced folate carrier protein (RFC) (Rothem et al., 2004) and in folylpolyglutamate synthetase (FPGS) decrease cellular uptake of antifolates or allow more rapid efflux of MTX (Zhao et al., 2000) respectively. Both