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ANTAGE-4578; No. of Pages 9

ARTICLE IN PRESS

International Journal of Antimicrobial Agents xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

New small-molecule inhibitors of dihydrofolate reductase inhibit *Streptococcus mutans*

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ARTICLE INFO

Article history:
Received 12 January 2015
Accepted 31 March 2015

Keywords:
Streptococcus mutans
Biofilm
Dihydrofolate reductase
Trimetrexate analogues

ABSTRACT

Streptococcus mutans is a major aetiological agent of dental caries. Formation of biofilms is a key virulence factor of *S. mutans*. Drugs that inhibit *S. mutans* biofilms may have therapeutic potential. Dihydrofolate reductase (DHFR) plays a critical role in regulating the metabolism of folate. DHFR inhibitors are thus potent drugs and have been explored as anticancer and antimicrobial agents. In this study, a library of analogues based on a DHFR inhibitor, trimetrexate (TMQ), an FDA-approved drug, was screened and three new analogues that selectively inhibited *S. mutans* were identified. The most potent inhibitor had a 50% inhibitory concentration (IC_{50}) of 454.0 ± 10.2 nM for the biofilm and 8.7 ± 1.9 nM for DHFR of *S. mutans*. In contrast, the IC_{50} of this compound for human DHFR was ca. 1000 nM, a >100-fold decrease in its potency, demonstrating the high selectivity of the analogue. An analogue that exhibited the least potency for the *S. mutans* biofilm also had the lowest activity towards inhibiting *S. mutans* DHFR, further indicating that inhibition of biofilms is related to reduced DHFR activity. These data, along with docking of the most potent analogue to the modelled DHFR structure, suggested that the TMQ analogues indeed selectively inhibited *S. mutans* through targeting DHFR. These potent and selective small molecules are thus promising lead compounds to develop new effective therapeutics to prevent and treat dental caries.

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1. Introduction

Dental caries is initiated by cariogenic bacteria under the carbohydrate-rich environment of the oral cavity. *Streptococcus mutans* is the principal causative agent for the development of dental caries. The ability of *S. mutans* to adhere to the tooth surface and to incorporate into a polymicrobial consortium is paramount for the development of the disease; thus, reducing the number of cariogenic bacteria in dental biofilms is the key to devising therapeutic and preventive strategies [1].

Various strategies aimed at preventing dental caries have been attempted on the basis of various criteria: increasing antimicrobial activity [2]; replacement of sucrose with other sweeteners [3]; inhibition of the key matrix-producing enzyme glucosyltransferases either by vaccine approaches [4] or enzymatic inhibitors [5]; and targeting another important surface protein antigen I/II using immunomodulatory monoclonal antibodies [6]. However, in vivo application of these promising approaches is uncertain.

Folate is essential for all organisms. It contributes to the production of cofactors required for the synthesis of DNA, RNA and amino acids. Dihydrofolate reductase (DHFR) is the key enzyme involved in the production of the cofactors, thereby playing a critical role in regulating folate metabolism [7].

The importance of DHFR in the folate cycle has been explored in order to develop drugs for tumour therapy as well as therapy against opportunistic infections of diverse origin [7–9]. Inhibition of DHFR leads to partial depletion of reduced intracellular folate, thereby limiting cell growth. Functionally, DHFR is highly conserved in all domains of life, however they are divergent in amino acid sequence, offering an opportunity to impart a high degree of selectivity for certain antifolate drugs against one organism versus another [10].

Antifolate drugs have been successful in the treatment of bacterial and parasitic infections and for cancer chemotherapy. A number of antifolate drugs have been developed to date [9,11–13]. Trimetrexate (TMQ) has been used clinically for treatment of the parasites *Pneumocystis carinii* and *Toxoplasma gondii* infections in acquired

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http://dx.doi.org/10.1016/j.ijantimicag.2015.03.015
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Please cite this article in press as: Zhang Q, et al. New small-molecule inhibitors of dihydrofolate reductase inhibit *Streptococcus mutans*. Int J Antimicrob Agents (2015), http://dx.doi.org/10.1016/j.ijantimicag.2015.03.015

immunodeficiency syndrome (AIDS) patients [13]. To date, no DHFR inhibitor has been evaluated for its selectivity against the cariogenic bacterium *S. mutans*. Therefore, it is imperative to design new inhibitors that have high selectivity against *S. mutans* DHFR (*Sm*DHFR). Considering that the primary sequence of *Sm*DHFR shares only 28% identity with the human DHFR (hDHFR) sequence, it is conceivable to rationally design selective and potent inhibitors of *S. mutans* based on the structure of TMQ.

The goal of this study was to identify small-molecule inhibitors of DHFR that are capable of inhibiting *S. mutans*. A library of ca. 100 small-molecule compounds, which were designed based on TMQ, a US Food and Drug Administration (FDA)-approved drug [13], was screened and compounds that potently inhibit *S. mutans* were identified. More importantly, these compounds were selective against *Sm*DHFR. Computer modelling and docking analysis supported the finding that the identified small molecule possesses much higher affinity for *Sm*DHFR than for hDHFR. The study to design more potent and selective compounds inhibiting *S. mutans* is ongoing, which should facilitate the development of potent therapeutic drugs against *S. mutans*.

2. Materials and methods

2.1. Synthesis of small-molecule compounds and assembly of a screening library

Protocols used to synthesise small-molecule compounds are described in the Supplemental material. The small-molecule library was assembled by the Velu Research Group.

The synthetic processes and schemes are described in the main text and the Supplementary material (Fig. 1A; Supplementary Fig. S1). The chemical structures of TMQ and its analogues are depicted in the results section (Fig. 1B).

2.2. Bacterial strains and culture conditions

Streptococcus strains, including *S. mutans* UA159, *Streptococcus sanguinis* SK36 and *Streptococcus gordonii* DL1, were grown as previously described [14].

2.3. Biofilm formation and inhibition assays

Exponentially grown *S. mutans* and *S. sanguinis* bacteria were inoculated at 1:100 dilution with chemically-defined biofilm medium (CDBM) containing 1% sucrose for biofilm assays, whilst *S. gordonii* was inoculated at 1:50 dilution. Compounds at indicated concentrations were added to the inoculated bacterial cultures. The incubation time was 16 h for *S. mutans* and *S. sanguinis* and 12 h for *S. gordonii* to obtain reproducible and comparable biofilms. For control cultures, the corresponding volume of dimethyl sulphoxide (DMSO) was added. Crystal violet staining measured at an optical density of 562 nm was used to monitor biofilm formation described previously [14]. Minimum inhibitory concentrations (MICs) of the compounds were examined using a previously described method [14]. The concentration of potent compounds that inhibited *S. mutans* biofilm formation by 50% (IC_{50}) was determined by serial dilution. Each assay was carried out with duplicate samples and was repeated three times.

2.4. Cloning, expression and purification of recombinant DHFR from Streptococcus mutans and human cells

The coding sequence for the *S. mutans* UA159 DHFR protein was amplified by PCR (GoTaq® DNA Polymerase; Promega) using genomic DNA as template and *Sm*DHFR-specific primers (CGC*GGATCC*ATGAACAATAAGCGAGAAAAG and GCCG*CTCGAG*TCATTCCTTTTTCTCAAGTAC). The resulting PCR product (513 bp) was digested with *Bam*HI and *Xho*I and was ligated into the pET28-SUMO vector to construct pET28-SUMO-*Sm*DHFR. The constructed plasmids were verified by DNA sequence analysis and were then transformed into *Escherichia coli* BL21. The pET28-SUMO-hDHFR was constructed using a similar experimental procedure with hDHFR-specific primers (CGC*GGATCC*ATGGTTGGTTCGCTAAACTGC and CCT*CTCGAG*TTAATCATTCTT CTCATATACTTCAAATTTGTAC) and human cDNA.

Expression, production and purification of recombinant *Sm*DHFR and hDHFR proteins were carried out using the experimental procedures described previously [15]. In brief, cell lysates prepared from induced recombinant strains were loaded into a Ni-NTA resin column (Novagen, USA) for initial purification and the 6×His-SUMO tag was removed from the fusion proteins with SUMO proteinase. The fractions were then subjected to size exclusion chromatography on a Superdex75 column (GE Life Science, USA). Concentrations of purified *Sm*DHFR and hDHFR were determined by the Bradford method (Bio-Rad Protein Assay).

2.5. Assay of enzymatic activity of SmDHFR and hDHFR

The activity of purified *Sm*DHFR and hDHFR was determined by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH to $NADP^+$ accompanying the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) (coupled $\varepsilon = 12.260\ M^{-1}\ cm^{-1}$) using a UV–visible spectrophotometer. Reaction velocities were measured at 25 °C for 1 min. A DMSO concentration of ≤0.125% was used in the assays as the effect of the solvent at this concentration is negligible. Under optimised conditions for each enzyme, *Sm*DHFR activity was measured in 50 mM Tris–HCl buffer (pH 7.0), whilst hDHFR activity was measured in 50 mM KH_2PO_4 (pH 7.3) containing 250 mM KCl and 5 mM β-mercaptoethanol [9]. The inhibitory activities of the compounds were determined by measuring reaction velocities at several fixed concentrations of DHF (15, 20, 25, 30 and 40 μM), NADPH (150 μM) and various concentrations of compounds (dissolved in 0.1% DMSO). The 200 μL reaction was initiated with 0.2 μg of purified *Sm*DHFR or 0.3 μg of hDHFR. Each measurement was performed in triplicate.

The double-reciprocal plots were used to calculate the reaction constant (K_m) and inhibitory constant (K_i) for inhibition of *Sm*DHFR and hDHFR by each compound. The concentration of each inhibitor required for inhibition of enzyme activity by 50% (IC_{50}) was determined using the following equation: $IC_{50} = C_i/(V_o/V_i - 1)$, where V_o and V_i are the initial velocities in the absence and presence of inhibitor, respectively, and C_i is the concentration of inhibitor. The selectivity index (SI) was calculated using the equation: $SI = K_i(hDHFR)/K_i(SmDHFR)$ or $IC_{50}(hDHFR)/IC_{50}(SmDHFR)$. The SI of each analogue was compared with that of TMQ to assess the selectivity of each compound.

2.6. Sequence alignment, homology modelling and validation

The *Sm*DHFR sequence was submitted to Phyre² (Protein Homology and Analogy Recognition Engine v.2.0) (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and Swiss-Model (http://www.swissmodel.expasy.org) for structural prediction and modelling. *Streptococcus pneumoniae* DHFR (*Sp*DHFR) (PDB code, 3ix9; resolution, 1.95 Å) was selected for structural modelling, and the whole chain B of *Sp*DHFR served as the template for the homology modelling of *Sm*DHFR using Phyre² normal mode. The model was superposed with the *Sp*DHFR template by VMD 1.9.1 to establish the agreed and disagreed regions [11]. The overall calculated root-mean-square deviation (RMSD) was taken

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