Over-production of dihydrofolate reductase leads to sulfa-dihydropteroate resistance in yeast

Article in FEMS Microbiology Letters · August 2004
DOI: 10.1016/j.femsle.2004.06.001 · Source: PubMed

CITATIONS
4

READS
60

3 authors, including:

Kuldeep D Karnik
Intas Pharmaceuticals Ltd.
2 PUBLICATIONS  4 CITATIONS

Ian G Macreadie
RMIT University
145 PUBLICATIONS  2,589 CITATIONS

Available from: Ian G Macreadie
Retrieved on: 17 September 2016
Over-production of dihydrofolate reductase leads to sulfa-dihydropteroate resistance in yeast

Onisha Patel a,b, Kuldeep Karnik a,b, Ian G. Macreadie a,b,*

a CSIRO Health Sciences and Nutrition, 343 Royal Parade, Parkville, Vic. 3052, Australia
b RMIT University, Bundoora, Vic., Australia

Received 4 March 2004; received in revised form 17 May 2004; accepted 1 June 2004
First published online 15 June 2004

Abstract

Dihydropteroate synthase (DHPS) can metabolise sulfa drugs into sulfa-dihydropteroate (sulfa-DHP), which inhibits cell growth through competition with dihydrofolate (DHF), possibly indicating dihydrofolate reductase (DHFR) as the target of sulfa-DHP. The effect of over-production of DHFR on sulfa-DHP resistance was examined in Saccharomyces cerevisiae using a strain that requires DHF for growth. This strain was transformed with a plasmid which encodes over-production of DHFR in the presence of CuSO4. Over-production led to resistance to sulfa-DHP suggesting that sulfa-DHP targets DHFR. Spontaneous mutants hyper-resistant to sulfa-DHP did not show any changes within DHFR.

© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Sulfa-DHP; DHFR; Resistance; Dihydrofolate; Over-production; Sulfa drug

1. Introduction

Sulfa drugs are well known as mimics of p-amino benzoate (pABA). In addition to their competitive inhibition of dihydropteroate synthase (DHPS) resulting in inhibition of folate synthesis sulfa drugs are metabolised to sulfa-containing folate analogs, sulfa-dihydropteroate (sulfa-DHP) that inhibits the growth of yeast and Plasmodium falciparum [8,9]. The production and utilisation of folate and sulfa-DHP is represented schematically in Fig. 1. The effect of sulfa-DHP in yeast was tested with the help of dihydrofolate synthase (DHFS) and DHPS deleted strains [8]. The inhibitory effect of sulfa-DHP and sulfa-pteridine (oxidised form of sulfa-DHP) was observed in vivo through competition with dihydrofolate (DHF), a substrate for dihydrofolate reductase (DHFR), suggesting DHFR as the likely target. However, study [8] failed to find any inhibitory effect of low concentrations of sulfa-pteridine in vitro against the yeast DHFR. Because of the poor solubility of sulfa-pteridine higher concentrations could not be tested and identification of DHFR as a target for sulfa-DHP could not be shown.

Sulfa-DHP resistant colonies were also obtained in our previous study [8] but they did not have any mutation in the sequences encoding the DHPS (an established target of sulfa drugs) or DHFR, encoded by DFR1 [8]. The lack of changes in DHFR does not prove that DHFR is not targeted by the sulfa-DHP, since resistance could conceivably occur through increased production of DHFR. In the case of P. falciparum, amplification of the DHFR-TS gene has been reported in laboratory strains resistant to pyrimethamine, a DHFR inhibitor [3,11,12]. Amplification of the DHFR gene has also been implicated as one of the causes of resistance to methotrexate (MTX) in mammalian cells [5].

To investigate the effect of over-production of DHFR on sulfa-DHP resistance, we used the DHFS deleted study at [8].

*Corresponding author. Tel.: +61-3-9662-7299; fax: +61-3-9662-7266.
E-mail address: ian.macreadie@csiro.au (I.G. Macreadie).
strain, LCY1, as a model system. This strain cannot synthesise DHF and hence the level of DHF can be controlled by adding it externally to the medium. This strain has a functional chromosomal \textit{DFR1} gene, however, the over-expression of \textit{DFR1} in this strain was achieved through the use of the plasmid-encoded \textit{DFR1} on pYEX-CHT.YDHFR, described previously [8]. This plasmid has the \textit{CUP1} promoter preceding the DHFR reading frame. By adding CuSO\textsubscript{4} in the medium the over-expression of \textit{DFR1} is induced in the yeast transformed with pYEX-CHT and pYEX-CHT.YDHFR [8] using the lithium acetate protocol [4]. The resulting transformants were selected on yeast minimal medium (0.67% Difco yeast nitrogen base without amino acids, 2% glucose) with supplements as required. The complete minimal media for LCY1 [pYEX-CHT] and LCY1 [pYEX-CHT.YDHFR], contained tryptophan at 20 \textmu g/ml. Because LCY1 could not grow on liquid minimal media it was essential that media for testing drug resistance were solidified by the addition of 2% Phytagar (Gibco-BRL). SMX and STZ were used at concentrations of up to 400 \textmu g/ml. MTX used was at 0.15–1 \textmu g/ml. DHF was added at a concentration of 10 \textmu g/ml.

2.2. Drugs

The sulfonamide drugs used were sulfamethoxazole (SMX) and sulfathiazole (STZ) bought from Sigma Chemical Company and MTX bought from Amersham. SMX and STZ were made fresh before use by dissolving in DMSO at 100 mg/ml stock concentration. MTX was made fresh at 10 mg/ml by dissolving in water and with minimal NaOH to aid solubilisation. The final concentration of NaOH (35 mM) did not affect the growth of the strain.

2.3. Drug medium

The yeast medium used was minimal medium (0.67% Difco yeast nitrogen base without amino acids, 2% glucose) with supplements as required. The complete minimal media for LCY1 [pYEX-CHT] and LCY1 [pYEX-CHT.YDHFR], contained tryptophan at 20 \textmu g/ml. Because LCY1 could not grow on liquid minimal media it was essential that media for testing drug resistance were solidified by the addition of 2% Phytagar (Gibco-BRL). SMX and STZ were used at concentrations of up to 400 \textmu g/ml. MTX used was at 0.15–1 \textmu g/ml. DHF was added at a concentration of 10 \textmu g/ml.

2.4. Drug assay

Strains LCY1 [pYEX-CHT] and LCY1 [pYEX-CHT.YDHFR] were grown overnight in YEPD liquid medium. The cells were pelleted and washed with water. The neat culture was then adjusted to an $A_{600}$ nm of 0.09–0.1. From the neat culture 1/10, 1/100 and 1/1000 dilutions were made and 10 \textmu l aliquots of the diluted cultures were spotted on each plate. Dilutions were necessary because thick cell suspensions appeared to be insensitive to drugs, as previously reported [2]. The plates were then incubated at 30 \degree C and aliquots with appropriate seeding densities were photographed.

3. Results and discussion

3.1. Effect of over-production of DHFR on MTX resistance in LCY1

In order to establish the validity of our yeast model system we examined the effect of DHFR over-production on MTX resistance. Our host strain, LCY1, has a DHFS deletion and is reliant upon exogenous DHF for growth [1]. LCY1 [pYEX-CHT.YDHFR] has a functional chromosomal \textit{DFR1} gene as well as a plasmid-encoded DHFR downstream of the \textit{CUP1} promoter. Over-production of plasmid-encoded DHFR sequences was enabled by the addition of CuSO\textsubscript{4}. LCY1 [pYEX-CHT] served as a control: it has only chromosomal DHFR sequences and
therefore cannot over-produce DHFR under the same condition. Both LCY1 [pYEX-CHT] and LCY1 [pYEX-CHT.YDHFR] were grown in the presence of 10 μg/ml DHF and up to 1.0 μg/ml MTX. The growth of the strains was analysed in the presence of 0, 50 and 75 μM CuSO\(_4\). Results of experiments carried out with STZ are shown in Fig. 3. For the strain LCY1 [pYEX-CHT] there was growth inhibition when the level of STZ reached 400 μg/ml. By comparison this level of STZ caused no growth inhibition of LCY1 [pYEX-CHT.YDHFR] in the presence of added copper. We conclude that increased production of DHFR led to STZ-DHP resistance.

For SMX, the growth of LCY1 [pYEX-CHT] was completely inhibited at concentrations of SMX above 75 μg/ml. Reasons why SMX inhibits at lower concentrations than STZ have not been addressed; we speculate that there may be better uptake of SMX or greater stability. In contrast to the growth of LCY1 [pYEX-CHT], SMX-resistant growth was apparent for LCY1 [pYEX-CHT.YDHFR] grown in the presence of added CuSO\(_4\) (Fig. 3). Drug resistant colonies were clearly visible at SMX concentrations of 200–400 g/ml. Thus the observations here also demonstrate that high levels of DHFR confer resistance to sulfa-DHP synthesised in vivo.

It can also be observed in Fig. 3 that the growth of LCY1 [pYEX-CHT.YDHFR] actually increased as

### 3.2. Effect of over-production of DHFR on sulfa-DHP resistance in LCY1

To demonstrate the effect of over-production of DHFR on sulfa-DHP resistance, LCY1 [pYEX-CHT] and LCY1 [pYEX-CHT.YDHFR] were grown in the presence of 10 μg/ml DHF and up to 400 μg/ml SMX or STZ. LCY1 has a functional DHPS activity and can synthesise sulfa-DHP in the presence of sulfa drugs [8]. The growth of the strains was analysed in the presence of 0, 50 and 75 μM CuSO\(_4\). Results of experiments carried out with STZ are shown in Fig. 3. For the strain LCY1 [pYEX-CHT] there was growth inhibition when the level of STZ reached 400 μg/ml. By comparison this level of STZ caused no growth inhibition of LCY1 [pYEX-CHT.YDHFR] in the presence of added copper. We conclude that increased production of DHFR led to STZ-DHP resistance.

For SMX, the growth of LCY1 [pYEX-CHT] was completely inhibited at concentrations of SMX above 75 μg/ml. Reasons why SMX inhibits at lower concentrations than STZ have not been addressed; we speculate that there may be better uptake of SMX or greater stability. In contrast to the growth of LCY1 [pYEX-CHT], SMX-resistant growth was apparent for LCY1 [pYEX-CHT.YDHFR] grown in the presence of added CuSO\(_4\) (Fig. 3). Drug resistant colonies were clearly visible at SMX concentrations of 200–400 g/ml. Thus the observations here also demonstrate that high levels of DHFR confer resistance to sulfa-DHP synthesised in vivo.
levels of SMX increased. The phenomenon has not been observed in wild-type strains [2] and appears unique to LCY1 [pYEX-CHT.YDHFR]. To explain this behaviour we consider the unique nature of the construct with DHFR production being linked to the CUP1 promoter. The CUP1 promoter is responsive, not just to copper and silver ions, but to a number of stimuli as well as to the heat shock transcription factor [10], which is itself responsive to a range of stresses [7]. If high levels of SMX plus copper were particularly effective in inducing the CUP1 promoter in our construct this would lead to more highly elevated DHFR levels and possibly more SMX resistance. Such a possibility deserves further consideration. The same effect may occur for other sulfa drugs like STZ, however, much higher levels of STZ would require examination since even at 400 μg/ml STZ no growth inhibition was observed. To see a reversal of inhibition we would obviously need levels much higher than those for inhibition.

3.3. Mutants resistant to sulfa-DHP

Spontaneous isolates that appeared hyper-resistant to SMX were observed in Fig. 3. Clonal purification and re-testing confirmed that these mutants do have a copper-inducible resistant nature that is more elevated than the parental strain. To further evaluate the cause of the resistance, total genomic DNA was extracted from these two drug resistant colonies and was back-transformed in bacteria to isolate the pYEX-CHT.YDHFR plasmids. Sequencing of the DHFR gene in these plasmids showed no changes from the wild-type DFR1 suggesting that resistance was not developed because of any mutations in the plasmid-encoded DFR1 gene. Resistance could also occur because of mutations in the chromosomal DHPS sequence, a target to sulfa drugs and involved in the synthesis of sulfa-DHP. However, previous experiments of sequencing of sulfa-DHP resistant colonies of LCY1 [8] showed no variations on the chromosomal genes suggesting that changes in DHPS or DHFR structure were not the mechanism for drug resistance in those cases. The most likely explanation for the resistance observed in this study is due to over-production of DHFR and not due to amino acid replacements within DHFR.

Further genetic analysis showed the elevated resistance to be due to multiple factors. Some resistance was of plasmid-mediated origin since a small degree of hyper-resistance was obtained when plasmid from hyper-resistant clones was re-transformed into a naive LCY1 host strain (data not shown). Since this is not due to changes in DFR1 sequence we presume that such changes may arise from the mutations that increase the strength of CUP1 promoter or increase plasmid copy number. The observation that re-transformants are not exactly like their parents suggests that there is also some non-plasmid, chromosomal gene involvement. Again these could include changes that increase the strength of CUP1 promoter or increase plasmid copy number to increase DHFR production. Further analysis of these possibilities is beyond the scope of the present study.

3.4. Implications

The work described here shows that over-production of DHFR causes resistance to sulfa-DHP in a yeast model system. Formation of sulfa-DHP has been described previously, however the mechanism of action of sulfa-DHP and its role in the folate pathway has remained of interest. Studies in yeast have shown that sulfa-DHP formed in vivo was inhibitory and showed competition with DHF suggesting DHFR to be the likely target [8]. This study has now successfully demonstrated that over-production of DHFR causes resistance to the sulfa-DHP.

This study has not determined whether DHFR binds sulfa-DHP directly: over-production of DHFR could simply have a protective role against some other toxic effect of sulfa-DHP. Similarly, we have not established whether there could be another target of sulfa-DHP. For example, DHFS remains as an obvious, possibly more important target.

Finally, the finding that DHFR affects resistance to sulfa-DHP has implications for chemotherapies that include sulfa drugs. While there has been substantial investigation of DHPS sequence changes leading to sulfa drug resistance it may now be more appropriate to consider changes to the levels of DHFR as also being important.

References


