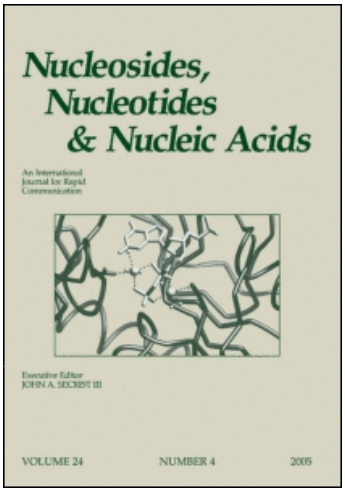

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Dihydroorotate Dehydrogenase mRNA and Protein Expression Analysis in Normal and Drug-Resistant Cells

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ABSTRACT

To follow the expression of the fourth enzyme of pyrimidine de novo synthesis dihydroorotate dehydrogenase (DHODH) in cells and tissues, we studied the DHODH mRNA expression by means of RT-PCR in rat tissues. Rabbit polyclonal anti-DHODH immunoglobulins were applied for immunochemical quantification of the enzyme protein by Western blotting. In mouse B-lymphocytes, which were adapted to tolerate up to a 50-fold concentration of the DHODH inhibitor leflunomide, a 20 fold protein overexpression was measured. Southern blotting indicated DHODH gene amplification.

Key Words: Dihydroorotate dehydrogenase; Drug-resistance; Gene amplification; mRNA; Pyrimidines.

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INTRODUCTION

Dihydroorotate dehydrogenase is located in the inner mitochondrial membrane and, via its electron acceptor ubiquinone, functionally coupled to the respiratory chain. The other enzymes of pyrimidine de novo synthesis are located in the cytosol. Since in contrast to those enzymes the expression and regulation of the gene encoding DHODH has not yet been investigated, we were specifically concerned to investigate DHODH mRNA expression in cells and tissues. Antibodies against DHODH have been successfully utilized for the purpose of protein identification throughout purification procedures and of enzyme localisation in cells.[1,2] It was the rationale of this study to apply such an immunological technique to reveal DHODH protein overexpression in a drug tolerating cell line in relation to the parental cells.

METHODS

The leflunomide tolerating cell line, A20R (IC_{50} 100 μM), was generated by gradually exposing the mouse B lymphocyte cell line A20N (IC_{50} 2 μM) to increasing drug concentrations over one year.[3] Cells were a gift from Dr. R. Bartlett and D. Thorn (Aventis, Germany). After harvesting, the cell pellets were transferred to Laemmli buffer and further processed for SDS-PAGE/Western Blotting using anti-DHODH IgG and peroxidase conjugated anti-rabbit IgG in combination with the enhanced chemiluminescence detection kit (Amersham).[2,4] 10 μg protein of each cell pellet was taken for the analysis. The content of DHODH was deduced from a calibration curve obtained with purified recombinant human DHODH protein (truncated version of 36 kDa versus 44 kDa of the mouse enzyme) as standard (0.5, 10, 15, 20, 25 ng per lane) on the same blot (HP DeskScanII and Sigma Scan). Southern blotting was performed for detection of genomic DNA (standard techniques). 3 μg DNA from A20N and A20R cells were digested, separated on agarose gels and transferred to QIAbrane Nylon Plus. Hybridization was with a DIG labeled 535 bp DNA probe (332–866) obtained from PCR with the truncated DHODH sequence in pFL61.[1] To show the DHODH mRNA, total RNA of rat testis was prepared and purified using deoxyribonuclease. Reverse transcriptase-PCR was performed according to Lee.[5,6] A partial DHODH sequence of 319 bp (755–1073) was amplified.

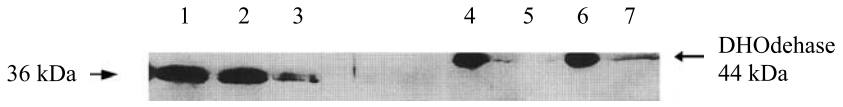

Figure 1. Western blot of DHODH protein expression in cells. Lane 1–3: 15 ng; 10 ng; 5 ng of recombinant DHODH protein (truncated) for standardization. Lane 5, 7: 610 μg total cell protein in parental B lymphocytes (A20N). Lane 4, 6: 10 μg total cell protein in drug tolerating cells (A20R).

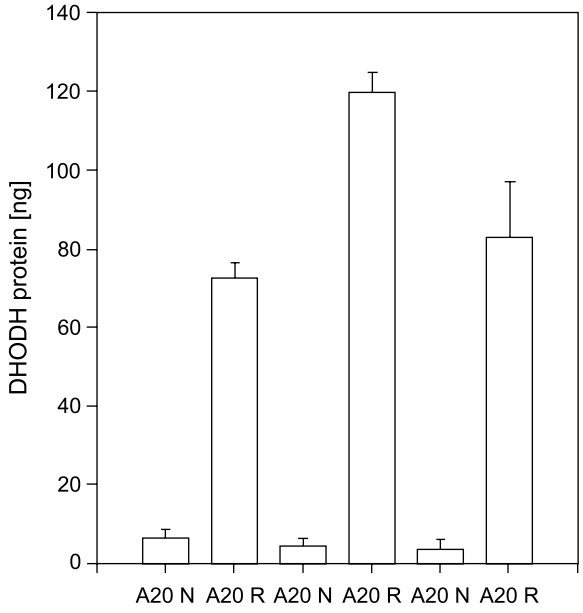

Figure 2. Evaluation of DHODH content. Semiquantitative determination from Westerns blots of A20N and A20R cells with recombinant DHODH as standard protein. Three different samples each.

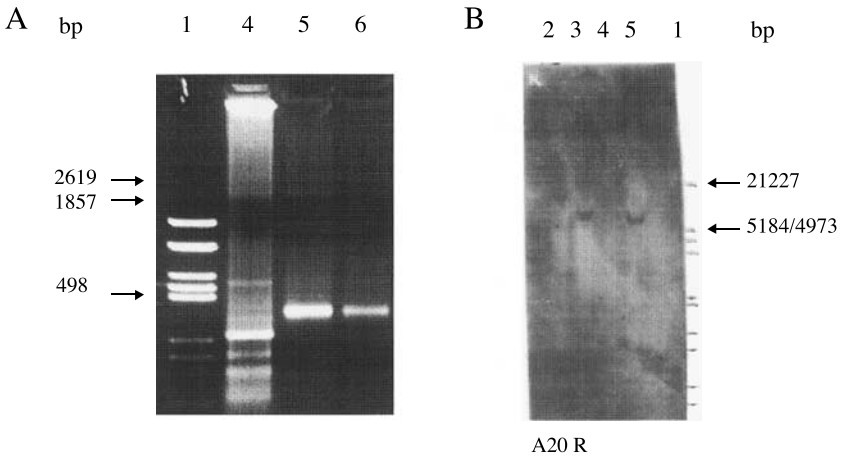

Figure 3. Genomic DHODH in A20R cells. A. DNA fragments after PCR. Lane 1: DNA standards of different length. Lane 4: unlabeled control fragment. Lane 5: 535 bp DIG-labelled probe. Lane 6: after treatment with Nucleotide Removal Kit. B. Southern blot with 535 bp probe. Lane 1: standards. Lane 2: following *Hin*dIII. Lane 3: *Bam*HI. Lane 4: *Sal*I. Lane 5: *Eco*RI.

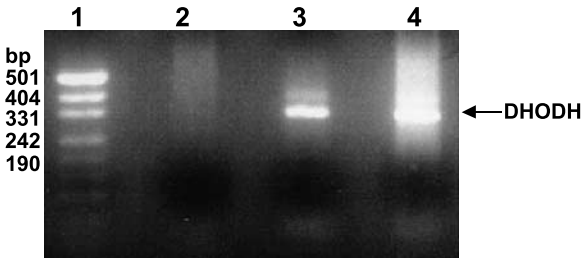

Figure 4. DHODH mRNA in rat testis RNA. Lane 1: marker. Lane 2: negative control Lane 3: 319 bp product in total RNA after removal of genomic DNA. Lane 4: 319 bp product without removal of DNA.

RESULTS AND DISCUSSION

A remarkable difference in the DHODH protein signal of A20N and A20R cells can be seen on Figure 1. The ratio of DHODH protein in A20N and A20R cells was 1:20, mean from three different samples (Figure 2).

It can be concluded that the 40–50 fold higher tolerance to leflunomide in A20R cells is considerably based on a higher expression of the drug target enzyme DHODH to bypass the inhibitory effect. The Southern blot shown in Figure 3B (lane 3 and 5) revealed specific signals for genomic DNA in A20R cells. Since these were not detected in the DNA blots of A20N cells (data not shown), DHODH gene amplification in A20R cells can be assumed following long-time drug exposure.

Other cell lines have been observed to develop drug-resistance to a much greater degree when exposed to transition-state analogues of key enzymes, such as PALA in the case of aspartate transcarbamoylase of the CAD enzyme. Therefore, it is not unreasonable to assume that the overexpression and proper location of an integral membrane protein could happen to a limited extent only. Thus DHODH rather than a cytosolic enzyme of pyrimidine biosynthesis would be a preferential target for drug development in view of putative therapy resistance. This should also be taken into consideration for the application of anti-pyrimidine agents with other species, e.g., insects, plants, fungi and parasites.

Figure 4 illustrates a 319 bp RT-PCR product of DHODH in rat testis.

In conclusion, the methods necessary for the detection of DHODH protein, mRNA and DNA are available now and can be utilized in forthcoming studies on gene and protein expression of the fourth enzyme of pyrimidine biosynthesis.

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