Inhibition of Dihydrofolate Reductase by Mofebutazon

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Mofebutazon, in contrast to phenylbutazon, inhibits dihydrofolate reductase in a concentration-dependent manner. An apparent K_i for mofebutazon and dihydrofolate reductase in the presence of NADPH as electron donor and dihydrofolate as electron acceptor of approximately 0.2 mM was calculated.

Introduction

Dihydrofolate reductase plays an important role in cellular metabolism by regenerating tetrahydrofolate which, in turn, transfers C-1 units necessary for the biosynthesis of purines, pyrimidines and certain essential amino acids such as methionine. It is understandable therefore, that, after inhibition of this enzyme cytostatic and thus antibacterial effects are observed [1, 2].

During enzyme catalysis, the transfer of a hydrid ion in the ternary dihydrofolate reductase-NADPH-dihydrofolate complex is preceded by the protonation of N-5 of DHF by glutamate-30 of the enzyme [3]. Methotrexate is the best known inhibitory DHF analogon. The pteridine ring system of methotrexate (N-(4-[[(2,4-diamino-6-pteridinyl-)-methyl-]methylamino-]benzoyl-)-L-glutamic acid) binding to the active centre of the enzyme, is surrounded by a hydrophobic protein moiety where the N-1 of the coenzyme is connected with a hydrogen bridge with Glu-30 of the active site [1]. Another potent inhibitor of this enzyme, methylbenzoprim, also forms hydrogen bonds between the diamino pyrimidine ring system and Glu-30, Thr-136, Ile-7 and Val-112 in the DHFR centre where both benzene rings of the inhibitor fit into the active site pockets of the enzyme [4].

Baggot *et al.* [2] recently showed that several non-steroidal antiinflammatory drugs (NSAIDs) significantly inhibited DHFR thus possibly explaining part of their biological, antiinflammatory effects. Under the compounds tested, phenylbutazone (PB) in contrast to compounds such as indomethazine (1-(4-chlorobenzoyl-)-5-methoxy-2-methyl-1 H-indole-3-acetic acid) or sulindac (5-fluoro-2-methyl-1-[[4-(methylsulfinyl-)-phenyl-]methylene-]-1 H-indene-3-acetic acid) showed no effect on the enzyme. Mofebutazon (MB) is structurally related to PB but contains only one benzene ring. Thus, one nitrogen of the pyrazole ring is unsubstituted opening a reactive N–H moiety in the neighbourhood of an electron withdrawing keto group (Fig. 1).

In this communication we report that MB inhibits DHFR whereas PB is inactive, as already reported by Baggot *et al.* [2].

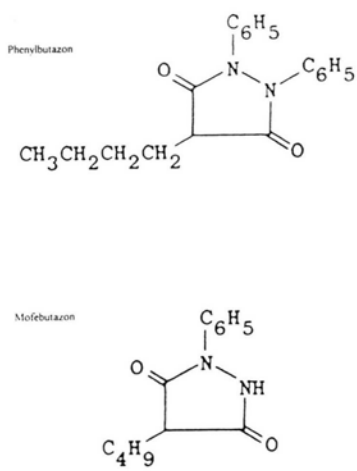

Fig. 1. Structures of phenylbutazon and mofebutazon.

Materials and Methods

Dihydrofolate, dihydrofolate reductase (EC 1.5.1.3) and NADPH were obtained from Sigma (Deisenhofen); all other chemicals were from Merck, Darmstadt.

Enzymic activity of DHFR was determined either spectrophotometrically by following NADPH oxidation at 340 nm or, in the case of testing strongly absorbing substances in the 340 nm region such as

Abbreviations: MB, mofebutazon; PB, phenylbutazon; DHF, dihydrofolate; DHFR, dihydrofolate reductase.

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PB, by the "Bratton-Marshall" reaction [5] *via* the reaction of the formed tetrahydrofolate (THF). Shortly, enzyme-catalyzed THF formation is stopped by H_2SO_4 and NaCl followed by the reaction of the acid-derived *p*-amino benzoylglutamate (pABG) with sodium nitrite, ammoniumsulfamate and *a*-naphthylethylene diamine. The extinction of the resulting red colour is proportional to the formed THF and can be determined at 540 nm [2]. The absorption coefficient E_{540} of the formed colour is 4.69×10^{-4} cm²/mol [5].

The reaction mixture of the test system contained in 1 ml: 25 mM phosphate buffer, pH 7.4; 100 μM NADPH; 0.01 U DHFR; 20–50 μM DHF and substance to be tested (MB or PB) in different concentrations. After preincubation of the test mixture for 10 min, the reaction was started by the addition of DHF. The reaction (4 h at 37 °C in the dark) was stopped by the addition of 0.6 ml of 5 M sulfuric acid and 1.1 ml of 0.3 M NaCl.

After transfer to the ice bath, 0.2 ml (0.1%) $NaNO_2$, 0.2 ml (0.5%) ammoniumsulfamate and 0.2 ml (0.1%) *a*-naphthylethylene diamine were added. After vigorous mixing, the reaction mixture was incubated for 75 min at room temperature in the dark. The extinction of the solution at 540 nm is compared to a corresponding calibration curve, where the extinctions obtained in the absence of a potential inhibitor are set as 100% reaction. All reactions were carried out at least in triplicate and were repeated with different concentrations of DHF (20, 30, 40 and 50 μM).

The K_i values were calculated according to the formula:

$$K_i = (E)(I) : (EI),$$

(E), representing the concentration of free enzyme; (I), the concentration of the inhibitor; (EI), the enzyme-inhibitor complex.

Furthermore, it was assumed that:

$$(EI) = (E_o) - (E_f),$$

where E_o represents the sum of enzyme in the reaction mixture, and E_f the concentration of the enzyme at the equivalent point. Thus, K_i is calculated by the equation:

$$K_i = (E_f)^2 : (EI).$$

The concentration of E_f was evaluated by enzyme titration according to Eisenthal and Cornish-Bowden [6].

Results and Discussion

As shown in Fig. 2, PB has no influence on DHFR activity whereas MB clearly inhibits the enzyme activity. 50% inhibition are obtained at approximately 1 mM MB.

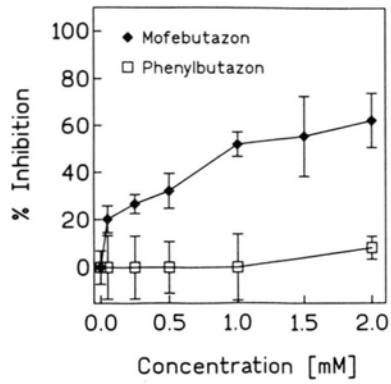

Fig. 2. Inhibition of dihydrofolate reductase by Mofebutazon.

With an other enzyme batch, maximal inhibition of 30–40% by 2 mM MB in the presence of 50 μM DHF were obtained where 2 mM PB also exhibited no effect (data not shown). From the deviation points of observed and theoretical inhibition curves using two different enzyme batches and DHF concentrations between 20 and 50 μM, K_i values of 0.20 (±) 0,003 mM and 0.261 (±) 0.07 mM were calculated.

MB is an antiinflammatory and antiphlogistic compound exhibiting similar activities as PB but lacking its undesired side effects. The pharmacological half lifetimes are also different: the half life in plasma for phenylbutazon is 78 h, that of MB less than 2 h [7].

The fact that MB is an inhibitor of DHFR and PB is without effect may be due to different chemical features of the compounds: the difference in the benzene substituents and/or the presence of a reactive, cyclic R–CO–NH–X group in MB. Therefore two independent effects may be brought about by these structural differences: a) the bulky second benzene substituent of PB may prevent localization and/or formation of hydrogen bonds [1] in the active centre and b) the reactive NH group may undergo such hydrogen bonds or interfere with the radical state of intermediary semiquinone structures of either the isoalloxazine moiety or the flavopro-

tein enzyme, DHFR, and/or its closely related reductant, the reduced pteridine. Investigations on this subject are currently underway in order to define further biochemical differences due to the individual structures of MB and PB.

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