The relation between inhibition of cell growth and of dihydroorotic acid dehydrogenase by Brequinar Sodium

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Summary

The growth inhibitory effects of Brequinar Sodium (DUP-785; NSC 368390) in 7 different cell lines were related to growth rates and to the inhibition of dihydroorotic acid dehydrogenase (DHO-DH) activity. IC₅₀ values were between 0.2 and 5.8 μ M; the fastest growing cell line was least sensitive. Despite a large variation in sensitivity, basal activity of DHO-DH showed little variation (only 2-fold) between the different cell lines. Residual activity of DHO-DH in the presence of Brequinar Sodium varied 30-fold. Drug sensitivity correlated with this residual DHO-DH activity; DHO-DH activity was only slightly inhibited by Brequinar Sodium in the most resistant lines, and almost completely in the most sensitive.

Keywords: pyrimidine de novo; dihydroorotic acid dehydrogenase; Brequinar Sodium (DUP-785); growth inhibition.

Introduction

DHO-DH is the fourth enzyme in de novo pyrimidine nucleotide synthesis, catalyzing the

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oxidation of L-dihydroorotic acid (L-DHO) to orotate. Unlike the other enzymes involved in this pathway, DHO-DH is located on the outer side of the inner mitochondrial membrane [2,7,13]. Brequinar Sodium is a potent inhibitor of de novo pyrimidine biosynthesis, by inhibition of DHO-DH [3,18]. The apparent K_i vs DHO is reported at 10–100 nM, and the mode of enzyme inhibition was described as linear mixed type [18].

In contrast with several other inhibitors of de novo pyrimidine biosynthesis, the structure of Brequinar Sodium does not resemble that of the substrate, the product or the co-factor of the reaction catalyzed by DHO-DH. N-phosphonacetyl-L-aspartate (PALA) for example, a potent inhibitor of aspartate transcarbamylase (ATC) [9,12], has structural similarity with aspartate [9]. As far as is known, Brequinar Sodium is not converted by any enzyme involved in pyrimidine or purine biosynthesis into an active compound, as is the case with e.g. pyrazofurin. This nucleoside analogue is phosphorylated to the monophosphate derivative which inhibits the orotate phosphoribosyl transferase-OMP decarboxylase complex [1].

As was shown in reversal studies with pyrimidine nucleosides, a block in the formation of UMP plays an important role in the in vitro effects of Brequinar Sodium [19]. However,

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neither the exact role of the target enzyme of Breqinar Sodium in the pyrimidine nucleotide synthesis nor the action of Breqinar Sodium towards DHO-DH are completely understood. Therefore, we studied the activity and the inhibition of DHO-DH by Breqinar Sodium in a panel of cell lines and correlated the findings with sensitivity to Breqinar Sodium.

Materials and methods

Breqinar Sodium was synthesized and obtained from the Medicinal Chemistry Section, DuPont Pharmaceuticals, Wilmington, Delaware, U.S.A. Orotic acid and L-DHO were from Sigma, Ohio. All other chemicals used were of standard analytical grade. The sources of the cell lines tested are in parentheses or have been described previously [16,17]. Suspension cultures of murine L1210 leukemia cells were routinely grown in RPMI-1640 medium. The monolayer cell lines rat hepatoma H35, the transformed human intestine cell line Intestine 407, and the human cell lines, WiDr colon carcinoma, M5 melanoma, 14C squamous cell carcinoma (University of Michigan, T.E. Carey) and MCF7 breast carcinoma (Michigan Cancer foundation, K. Cowan) were routinely cultured in Dulbecco's Medium. Both types of media were supplemented with 10% heat-inactivated non-dialyzed fetal calf serum, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) [16,18,19]. All cultures were maintained in logarithmic growth. Growth inhibition studies were performed in 6-well cluster plates [16], using a Sysmex electronic microcell counter (TOA Medical Electronics Co., Ltd, Kobe, Japan) to enumerate treated and control cells at the time of addition, and after 48-h exposure to Breqinar Sodium.

For DHO-DH assays, cells in the logarithmic growth phase were harvested 2 days after transfer, counted, spun down and cell pellets were stored at -70°C . Enzyme activity was measured in total cell extracts after suspension of the pellet in 0.1 M Tris-HCl, pH 8.0 ($1-2 \times 10^6$ cells/ml) and subsequent sonication as

described [16,17]. In a total reaction volume of 500 μl , the enzyme was assayed at a final concentration of 158 μM L-DHO at 37°C . After 10–20 min the reaction was terminated by addition of 50 μl 40% ice-cold trichloroacetic acid. The amount of orotic acid was determined after neutralization [14] using a sensitive HPLC method [17].

Results

Growth inhibition by Breqinar Sodium on 7 cell lines was estimated after 48 h continuous exposure (Fig. 1). In Table 1 cell lines are ranked in order of sensitivity. The most sensitive and resistant cell lines, 14C and L1210, respectively, displayed approximately a 30-fold difference in IC_{50} , and a 3-fold difference in growth rate. Four cell lines with a comparable growth rate displayed a rather small variation in sensitivity from 0.32 to 0.45 μM (Table 1).

DHO-DH assays were linear with respect to the amount of protein and time up to at least 20 minutes at optimal substrate concentrations. Inhibition of DHO-DH was determined at 1.3 μM Breqinar Sodium, a concentration which is present in plasma of patients treated with Breqinar Sodium for several days [20]. Under these conditions values were not below detection limit [17,18] and a marked decline in DHO-DH activity was observed in most cell lines (Table 1). Basal enzyme activity in 14C was approximately 2 times lower than in most other cell lines. However, a considerable variation in the extent of inhibition by Breqinar Sodium was observed. A relatively high residual activity was found in H35 and L1210, while 14C, the most sensitive line, had a very low residual activity.

Discussion

In a study of a series of different cell lines, our findings suggest a correlation between growth inhibition by Breqinar Sodium and the residual activity of the target enzyme DHO-DH in the presence of the drug. The role of

Fig. 1. Growth inhibitory effect of Brequinar Sodium on 7 cell lines. One curve for each cell line representing the mean of 3–4 separate experiments is shown. S.E. was less than 20%. Values of M5 and L1210 are from previous studies [5]. I407, Intestine 407.

inhibition of DHO-DH in growth-inhibition effects, had not yet been studied.

DHO-DH activity as a determinant for effectiveness of Brequinar Sodium might only be important at very low or high enzyme activities. Due to a relatively small variation in activity (2-fold) in this study, the possibility that a very high activity would confer increased resistance, could not be examined. By

contrast, for PALA, another antiprimidine drug, a correlation between activity of the target enzyme, ATC, and sensitivity was established [6,10]. Moreover, mutant resistant cells appeared to have increased enzyme activities [8,9]. Elevated levels of target enzyme activity were also observed in cell lines with induced resistance against pyrazofurin [22].

Sensitivity to Brequinar Sodium correlated

Table 1. Growth rates of 7 cell lines; inhibition of growth and of DHO-DH by Brequinar Sodium.

Cell line	Doubling time (h)	IC ₅₀ (μM)	DHO-DH activity	
			Control	+ DUP-785
14C	39 ± 1	0.19 ± 0.07	7.6 ± 0.5	0.2 ± 0.1
WiDr	27 ± 2	0.32 ± 0.06	17.9 ± 2.5	2.2 ± 0.5
M5	23 ± 1	0.39 ± 0.06	10.0; 8.2	0.5; 0.35
MCF7	23 ± 2	0.42 ± 0.09	16.5 ± 2.7	0.4 ± 0.1
I407	27 ± 2	0.45 ± 0.09	16.1 ± 2.1	2.0 ± 1.1
H35	19 ± 1	2.53 ± 0.29	15.0 ± 1.4	7.1 ± 1.9
L1210	13 ± 1	5.81 ± 2.10	16.3 ± 1.2	8.6 ± 1.9

IC₅₀ values (concentrations that cause 50% growth inhibition) were calculated from the separate experiments. Enzyme activity is given as nmol/h per 10⁶ cells. All values (unless otherwise indicated) are shown as means ± S.E. of 3–5 separate experiments. Inhibition of DHO-DH was assayed at 1.3 μM Brequinar Sodium (DUP-785).

better with the extent of DHO-DH inhibition by Brequinar Sodium. Possibly, the difference in levels of residual uninhibited activity is an aspect of the interaction between DHO-DH and Brequinar Sodium in cell lines with comparable basal activities. To date, neither the precise interaction between Brequinar Sodium and its target enzyme, nor the significance of DHO-DH in cells, are clear. During hepatocarcinogenesis, DHO-DH activity declined [4] while mutant deficient cells did not show a requirement for exogenous uridine [21]. In L1210, we demonstrated that the activity of ATC was several times higher than that of DHO-DH [10], while the activity of the following enzyme in *de novo* pyrimidine synthesis, orotate phosphoribosyl transferase, was somewhat lower than that of DHO-DH [15] in the tested lines. DHO-DH might be involved in superoxide formation [5], while reduced activity under hypoxic conditions has been observed [11]. In tumors which might have a limited oxygen supply, DHO-DH might be rate-limiting in *de novo* pyrimidine synthesis. Unlike other anti-metabolites, the target enzyme of Brequinar Sodium is located in the mitochondria, thus requiring passage of the drug through the plasma and mitochondrial membranes in order to be active. Retention of Brequinar Sodium in the mitochondrion is most likely a determinant of growth inhibitory effects.

In our growth inhibition studies, we observed another biological parameter which might be related to sensitivity to Brequinar Sodium; the rate of cell proliferation correlated with the degree of sensitivity to Brequinar Sodium. Cell lines with high growth rates are less sensitive. Although not as clearly as in the present study, this phenomenon was previously reported for PALA [6,10]. Cells continuously exposed to Brequinar Sodium showed accumulation in the S-phase [19], which appeared to be higher in WiDr than in the most resistant and fastest growing line L1210. Sensitivity might be related to the capability of cells to escape from the S-phase block in the cell-cycle, caused by the drug.

In conclusion, our results demonstrate that the sensitivity to Brequinar Sodium *in vitro* is not directly related to the activity of DHO-DH. However, the residual DHO-DH activity in the presence of Brequinar Sodium showed a good correlation with its growth inhibitory effects. The results might be of importance for the clinical trials which are currently ongoing with this new drug [20].

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