THE RELATIONSHIP BETWEEN DIHYDROOROTIC ACID DEHYDROGENASE AND *IN VITRO* AND *IN VIVO* CYTOSTATIC EFFECTS OF BREQUINAR SODIUM (DUP-785; NSC 368390)

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INTRODUCTION

Dihydroorotic acid dehydrogenase (DHO-DH) is a critical enzyme in the *de novo* pyrimidine nucleotide synthesis (Fig. 1). It is the fourth enzyme in this pathway, and is located on the outer site of the inner membrane of the mitochondrion [1,2]. Brequinar Sodium (BS; DUP-785; NSC 368390) is a novel potent inhibitor of DHO-DH [3,4] with a Ki varying between 10 and 100 nM. Treatment of cells led to an accumulation in the S phase [4]. The growth-inhibitory effects of BS could be prevented and reversed by addition of uridine or cytidine to the cell culture medium [4,5], but not by thymidine or deoxycytidine. So, a selective depletion of one of the pyrimidine deoxynucleotides is not likely to be responsible for the growth-inhibitory effects of BS. Depletion of pyrimidine deoxyribonucleotides apppeared to be proportional to that of the ribonucleotides [5], and could be reversed by uridine. Inhibition of UMP synthesis appears to be crucial for the *in vitro* action of BS. To get more insight in the role of DHO-DH in the synthesis of pyrimidine nucleotides and in the action of BS we measured the activity of DHO-DH and the inhibition by BS in several cell lines from different histological origin and determined their sensitivity to BS.

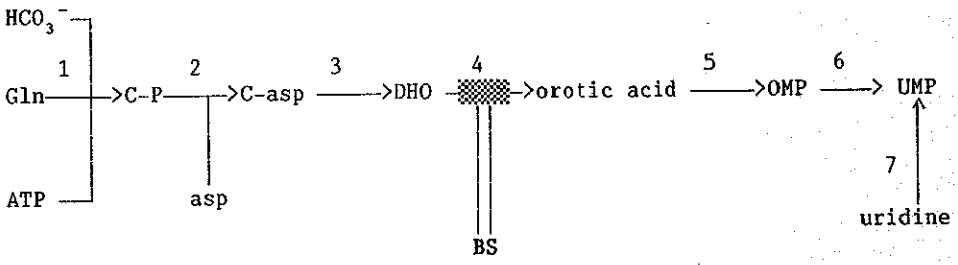

Fig. 1: Pyrimidine *de novo* nucleotide synthesis. The enzymes are; 1, carbamyl-P synthetase II; 2, aspartate transcarbamylase (ATC); 3, DHOase; 4, DHO-DH; 5, orotate phosphoribosyltransferase (OPRT); 6, OMP decarboxylase; 7, uridine kinase. Gln, glutamine; C-P, carbamyl-P; asp, aspartate; C-asp, carbamyl-asp; DHO, dihydroorotic acid. The bar represents inhibition by BS.

In vitro we demonstrated that a short term exposure of cells to BS followed by culture in drug-free medium did not affect growth rate of the cells [4,5]. This appeared to be related to the retention of inhibition of DHO-DH [4] and depletion of pyrimidine nucleotides [5]. A prolonged presence of BS was essential to maintain both enzyme inhibition and nucleotide depletion. In vitro it is easily possible to manipulate the levels of possible rescue compounds in the medium, e.g. by the use of dialyzed serum. Cell lines cultured in dialyzed serum were more sensitive to BS than when cultured in non-dialyzed serum [4]. In vivo uridine is present at relatively high concentrations in tissues, compared to e.g. plasma [6,7], which might affect the antitumor activity of BS. In order to study the in vivo antipyrimidine effects of BS we used two murine colon tumors as models in which we measured the retention of the inhibition of DHO-DH and the effect of BS on nucleotide pools.

MATERIALS AND METHODS

BS was synthesized and obtained from the Medicinal Chemistry Section, DuPont Pharmaceuticals, Wilmington, Delaware, USA. Pyrimidine compounds were obtained from Sigma, Ohio, while other compounds were of analytical grade quality. The origin of cell culture media and sera has been described previously [4,5,8]. Female C57Bl/6 and Balb-c mice were obtained from the animal breeding station "Proefdieren-bedrijf TNO", Zeist, the Netherlands and were kept in an area with standardized light-dark cycle for at least 10-14 days prior to the beginning of an experiment. Mice had excess to food and water ad libitum. The sources, growth characteristics and evaluation of antitumor activity of Colon 26 and Colon 38 (both murine colon adenocarcinomas) have been described previously [9, 10]. Colon 26 was maintained in Balb-c and Colon 38 in C57Bl/6 mice, both subcutaneously in the thoracic region.

Cell lines were maintained as described previously in 10% heat-inactivated non-dialyzed fetal bovine serum [4,5,8]. Growth inhibition was determined by comparison of cell number at the start and the end of culture. Only cells in logarithmic phase were used for enzyme assays. Cells were harvested, counted, centrifuged and frozen at -70°C. The cell pellets were suspended in 0.1 M Tris-HCl (pH 8.0) and lysed by sonication, then DHO-DH was assayed as described using a sensitive HPLC method [12]. The enzyme was assayed at 158 μM L-DHO and inhibition was measured at 1.3 μM BS. The antitumor activity of BS was determined by i.p. injec-

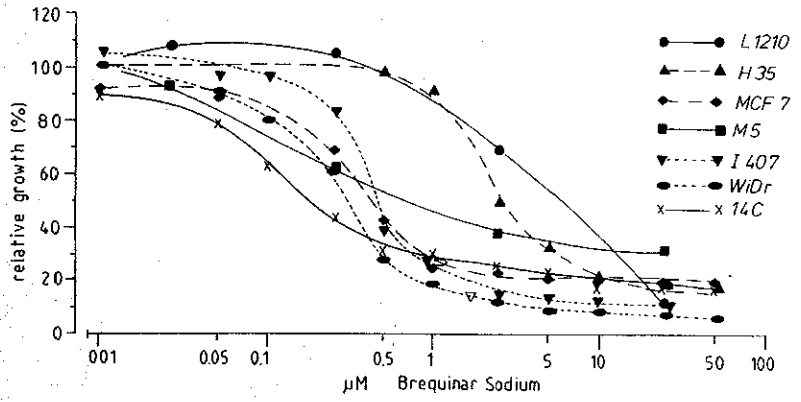

Fig. 2. Growth-inhibition of 7 cell lines by BS. Cells were continuously exposed to BS for 48 hr and relative growth was determined as described previously [7]. Mean values of 3-4 separate experiments are shown. Values of M5 and L1210 are from [4].

tion of BS; mouse weight and tumor size was measured twice weekly; tumor size and doubling times were calculated relative to that of day 0 and used for evaluation of antitumor effect by means of the growth delay factor (GDF) as described [9,10].

The effects of BS on the activity of DHO-DH and the levels of pyrimidine nucleotides were determined after a single i.p. injection of BS at 50 mg/kg. Mice were killed by cervical dislocation and tumors were removed immediately. Mitochondria were prepared [11] and used for the measurement of DHO-DH essentially as described above for cell lines. Other tumors were immediately frozen in liquid nitrogen and used for the measurement of pyrimidine nucleotides with anion-exchange HPLC as described [6] after pulverization using a microdismembrator [6,12].

RESULTS

In vitro sensitivity and DHO-DH activity

Seven cell lines from different histological origin were tested for their sensitivity to BS (Fig. 2). Cells were continuously exposed to BS, since we had demonstrated previously that at short term exposure the growth-inhibition was not retained [4,5]. 14C was the most sensitive and L1210 and H35 were the most resistant cell lines with a more than 14-fold difference in sensitivity. However, in none of the cell lines a complete growth-inhibition was observed at higher concentration, even at 50 μM BS some cell growth was observed. A relationship was observed between growth rate and sensitivity. The most sensitive line, 14C, was also the most slowly growing line, while the most resistant line, L1210, was the fastest growing cell line (Fig. 3).

DHO-DH activity was measured at a saturating substrate concentration and the inhibition of 1.3 μM BS was determined. The activity was lowest in 14C, intermediate in M5, and about 2-times higher in the other cell lines. The extent of inhibition by BS varied considerably among the tested cell lines. The inhibition was highest in 14C and MCF7 cells, intermediate in WiDr and Intestine 407, and moderate in H35 and L1210 cells. It appeared that the most sensitive cell line also displayed the lowest activity of DHO-DH. However, the other cell lines had a comparable activity although there was more than ten-fold difference in sensitivity. A good correlation was observed between sensitivity to BS and the remaining DHO-DH activity in the presence of BS. For 14C the highest enzyme inhibition was observed, while H35 and L1210 showed a slight inhibition.

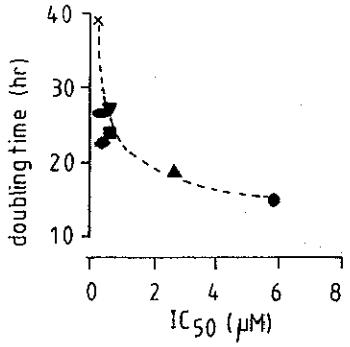

Fig. 3. Relationship between growth rate of the cell lines and sensitivity to BS. Symbols are the same as in Fig. 2.

Table 1. IC50 FOR BS AND ACTIVITY OF DHO-DH IN CELL LINES.

Cell line	Origin	IC50 (μM)	DHO-DH activity	
			− BS	+ BS
14C	squamous cell ca.	0.19 ± 0.07	7.6 ± 0.5	0.2 ± 0.1
WiDr	colon ca.	0.32 ± 0.06	17.9 ± 2.5	2.2 ± 0.5
M5	melanoma	0.39 ± 0.06	10.0; 8.2	0.5
MCF7	breast ca.	0.42 ± 0.09	16.5 ± 2.7	0.4 ± 0.1
I407	tr.epithelial intestine	0.45 ± 0.09	16.1 ± 2.1	2.0 ± 1.1
H35	rat hepatoma	2.53 ± 0.29	15.0 ± 1.4	7.1 ± 1.9
L1210	murine leukemia	5.81 ± 2.10	16.3 ± 1.2	9.5

Enzyme activity is given as nmol/hr per 10^6 cells. Values are means ± SE of 3-5 separate experiements. Inhibition of DHO-DH was determined in the presence of 1.3 μM BS. Ca., carcinoma; tr, transformed; I407, Intestine 407. All cell lines were from human origin, unless otherwise indicated.

Table 2. ANTITUMOR EFFECT OF BS ON COLON 26 AND COLON 38, IN RELATION TO DHO-DH ACTIVITY AND INHIBITION BY BS

Tumor	Doubling time (days)	GDF		DHO-DH activity	
		5xD	Dx5	control	+ 1.3 μM BS
Colon 26	2.7	0.04	0.69	551 ± 71	28.5 ± 3.4
Colon 38	5.1	0.62	2.12	123 ± 24	12.2 ± 3.8

BS was injected i.p. at 50 mg/kg at day 0, 4, 8 and 12 (5xD) or daily at 25 mg/kg at day 0-5 (Dx5); GDF < 1, insensitive; > 1, sensitive. DHO-DH activity (nmol/hr per mg mitochondrial protein) was determined in untreated tumors; values on enzyme activity are means ± SE of 4 tumors.

In vivo sensitivity and antipyrimidine effects of BS

The in vivo effects of BS were studied in two murine colon carcinomas. A better antitumor effect was observed at at daily administration in both tumor lines (Table 2). Colon 26 was not sensitive to BS and had a high basal activity of DHO-DH, while inhibition of DHO-DH was more than 80%. Colon 38 was more sensitive to BS than Colon 26 and showed a lower basal activity of DHO-DH. In these tumors we also studied the retention of antipyrimidine effects, i.e. retention of inhibition of DHO-DH and the effect of BS on pyrimidine nucleotide pools in the tumors. In both tumor lines a single injection of 50 mg BS/kg resulted in an almost complete inhibition of DHO-DH after 4 hr (Fig. 4), but was recovered considerably after 1 day in both tumor lines. In Colon 38 DHO-DH activity returned to basal levels after 4 days. In Colon 26 DHO-DH activity remained partially inhibited and did not return to pretreatment levels within 4 days, although the absolute activity in treated tumors was never lower than in treated Colon 38 at the same day.

The in vivo antipyrimidine effects of BS were also studied by measurement of the pyrimidine nucleotide levels at several time-points after one injection of BS. Only total pyrimidine nucleotides (UΣP and CΣP) were measured, by degradation of UTP, UDP and UDP-sugars to UMP and CTP, CDP and cytidine nucleotide sugars to CMP [6]. CΣP were comparable in Colon

26 and Colon 38 and amounted to about 200 pmol/g wet weight. BS had not effect on CEP in both tumor lines (data not shown). However, in Colon 38 BS caused a depletion of UEP after 1 day, followed by a rebound after 2 and 4 days (Fig. 5). In Colon 26 an increase in UEP was observed after 2 and 4 hr, after 1 day UEP levels decreased to about 80% of control levels followed by a rebound after 2 and 4 days.

DISCUSSION

Seven different cell lines from human and animal origin differed widely in their sensitivity to BS. It appeared that the activity of DHO-DH is not an important determinant of the sensitivity to BS. The most sensitive cell line had the lowest DHO-DH, while the other cell lines showed a higher enzyme activity, but still displayed a large variation in

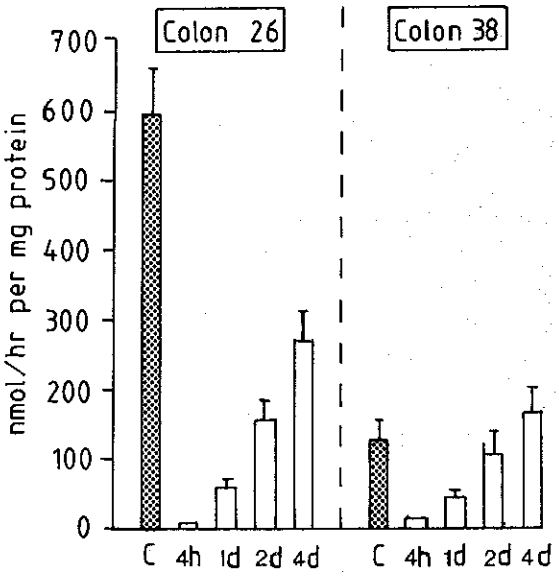

Fig. 4. Effect of one injection of BS at 50 mg/kg on the DHO-DH activity of Colon 26 and Colon 38 tumors. Values represent means ± SE of 3-7 tumors.

sensitivity. It might be that only very low or very high activities are determinants for sensitivity or resistance, respectively. However, the latter could not be investigated in the present study, because of the relatively low variation in DHO-DH activity. For another pyrimidine anti-metabolite, N-phosphonacetyl-L-aspartate (PALA) an inhibitor of ATC, a relationship between enzyme activity and sensitivity could be demonstrated [13,14]. Induced resistance against PALA appeared to be associated with increased enzyme activity [15,16]. A better correlation was observed between the activity of DHO-DH in the presence of BS and the sensitivity of these cell lines to BS. The most sensitive cell line, 14C, had the lowest remaining activity. Also in vivo the enzyme activity and its inhibition correlated with the antitumor activity, although only data of two tumor lines are presented. Another determinant for the sensitivity to BS appears to be the proliferation rate of the cells. This was also observed for the two tumor lines. A less clear relationship between proliferation rate and sensitivity was observed for the effect of PALA

[13,14]. We recently demonstrated that BS caused an accumulation of cells in the S-phase [5], but this block was less for the fast growing L1210 cells. So more cells might pass through the cycle and (partly) explain the lower sensitivity.

In cell lines treatment with BS resulted in a marked reduction of pyrimidine nucleotides [3,5]. Retention of depletion of UTP and CTP and of DHO-DH inhibition [4] appeared to be determinants of the extent and duration of growth-inhibition. Data shown in Fig. 5 suggest that in vivo nucleotide depletion is related to the antitumor effect of BS. In Colon 38, which is more sensitive than Colon 26, UΣP decreased. This decrease, however, was followed by a rebound in nucleotide pools. Also in Colon 26 the pyrimidine nucleotide pool was higher after 4 days. The effects of BS

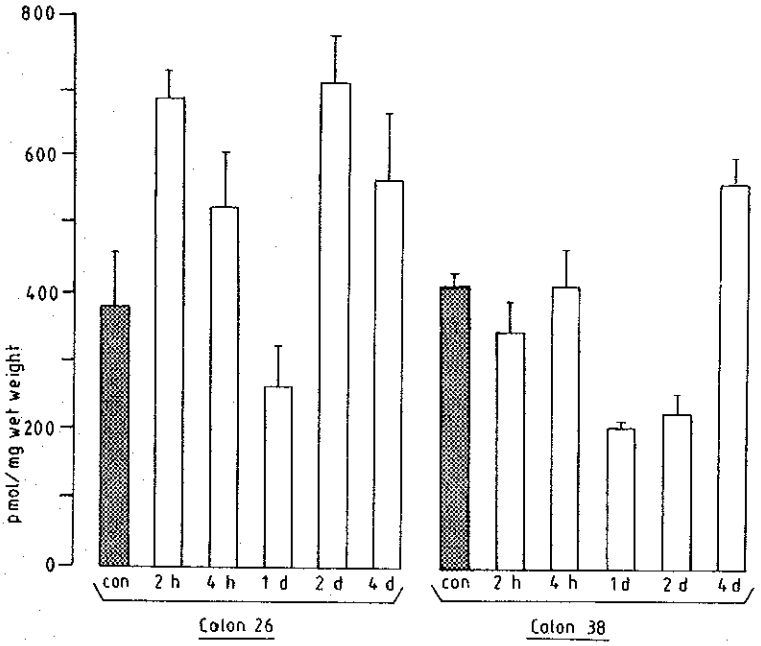

Fig. 5. Effect of BS on the levels of UΣP in Colon 26 and Colon 38. Values are means ± SE of 3-14 tumors.

are unlike to those observed with PALA [16-19] or pyrazofurin, an inhibitor of OPRT [20]. Extent of depletion, observed with BS was less, while the rebound was not observed with PALA. In most studies pools were only measured at 24 hr. However, we observed in B16 cells that both UTP and CTP remained depleted for 5 days [19]. Furthermore, in Lewis lung carcinoma one injection of PALA resulted in inhibition of ATC which was retained for 6 days in a sensitive line [16]. Retention of DHO-DH inhibition was only a few days in the murine tumors and parallelled the effects on UΣP. It should be noted that although the extent of inhibition in the Colon 26 was higher than in Colon 38, that the absolute DHO-DH activity in Colon 26 was never lower than in Colon 38. So, it might be concluded that also in vivo the absolute activity in the presence of or after BS is important. Recently we demonstrated that in lymphocytes of patients trea-

ted with BS that DHO-DH was inhibited for up to one week [21]. The extent of antipyrimidine effects appeared to be related to toxicity.

Sensitivity of cells and tumors to BS might also be influenced by other factors than activity of DHO-DH. High pools of intracellular or intratumoral salvage precursors and/or an effective salvage pathway might result in a bypass of pyrimidine de novo nucleotide inhibition. In cell lines the pool of precursors can be regulated by the use of dialyzed serum. However, in vivo it has been demonstrated that tissues can have high uridine pools [6,7]. In Colon 38 we measured a uridine pool of about 100 μmol/kg and in Colon 26 of about 10 μmol/kg, which are both higher than the plasma level. Although this might imply an effective rescue in Colon 38, this is apparently not the case, since this tumor is more sensitive than Colon 26. This might be related to the difference in uridine metabolizing enzymes. Elsewhere in these proceedings [22] we demonstrate that the activities of these enzymes in Colon 26 are several times higher than in Colon 38.

Despite the limited antipyrimidine effects (at least in comparison to PALA or pyrazofurin) BS shows a broad antitumor activity against both experimental tumors and human xenografts [23]. The role of DHO-DH in cellular function is not clear. The activity of DHO-DH in the M5 and WiDr is higher than that of OPRT [24]. During hepatocarcinogenesis DHO-DH is decreased in activity [25], while cells with defective DHO-DH did not require exogenous uridine for growth [26]. DHO-DH is an unique enzyme in pyrimidine pathway since it is located in the mitochondrion [1,2] and it is coupled to the electron transport chain. Thus, it has been demonstrated that under hypoxic conditions DHO-DH activity is decreased [27], while hypoxic conditions also appeared to be synergistic with another inhibitor of DHO-DH. Since most tumors are more or less hypoxic this might be an important determinant of the antitumor activity of BS. E.g. the Colon 38 is a very necrotic tumor and thus hypoxic. DHO-DH might represent an unique target for cancer chemotherapy.

In conclusion, the sensitivity to BS is related to the extent of DHO-DH inhibition in vitro. In vivo retention of DHO-DH inhibition paralleled the effects on UTP. However, the observed antipyrimidine effects might only be a marker but not a determinant of the effects of BS.

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