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THE "ANTI-PYRIMIDINE EFFECT" OF HYPOXIA AND BREQUINAR SODIUM (NSC 368390) IS OF CONSEQUENCE FOR TUMOR CELL GROWTH

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Abstract—The rationale of the present study was to investigate the simultaneous effect of hypoxia and drugs with an "anti-pyrimidine effect" on tumor cell proliferation to evaluate putative changes in the sensitivity of cells to these kinds of chemotherapeutic treatment on reduced O_2 tension. Pyrimidine *de novo* biosynthesis, at the stage of respiratory chain-dependent dihydroorotate dehydrogenase, was found to be a biochemical target site for oxygen deficiency as well as for Brequinar Sodium™ (6-fluoro-2-(2'-fluoro-1,1'-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt) (Brequinar). Increasing drug concentrations (0.1–50 μM) reduced the proliferation rate of *in vitro* cultured Ehrlich ascites tumor cells (IC_{50} = 0.25 μM). Decreasing concentrations of O_2 reduced the proliferation rate (50% at ~3.5% O_2). Brequinar at 2.5 and 12.5 μM stimulated the incorporation of exogenous [^{14}C]uridine into RNA to 140 and 190% of controls, respectively, as a result of active salvage pathways, whereas it decreased the incorporation of [^{14}C]$NaHCO_3$ by the *de novo* pathway (to 20 and 5% of controls, respectively). Cells routinely grown in glucose-free, uridine-supplemented medium were resistant to 12.5 μM of the drug. The complete growth pattern of the tumor cells (increase in cell number and protein, RNA and DNA content of cultures during a 24-hr culture period) was examined (i) on reducing the O_2 tension of the atmosphere stepwise from 20 to 1% O_2; (ii) on addition of 0.125 μM Brequinar; and (iii) under both conditions. The combination was found to give an additive inhibitory effect under moderate hypoxia (5–20% O_2) and a greater than additive effect if the oxygen tension was further reduced (1–5%).

Since hypoxic cells in solid tumors are thought to be an obstacle to effective cancer treatment by chemotherapy or radiotherapy, considerable effort has been expended in identifying their biochemical, physiological and rheological characteristics, which could be exploited in a therapeutic attack on such cells (for review see Refs 1–3). Although the enhancing effect of low oxygen tension on the cytotoxicity of diverse drugs has been established in a great number of studies, the underlying mechanisms seem to be different for each drug and have often not been completely identified [4–7]. Relatively little attention has been directed to hypoxia as a modulator of drugs interfering with RNA and DNA precursor metabolism. An interference with these biosynthetic pathways by oxygen deficiency at the stage of the respiration-dependent mitochondrial enzyme dihydroorotate dehydrogenase (DHO-DH†) (EC 1.3.3.1) and of the oxygen-dependent ribonucleotide reductase (EC 1.17.4.1) could entail some material restrictions of substrates necessary for the doubling of cell mass resulting in the cessation of growth and proliferation [8–11]. In the present study the individual growth-inhibitory effects of hypoxia and Brequinar on *in vitro* cultured tumor cells were examined and compared with the simultaneous influence of the two treatments. Brequinar is an antimetabolite that inhibits the pyrimidine *de novo* synthesis by acting at the level of DHO-DH with a resulting depletion of the pyrimidine precursor pools ("anti-pyrimidine effect") [12, 13]. The present studies involved incorporating labeled precursors into cellular RNA and flow cytometric cell cycle analysis of EAT cells. The results suggest the possibility of a synergistic effect in the sense that hypoxia could modify the effectiveness of "anti-pyrimidine" drugs.

MATERIALS AND METHODS

Materials. Chemicals, buffer and media components were obtained from either Bayer (Leverkusen, F.R.G.), Boehringer (Mannheim, F.R.G.), Merck (Darmstadt, F.R.G.), Serva (Heidelberg, F.R.G.) or the Sigma Chemical Co. (Deisenhofen, F.R.G.). Brequinar Sodium™ was from Du Pont Pharma GmbH (Bad Homburg, F.R.G.). 5-Aza-DHO was from a laboratory synthesis. Radiolabeled substances were from Amersham-Buchler (Braunschweig, F.R.G.).

Cell cultivation. Hyperdiploid EAT cells, serially passaged in female NMRI mice, were grown in suspension culture using Eagle's medium containing 10% horse serum, 10 mM Hepes buffer, Securopen™ Bayer (100 mg/L) and streptomycin (30 mg/L). For

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† Abbreviations: Brequinar, Brequinar Sodium™, 6-fluoro-2-(2'-fluoro-1,1'-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt; DHO, dihydroorotate; DHO-DH, dihydroorotate dehydrogenase, dihydroorotate-ubiquinone oxidoreductase; EAT, Ehrlich ascites tumor; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

in vitro culture the seeding density was approximately 2×10^5 cells/mL. Change of medium was every 24 hr. The cultures were gently agitated every hour. The culture experiments were performed in the second *in vitro* passage; the cell cultures studied in parallel were of the same origin. The viability of cells was assessed by phase-contrast microscopy and the dye-exclusion test. For some experiments cells were routinely grown in glucose-free, 2 mM uridine-supplemented culture medium. As described previously [14], their growth rates were usually 70% that of cells grown in 10 mM glucose-supplemented medium. For inhibitor studies, Brequinar was added to cell cultures at a final concentration of 0.125–50 μM. To maintain cells under hypoxic conditions the culture flasks were flushed continuously (60 mL/min) with a gas mixture of argon/5% CO_2 containing 1, 3, 5, 7 or 20% oxygen [Messer-Griesheim, F.R.G.]. All gases were humidified by bubbling through a water column at 37°. Oxygen concentrations in the protective atmosphere were controlled using an Orbisphere High Sensitivity O_2-Detector Model 2717 (Orbisphere Corporation, Switzerland) [15].

Determination of growth and metabolic parameters. Cell number was measured by an electronic cell counter (Sysmex CC 100, Digitana, Hamburg, F.R.G.). The relative increase in cell number during a 24-hr culture period was calculated and expressed as "growth (%)" = [(number of cells)$_{24}$ − (number of cells)$_0$]/(number of cells)$_0$ × 100. Flow cytometric analyses of cell cycle distribution were performed using the PAS II system [Partec, Münster, F.R.G.] equipped with an ACAS cytometry analysis system [Ahrens, Bargteheide, F.R.G.]. Processing of cell samples by fixation with 95% ethanol and staining with diamidino-2-phenylindole has been described in detail elsewhere [15]. The increase in the protein content of cultures was estimated after a 3-fold washing of aliquots of cells in 0.9% NaCl, employing the Folin–Lowry method with bovine serum albumin as standard. DNA content was determined in 0.5-mL aliquots, after the cells were washed, by measuring the fluorescence of the DNA-specific stain Hoechst 33258 (Jasco FP 770, Biotronik, Maintal, F.R.G.) with thymus DNA as standard. Spectrophotometric determination of total RNA content was performed with orcinol, after extraction of cells with hot perchloric acid and RNA as standard, as described in detail previously [11]. The calculation of the relative increases in protein, DNA and RNA content during a 24-hr culture period was the same as outlined already for the number of cells. For incorporation of preformed precursors into RNA: a 2-mL cell suspension was incubated for 15 min at 37° with 0.5 μCi [2-^{14}C]uridine (sp. act. 59 mCi/mmol); and a 10-mL cell suspension was incubated for 60 min with 5 μCi [^{14}C]$NaHCO_3$ (sp. act. 53 mCi/mmol). The extraction of total RNA was according to Crandall and Tremblay [16], which implies a modified Schmidt–Thannhauser procedure. After washing three times, the cells were precipitated in 0.6 N perchloric acid. The supernatant containing acid-soluble, labeled metabolites was removed; the pellet was washed twice with 0.6 N perchloric acid. The remaining precipitate of DNA, RNA and protein was then treated with 0.3 N KOH for 15 hr at room temperature. This procedure catalyses the cleavage of all phosphodiester bonds in RNAs and yields intermediate cyclic-2′,3′-phosphonucleosides that hydrolyse to nucleoside-2′-(or 3′-)phosphates. Re-acidification precipitated the alkali-stable DNA, proteins and lipids. Aliquots of the supernatant containing labelled nucleotides from total RNA (here named "RNA-fraction") were scintillation counted. No label was found in this fraction if cells in control experiments were incubated with [2-^{14}C]-thymidine as a marker for DNA. Only re-precipitated material of cells was found to be labeled by thymidine. Acid-insoluble material from unfractionated cell pellets contained labels from thymidine, uridine or HCO_3^-.

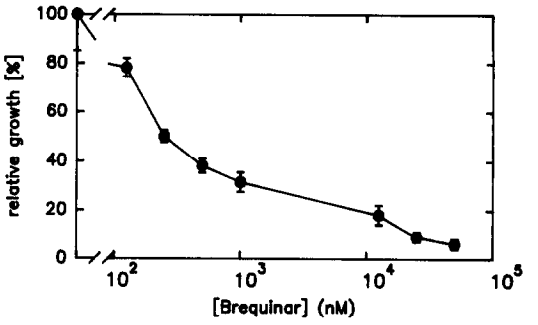

Fig. 1. Growth of EAT cells during a 24-hr culture period. The cells were exposed continuously to different concentrations of Brequinar. Relative growth (%) = values for increase in cell number expressed as percentages of untreated controls (100%). Values are means of results from six separate culture experiments, each treatment in duplicate.

RESULTS

Inhibition of cell proliferation by Brequinar

In the present set of culture experiments, the increase in cell number during a 24-hr period was in the range of 145 to 165%. Concentration-dependent inhibitory effects of Brequinar on cultured EAT cells are shown in Fig. 1. The IC_{50} was 0.25 μM. The addition of 25 μM resulted in about a 90% reduction of proliferation. Viability of cells, as checked by the dye-exclusion test, was not affected up to 12.5 μM Brequinar during a 24-hr culture period. After removal of the inhibitor, by a change of medium after 24 hr, EAT cells did not resume proliferation during an observation period of a further 24 hr. On the addition of higher concentrations of Brequinar (≥25 μM) the residual increase in cell number was found to be dependent upon the number of individual G_2 cells in the population. This could be explained by analysis of the cell cycle effect of Brequinar. Figure 2 shows the cell cycle distribution of EAT cells sampled before (DNA histogram A) and after 24 hr of 12.5 μM drug application (histogram B) in comparison with control cells (E). A characteristic retardation or even accumulation of cells at the G_1/early S stage can be seen in these histograms. G_2 cells were apparently less susceptible to the drug,

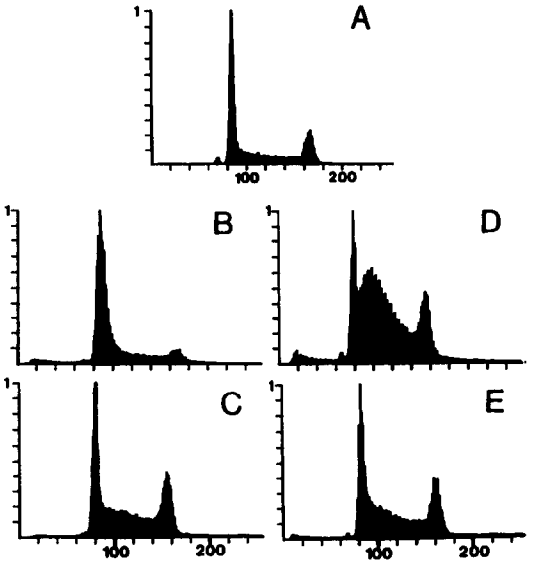

Fig. 2. Flow cytometric DNA distribution of EAT cells. Each histogram represents the typical cell cycle distribution at the indicated hour of cell culture. Abscissa: fluorescence intensity was equivalent to DNA content of cells. Ordinate: number of pulses per channel was equivalent to cell number (relative units). First peak, 2c DNA = G_1 cells; second peak, 4c DNA = G_2 cells; in between are the S cells. Fluorescence of early S cells can overlap with that of G_1 cells; fluorescence of late S cells can overlap with that of G_2 cells. (A) 0 hr; after 24 hr of treatment with (B) 12.5 μM Brequinar; (C) 12.5 μM Brequinar + 100 μM uridine; (D) 12.5 μM Brequinar + 100 μM deoxycytidine; (E) untreated controls.

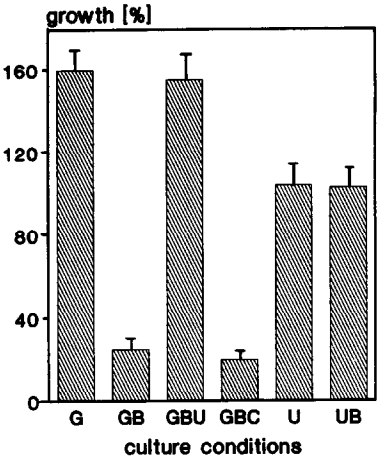

Fig. 3. Growth of EAT cells cultured in glucose-supplemented or glucose-free/uridine-supplemented medium. Cells were exposed continuously to 12.5 μM Brequinar for 24 hr. Growth (%) = [(number of cells)$_{24}$ − (number of cells)$_0$]/(number of cells)$_0$ × 100. Values are means of results from three separate culture experiments, each treatment in duplicate. G, Glucose-medium (10 mM) (gluc+); GB, gluc+ and Brequinar; GBU, gluc+ and Brequinar and 0.2 mM uridine; GBC, gluc+ and Brequinar and 0.2 mM cytidine; U, 2 mM uridine-medium, without glucose (uri+); UB, uri+ and Brequinar.

completed the cycle (as was also concluded from the increase in cell number) and were delayed in the G_1/early S phase of the next cycle (histogram B, much reduced second peak). It is evident from the bar diagram in Fig. 3 that the growth-inhibiting effect of Brequinar was neutralized by the addition of uridine to the cell cultures. Confirmation comes from the DNA histogram C (Fig. 2), which is very similar to that of cells which were not treated with Brequinar (histogram E). Cytidine was ineffective in treated EAT cells (Fig. 3) because these are unable to metabolize cytidine via deamination to refill the uridylate pool [17]. The addition of deoxycytidine was unable to stimulate division of EAT cells (data not presented in Fig. 3); however, it is obvious from the histogram D in Fig. 2, that this DNA precursor had a considerable promoting effect on the G_1/S transition. A stimulating effect of uridine on cell proliferation was also observed when growth of EAT cells had been inhibited by aza-DHO, a competitive inhibitor of DHO-DH [11, 18]. If EAT cells were grown in glucose-free, uridine-supplemented medium, they also became insensitive to Brequinar (Fig. 3). As can be expected from the findings for active salvage of exogenous uridine, the incorporation of labeled uridine into the RNA-fraction was greatly enhanced in cells which had been treated for 8 hr with 2.5 and 12.5 μM Brequinar (140 and 190% of untreated control cells, respectively). Similar effects were observed when EAT cells were kept in the presence of 2 mM aza-DHO or of inhibitors interfering with the electron transfer from DHO to oxygen, via the mitochondrial respiratory chain [11, 19]. In contrast, the incorporation of labeled HCO_3^- as a *de novo* precursor of pyrimidine bases into the RNA-fraction of EAT cells was greatly reduced in the presence of the same Brequinar concentrations (20 and <5% of untreated controls, respectively).

Comparison of growth impairment by hypoxia and Brequinar

The set of bar diagrams in Fig. 4 gives a comprehensive survey of the increase in cell number (G), protein (P), DNA (D) and RNA (R) content of cultures under different oxygen tensions and/or in the presence of Brequinar. Values are expressed as percentages of normoxic controls (20% O_2, no drug). A drug concentration of 0.125 μM was chosen because the residual growth of 75–80% of untreated controls (Fig. 1) allowed a study of the additional influence of oxygen shortage. A 50% reduction in cell growth under an atmosphere of about 3.5% O_2 could be deduced from these and other data. All the graphs obtained from separate sets of culture experiments show a very similar influence of Brequinar on the individual growth parameters. The increase in cell number was reduced to 70–80%, protein to 80–90%, RNA to 75–80% and DNA to 70–75% of untreated controls. These data reveal that the production of protein was less affected by

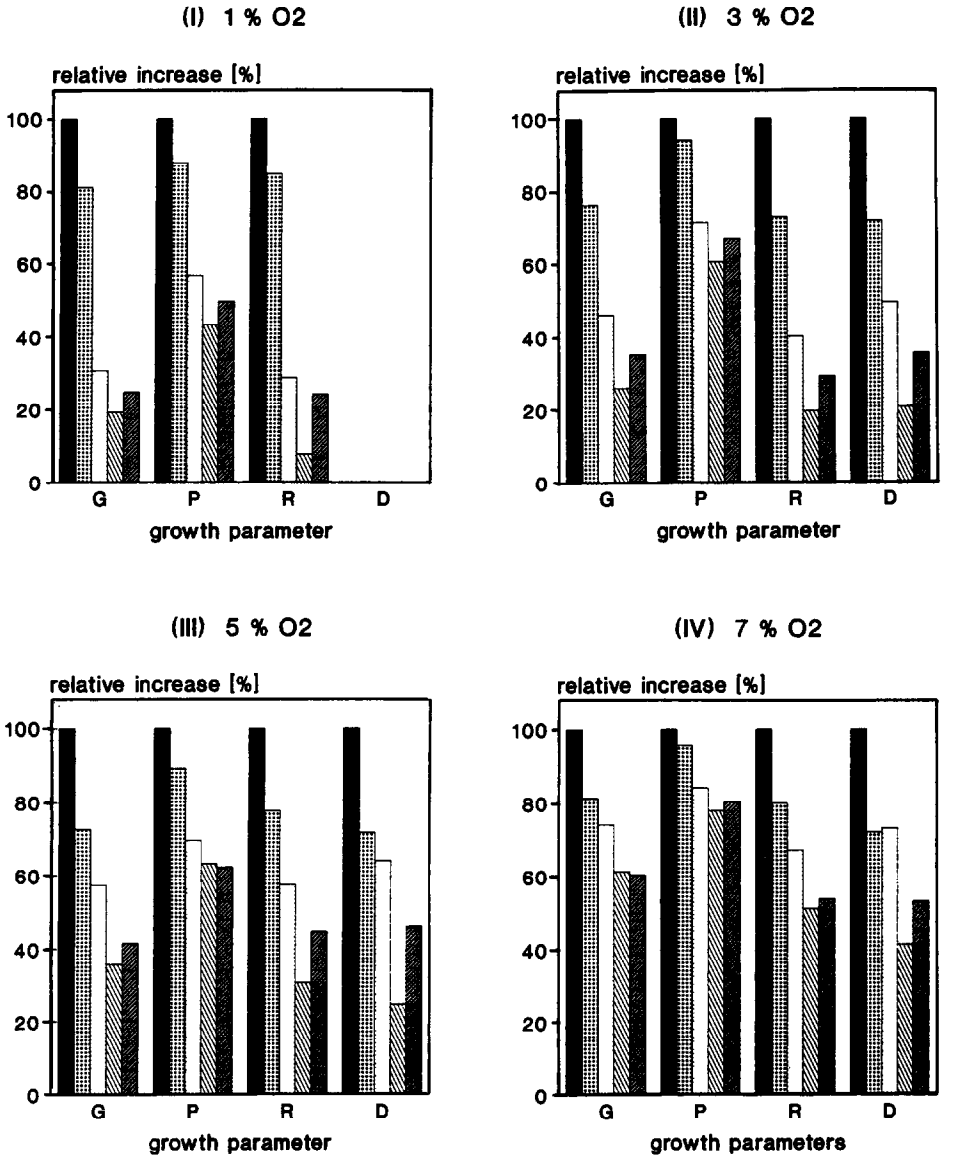

Fig. 4. Comparison of growth parameters of EAT cells treated with hypoxia, Brequinar or hypoxia + Brequinar. Cells were cultured under different O_2 tensions (as indicated at the top of the individual bar diagram, I–IV) and/or in the presence of 0.125 μM of the drug. The calculation of the increases in protein, RNA and DNA content of cultures was the same as that of cell growth (see legend to Fig. 3). The values are means of results from eight separately initiated culture experiments (which differed from each other by between 5 and 15% of the mean) and are expressed as percentages of normoxic, untreated controls. B, Brequinar; H, hypoxia; C, control. (■) Control; (▦) B/C; (▩) H/C; (▨) (B + H)/C; (▨) (B/C) × (H/C) as calculated; G, increase in cell number (growth); P, protein; R, RNA; D, DNA.

the drug concentration used. In addition, Fig. 4 gives information on the effectiveness of Brequinar plus hypoxia [20]. The bars on the right of each group represent the calculated product of the individual effects of Brequinar and hypoxia (Fig. 4, legend). At 7% O_2 and for the parameters of increase in cell number, protein and RNA, the bars are of similar size to the adjacent bars which represent the simultaneous effect of the two treatments. This finding indicates an additive effect of Brequinar plus hypoxia on EAT cells. At lower oxygen concentrations (1–5%), the size of the bars is greater, especially for the RNA and DNA parameters. This comparison gives evidence of a greater than additive effect of the two treatments on the growth parameters. Protein synthesis was targeted less by moderate hypoxia [21] and is not known to be a specific target of Brequinar. Furthermore, the drug does not interfere directly with the energy metabolism of cells. The direct impairment of pyrimidine *de*

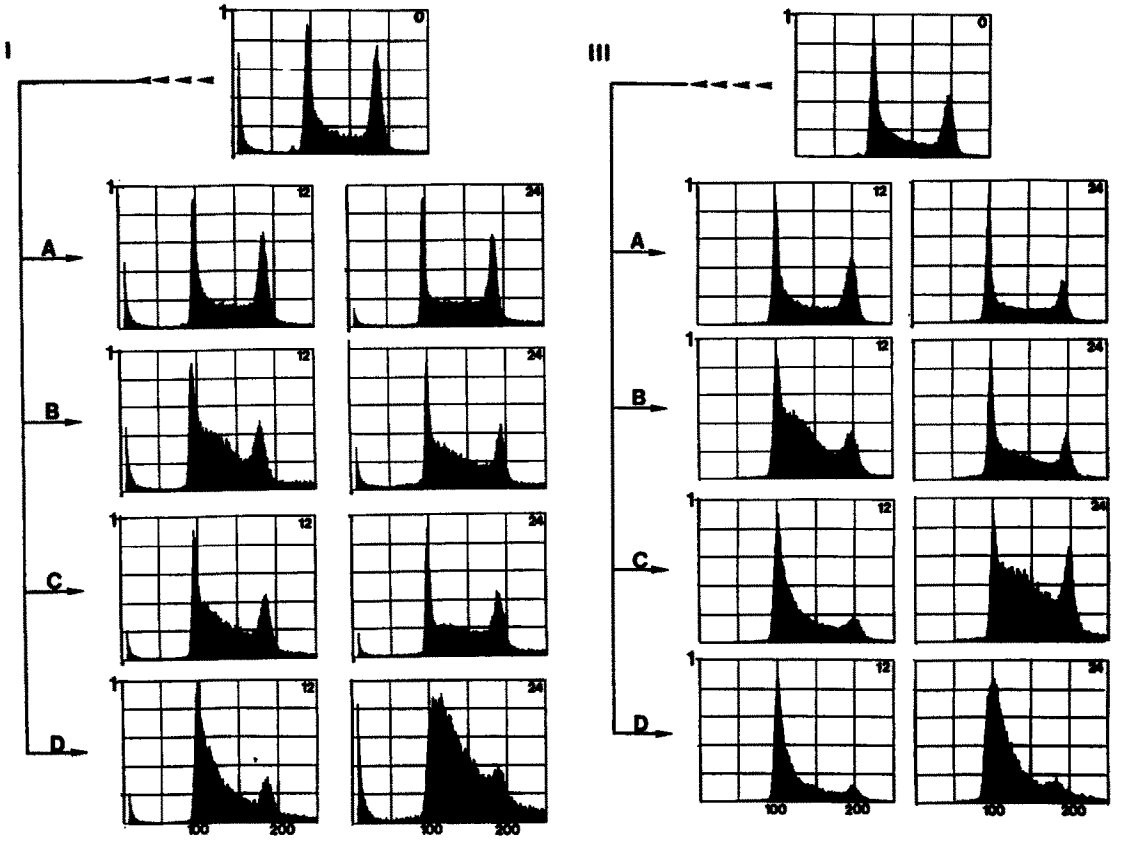

Fig. 5. Flow cytometric DNA distribution of EAT cells. Each histogram represents the typical cell cycle distribution after 12 and 24 hr of cell culture. Abscissa: fluorescence intensity was equivalent to DNA content of cells. Ordinate: number of pulses per channel was equivalent to cell number (relative units). Samples were from the set of cultures presented in Fig. 4. Chart I: 1% O_2; Chart III: 5% O_2. (II and IV are not presented). Line A: normoxic control; line B, 0.125 μM Brequinar; line C: hypoxia; line D: hypoxia + 0.125 μM Brequinar.

novo synthesis is the only specific and primary effect known so far.

The results of flow cytometric analyses of samples from cells kept under 1 and 5% oxygen were selected here to demonstrate the alterations in cell cycle progression induced by hypoxia, Brequinar and hypoxia plus Brequinar (histograms of cells kept at 3 and 7% O_2 not shown). The DNA histograms in Fig. 5 (I and III), line B show a shift in the cycle distribution towards the G_1/early S area that can be interpreted as a delay in cycle progression after applying Brequinar, as well as after establishing hypoxic conditions (line C), as compared with untreated controls (line A). An additional retardation can be seen as soon as cells are treated with the combined regimen (line D).

DISCUSSION

The results of the present *in vitro* study underscore the potent growth-inhibitory effect of Brequinar which was additive to the inhibition of cell proliferation by oxygen depletion. Because of a common target, the mitochondrially bound DHO-DH, both conditions, i.e. the presence of the drug and hypoxia, were found to cause depletion of RNA and DNA precursors [10, 12, 13, 22, 23].

The IC_{50} of 0.25 μM Brequinar for EAT cells (population doubling time 16–18 hr) was in the lower range of the scale for other cell lines, i.e. 0.2–6 μM [12, 22, 24]. This value does not fit in with the explanation that cell lines with high growth rates *per se* are less sensitive to Brequinar [24]. The kind of culture used here could also play a crucial role. Suspension culture allows a fairly uniform exposure of all cells to medium ingredients and also to Brequinar which was found to be tightly bound to plasma proteins [25]. This was of great advantage for the study of the basic mechanisms of the influence of hypoxia on the efficacy of drugs affecting cell proliferation [15, 21].

The tolerance towards the growth-inhibitory effect of Brequinar in the presence of exogenous uridine seems to be characteristic of those cells and tissues capable of salvaging preformed nucleic acid precursors [11, 12, 22, 26] and which do not use

UMP *de novo* synthesis, e.g. EAT cells grown in glucose-free, uridine-supplemented medium [14].

The incorporation rate of $[^{14}C]HCO_3^-$ into precursors and nucleic acids [13, 16] was also taken in EAT cells as evidence for the function of a *de novo* pyrimidine pathway. The uptake of label into the RNA-fraction was found to decrease following exposure to hypoxia of different degrees [21]. Likewise, it was inhibited in the presence of increasing Brequinar concentrations. This finding of adaptation of the pyrimidine biosynthetic rate in EAT cells is in contrast to that of Anderson *et al.* [22], who observed an appreciable release of DHO into the medium on the addition of Brequinar to L1210 cells.

It was shown for human colon adenocarcinoma cells and L1210 cells [12, 26], as well as for EAT cells in the present study, that the addition of deoxycytidine did not circumvent the cytotoxicity of Brequinar. The fact that this nucleoside considerably speeded up the traverse from G_1/early S blocked cells (Fig. 2) was overlooked by others. However, the cycle-specific delay of Brequinar-treated cells was neutralized by 0.1 mM deoxycytidine without an appropriate effect on RNA and protein synthesis (data not shown). These results allow two conclusions to be drawn: (i) DNA synthesis in Brequinar-treated cells can be supported independently of impaired RNA production, solely by the salvage of exogenous deoxycytidine (the thymidine necessary for DNA synthesis can be produced from deoxycytidine but thymidine is not metabolized to deoxycytidine; deoxycytidine is not, or not to a great extent, metabolized to uridine); and (ii) the inhibition of RNA synthesis did not allow the doubling of cell mass and, therefore, seemed to prevent the cell division of Brequinar-treated cells.

Our previous studies have shown that pyrimidine *de novo* synthesis plays a key role in the modulation of cell growth by hypoxia. On the other hand, low oxygenation can alter the tumoricidal activity of drugs either by enhancement or reduction of their efficacy [3–7]. From the results obtained in this first study of the effect of hypoxia on Brequinar-treated cells, it can be concluded that the effectiveness of drugs interfering with the UMP *de novo* pathway may not be minimized by oxygen shortage, rather, it may be enhanced. For an explanation of these observations based on the present state of knowledge, the changes in mitochondrial structure and integrity as observed under more severe hypoxia have to be considered [27, 28]. Impairment of oxidative phosphorylation could alter the membrane potential and also the charge distribution within the inner mitochondrial membrane [29]. This could entail changes in the conformation of membrane proteins, e.g. DHO-DH, resulting in a modified exposure of the enzyme to drugs of the quinoline carboxylic acid type. Another possible explanation comes from unpublished observations on increased oxygen consumption (in comparison to untreated controls) after a 24-hr application of 2.5 μM Brequinar to EAT cells. This was not expected in view of the reduced growth rate of the cells (Fig. 1). This high oxygen consumption could be explained either by non-specific uncoupling of functional oxidative phosphorylation in the mitochondrial membrane or as being a specific metabolic adaptation of cells to remove the drug by increasing the activity of the energy-dependent efflux pump [30]. As matter of speculation, particularly as lower Brequinar concentrations were used in the present experiments, our finding of a greater than additive effect of Brequinar plus hypoxia could be interpreted as low oxygen concentrations robbing the cells of their capability to counter the stress of Brequinar attack. This could result in an increased potency of the same drug concentration.

Concerning the *in vivo* situation of tumors, the present study also supports the fact that a putative synergistic effect of hypoxia and the drug might not be generated if preformed precursors became available from plasma or from necrotic cells arising in hypoxic areas of solid tumors [2, 21, 23]. In addition, the parallel experiments of this research group on the purified rodent DHO-DH give evidence that under certain conditions the enzyme can use molecular oxygen instead of ubiquinone as a proximal electron acceptor [18]. In analogy to the change in xanthine dehydrogenase/oxidase activity described for animal tissues [31], the DHO-DH activity could possibly switch to increased oxidase activity on the reoxygenation of tumor areas following ischemia. Since *in vitro* the oxidase activity was found to be marginally inhibited by Brequinar, fluctuations in oxygenation of a tumor could counteract the synergistic attack of Brequinar and hypoxia on cell growth and proliferation as described in the present study. The complex nature of an enzyme with dehydrogenase and/or oxidase activity, and its relevance for antitumor drugs, such as Brequinar, with respect to drug resistance [32–34] deserves additional study.

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