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Quinazoline Antifolates as Inhibitors of Growth, Dihydrofolate Reductase, and Thymidylate Synthetase of Mouse Neuroblastoma Cells in Culture

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SUMMARY

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Correlations between inhibition of growth and of enzyme activity by several quinazoline analogues of folic acid have been made with two lines of C1300 mouse neuroblastoma cells, one sensitive and one resistant to quinazoline analogues. The quinazoline analogues studied fell into two classes: 2,4-diamino and 2-amino-4-hydroxy derivatives. The 2,4-diaminoquinazoline analogues DAQ (*N*-[*p*-[[(2,4-diamino-6-quinazolinyl)methyl]methylamino]benzoyl]-L-glutamic acid) and methasquin (*N*-[*p*-[(2,4-diamino-5-methylquinazolinyl)methylamino]benzoyl]-L-aspartic acid) effectively inhibited both cell growth and dihydrofolate reductase activity, whereas the 2-amino-4-hydroxyquinazoline analogue AHQ (*N*-[*p*-[[(2-amino-4-hydroxy-6-quinazolinyl)methyl]methylamino]benzoyl]-L-glutamic acid) was less potent. Nevertheless, AHQ was more inhibitory toward thymidylate synthetase than DAQ. With the 2,4-diaminoquinazoline analogues there was a good correlation between inhibition of growth and dihydrofolate reductase, but with AHQ growth inhibition seemed to depend upon the inhibition of both dihydrofolate reductase and thymidylate synthetase. Sensitive cells could be completely protected against the toxic effects of AHQ by either leucovorin or thymidine; the concentration of leucovorin (0.56 μM) necessary to provide 50% protection against a lethal dose of AHQ (30 μM) was 38 times less than the amount of thymidine (21 μM) required for 50% protection. Because of its unique potency toward thymidylate synthetase, AHQ may be useful in the chemotherapy of neuroblastoma.

INTRODUCTION

Neuroblastoma is probably the most common of all the solid cancers of children (1). The most effective chemotherapeutic management of metastatic neuroblastoma has been demonstrated to be alternate weekly administration of vincristine sulfate and cyclophosphamide. The over-all response rate in neuroblastoma patients of all ages is still quite low; reported findings show remission rates of only 32–38% with a mean duration

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of 3–12 months (2–4). It is clear that new chemotherapeutic approaches are necessary to manage this highly malignant tumor.

The mouse neuroblastoma C1300 cell line has provided much information about the morphological and enzymatic differentiation of transformed neuroblasts in cell culture and might become similarly useful in designing new pharmacological approaches to metastatic neuroblastoma in children (5, 6). In searching for enzyme induction as a marker in neuroblastoma cells grown in cell culture, it was demonstrated that the specific activity of thymidylate synthetase was 2.5-fold higher in rapidly dividing cells than in stationary cells (5), suggesting that thymidylate synthetase plays an important role in the growth of neuroblastoma.

We therefore initiated studies with a 2-amino-4-hydroxyquinazoline antifolate recently reported by Bird *et al.* (7) to be a potent inhibitor of thymidylate synthetase as well as of dihydrofolate reductase; this antifolate, called compound II by Bird *et al.* (7), is here designated Parke, Davis compound 2 or PD-2. We derived a neuroblastoma cell line resistant to PD-2 and also studied the effects of PD-2 on the growth of sensitive and PD-2-resistant neuroblastoma cells in culture and on the activities of thymidylate synthetase and dihydrofolate reductase in extracts from sensitive and PD-2-resistant cells. Further studies were carried out with other related quinazoline antifolates, such as the 2,4-diaminoquinazoline analogue corresponding to PD-2; this antifolate, called compound V by Bird *et al.* (7), is here designated DAQ,[1] for diaminoquinazoline.

An important quinazoline derivative used in our studies was a preparation of the 2-amino-4-hydroxyquinazoline antifolate (5,8-deaza-10-methylfolate) which was obtained by a synthetic route different from the Parke, Davis synthesis (8). This preparation (hereafter called aminohydroxyquinazoline or AHQ to distinguish it from the Parke, Davis preparation, PD-2) was made available to us by Dr. Laurence Plante.[2] In the new synthesis, possible contamination by a 2,4-diamino compound is precluded by avoiding the use of a 2,4-diamino intermediate.

From a comparison of the effects of PD-2, DAQ, and AHQ on the growth of neuroblastoma and on the enzyme activities of thymidylate synthetase and dihydrofolate reductase, it was concluded that even after purification by preparative chromatography PD-2 contained 93% AHQ and 7% DAQ.[3] We therefore focused our attention on AHQ, studying the relationship between the inhibition of growth and of thymidylate synthetase and dihydrofolate reductase. We also studied the protective effects of leucovorin and thymidine. It is concluded that although AHQ is less potent than the diaminoquinazolines as an inhibitor of neuroblastoma growth in culture, it may be useful in the chemotherapy of neuroblastoma because of its unique potency toward thymidylate synthetase.

MATERIALS AND METHODS

Chemicals. A Parke, Davis preparation (PD-2) containing 93% AHQ (5,8-deaza-10-methylfolic acid) and 7% of the corresponding 2,4-diamino analogue, DAQ (*N*-[*p*-[[(2,4-diamino-6-quinazolinyl)methyl]methylamino]benzoyl]-L-glutamic acid), as well as pure DAQ and methasquin (*N*-[*p*-[(2,4-diamino-5-methylquinazolinyl)methylamino]benzoyl]-L-aspartate), were gifts from Dr. John R. Dice (Parke-Davis and Company), to whom we are greatly indebted. Methotrexate (amethopterin) was obtained from Lederle; its structure is *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]-benzoyl]-L-glutamate.

Crude PD-2 was purified by paper chromatography as follows: 10 mg of compound were dissolved in 1 ml of ethanol–glacial acetic acid-water (2:1:1). Ascending chro-

[1] The abbreviations used are: DAQ, diaminoquinazoline (*N*-[*p*-[[(2,4-diamino-6-quinazolinyl)-methyl]methylamino]benzoyl]-L-glutamic acid); AHQ, aminohydroxyquinazoline (*N*-[*p*-[[2-amino-4-hydroxy-6-quinazolinyl)methyl]methyamino]-benzoyl]-L-glutamic acid).

[2] Details of the synthesis of AHQ will be published elsewhere.

[3] Calculated by equating the presumed fraction DAQ (X), times its potency (Table 1), plus the presumed fraction of AHQ ($1 - X$), times its potency, with the potency of chromatographed PD-2.

matography was carried out with Whatman No. 1 paper in 1-butanol–glacial acetic acid–water (2:1:1). PD-2 with an R_F of 0.81 was eluted from the paper with 0.15 N NH_4OH. The eluate was evaporated to dryness, and the residue was dissolved in 0.33 N HCl. The compound was precipitated by the addition of concentrated NH_4OH to pH 4. The precipitate was washed by resuspension and centrifugation with water. Chromatographed and original PD-2 were diluted with 0.005 N NaOH to equal absorbance at 310 nm to compare their inhibitory effectiveness. Chromatographed PD-2 was more potent than crude PD-2 (presumably because of the removal of noninhibitory ultraviolet-absorbing impurities), but chromatographed PD-2 still contained 7% DAQ. At pH 7.0 chromatographed PD-2 has maxima at 307 and 227 nm. At pH 10.5 the maxima are at 311, 275, and 227 nm. The ultraviolet absorption spectrum of AHQ is identical with that of PD-2.

Details of the novel synthesis of pure AHQ by Dr. Laurence Plante will be published elsewhere. It is important to emphasize again that a 2,4-diaminoquinazoline is not an intermediate in the synthesis, as is the case for the Parke-Davis preparation.

Cell lines. The mouse (Ajax strain) neuroblastoma C1300 tumor was originally obtained from the Jackson Laboratories, Bar Harbor, Maine, and clone C46 was derived from a single cell cloned from tissue culture-adapted cells. Clone C46 was a gift of Dr. Gordon H. Sato, University of California, San Diego.

A PD-2-resistant cell line was derived from neuroblastoma C46 cells and is designated (C46)PD-2R. Sensitive (control) C46 cells were exposed to 2 μM PD-2 in Dulbecco's modified Eagle's medium with 20% fetal calf serum, and the medium containing PD-2 was changed daily, resulting in the loss of nearly 99% of C46 cells. One month after the initial exposure to PD-2, small colonies of cells began to form about the few remaining cells, and 6 weeks after the initial exposure cells maintained in medium with 2 μM PD-2 began to divide rapidly with a generation time of 24 hr. These (C46)PD-2R cells were maintained in 2 μM PD-2 for 40 generations and continued to divide rapidly, showing no cytotoxic effects. Cultures appeared to be 100% viable by exclusion of nigrosin (0.5%) stain. Karyotype analysis of control and PD-2-resistant C1300 cells showed both cell lines to have a tetraploid number of chromosomes; no marker chromosomes were noted.

Culture conditions. Clone C46 of mouse neuroblastoma C1300 was grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum in Falcon flasks or Petri dishes at 37° in an atmosphere of 10% CO_2–90% air at 100% humidity. Cultures were routinely checked for contamination by pleuropneumonia-like organisms; all experiments were performed on cultures that were free of this contamination. To prevent contamination, cells were grown in the presence of kanamycin (200 μg/ml) and spectinomycin (125 μg/ml); neither antibiotic alone was effective. The presence of these antibiotics in the medium did not affect the growth rate of the neuroblastoma cells.

For the cell culture experiments, cells were kept rapidly dividing in monolayer culture adherent to the surface of Falcon flasks (75 cm^2). To ensure that cells for experiments were rapidly dividing, 1×10^6 cells were plated per flask, and 4 days later, when the cells were in the logarithmic phase of growth, they were removed from the surface by a 10-min exposure to 0.25% trypsin in Dulbecco's modified Eagle's medium. Before exposure to trypsin the medium was removed from the flasks by aspiration, and the cells were rinsed once with Dulbecco's modified Eagle's medium salts to remove most of the fetal calf serum. Trypsin digestion was carried out by adding 5 ml of 0.25% trypsin in Dulbecco's modified Eagle's medium to the flask and then aspirating the solution immediately. The flask was then incubated for 10 min at 37°, and the cells were tapped off in 10 ml of fresh medium containing 10% fetal calf serum. The dispersed cells were counted with a Coulter model Z_{BI} electronic cell counter. An appropriate dilution was then made with serum-containing medium to give a suspension from which aliquots for plating the appropriate number of cells were taken. Except where noted, the medium was changed every third day.

Determination of concentration of drug inhibiting cell growth by 50%. The relative

growth-inhibitory potencies of the various antifolates were compared in terms of EC_{50} values (9). EC_{50} refers to the effective concentration of drug that produces 50% inhibition of growth in the following standard 3-day assay. For each drug 5×10^5 sensitive or PD-2-resistant cells are inoculated into each of a series of 60-mm Falcon tissue culture dishes containing graded concentrations of drug in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Each series consists of six or more drug concentrations and a control with no drug; four plates are inoculated at each concentration. Cells are grown for 3 days and then treated with trypsin, dispersed by pipetting up and down at 0°, and counted in a Coulter counter. The average cell number at each drug concentration, expressed as percentage of control, is plotted against the logarithm of the drug concentration to determine the EC_{50} value.

Assays of neuroblastoma thymidylate synthetase and dihydrofolate reductase in the presence and absence of drugs. Determination of the effect of quinazoline antifolates on thymidylate synthetase and H_2-folate reductase in crude extracts obtained from clone C46 cells was carried out as follows. A crude extract was prepared from rapidly dividing C46 cells grown in 150-mm tissue culture dishes. The cells were washed once with 0.9% NaCl and then scraped off the dishes with a rubber policeman; the cells were suspended in a small volume of 50 mM Tris buffer, pH 7.4, containing 10 mM 2-mercaptoethanol, 1 mM sodium versenate, and 25% sucrose, and freeze-thawed three times (−78°–37°). The suspension of disrupted cells was centrifuged in a Sorvall SS-34 rotor at 17,000 × *g* for 10 min at 4°, yielding a clear supernatant fluid.

Thymidylate synthetase was assayed by a modification of the method of Roberts (5, 10). In the assay, a typical incubation mixture included 200 μl of crude neuroblastoma cell extract, 5–25 μl of diluted antifolate solutions, substrates, and the Tris–mercaptoethanol–versenate–sucrose buffer described above, up to a volume of 315 μl. Dihydrofolate reductase in neuroblastoma extracts was assayed spectrophotometrically (11). Because of the low activity of the extracts from sensitive neuroblastoma cells and the turbidity caused by using large amounts of protein in the H_2-folate reductase assay, it became necessary to purify the H_2-folate reductase from sensitive cells before assay; the H_2-folate reductase from PD-2-resistant neuroblastoma cells was sufficiently active to assay directly. The crude extracts were purified by first adding streptomycin sulfate to 0.7%; after centrifugation of the resultant white precipitate, ammonium sulfate was added to the supernatant fluid to 40% saturation and centrifuged again. Ammonium sulfate was then added to 80% saturation, which precipitated the H_2-folate reductase activity. The precipitate was dissolved in 2 volumes of 0.05 M Tris, pH 7.5, containing 0.01 M mercaptoethanol. Protein was determined by the method of Lowry *et al.* (12).

RESULTS

Relative potencies of quinazolinyl antifolates as inhibitors of sensitive and PD-2-resistant neuroblastoma growth in culture and thymidylate synthetase and dihydrofolate reductase activities in vitro. As shown in Table 1, the 2,4-diaminoquinazolinyl antifolates DAQ and methasquin are much more potent inhibitors of sensitive cell growth and H_2-folate reductase than PD-2. Methotrexate, a 2,4-diaminopteridinyl antifolate, inhibits cell growth somewhat less than the 2,4-diaminoquinazolinyl antifolates, but it is still 3.2 times as potent as chromatographed PD-2, which, in turn, is somewhat more effective than crude PD-2 (presumably because of the chromatographic removal of ultraviolet-absorbing material that is not inhibitory). Methotrexate has about the same potency as methasquin and DAQ as an inhibitor of H_2-folate reductase. AHQ, the independently synthesized pure 2-amino-4-hydroxyquinazolinyl compound, is 88 times less potent than chromatographed PD-2 in inhibiting sensitive cell growth. AHQ is at least 15 times as inhibitory as DAQ toward neuroblastoma thymidylate synthetase and 95-fold less so toward H_2-folate reductase. A similar pattern of potencies is found in the PD-2-resistant cells.

The cells made resistant to crude PD-2 are more resistant to both methasquin (20-fold) and methotrexate (11-fold) than to PD-2 (7.5-fold). The degrees of resistance to

TABLE 1

Quinazoline antifolates as inhibitors of sensitive and PD-2-resistant neuroblastoma growth in culture and of thymidylate synthetase and dihydrofolate reductase activities in vitro

Inhibitor	Basic ring structure	Substituents[a]					EC_{50} for cell growth[b,c]			EC_{50} for thymidylate synthetase[b,e]		EC_{50} for dihydrofolate reductase[b,g]		
		2	4	5	10	17	Sensitive cells	Resistant cells	Resistance[d]	Sensitive neuroblastoma	Resistant *L. casei*[f]	Sensitive neuroblastoma	Resistant neuroblastoma	Resistant *L. casei*[f]
							μM	μM	*-fold*	μM	μM	μM	μM	μM
Methasquin	Quinazolinyl	NH_2	NH_2	CH_3	H	Asp	0.008 (1750)	0.160 (312)	20			0.004 (45)	0.0035 (71)	0.0022 (364)
DAQ	Quinazolinyl	NH_2	NH_2	H	CH_3	Glu	0.011 (1272)	0.080 (625)	7.3	2.0 (1)	5.6 (1)	0.0019 (95)	0.0024 (104)	0.0014 (559)
Methotrexate	Pteridinyl	NH_2	NH_2	H	CH_3	Glu	0.047 (298)	0.54 (93)	11			0.0024 (75)	0.0038 (66)	0.0019 (421)
Crude PD-2	Quinazolinyl	NH_2	OH (NH_2)	H	CH_3	Glu	0.57 (25)	4.3 (12)	7.5					
Chromatographed PD-2	Quinazolinyl	NH_2	93% OH, 7% NH_2	H	CH_3	Glu	0.16 (87.5)	1.2 (42)	7.5	0.18 (11)	0.248 (23)	0.02 (9)	0.024 (10)	0.02 (40)
AHQ	Quinazolinyl	NH_2	OH	H	CH_3	Glu	14.0 (1)	50.0 (1)	3.6	0.14 (14.3)	0.21 (27)	0.18 (1)	0.25 (1)	0.80 (1)

[a] Substituents are numbered as in methotrexate.

[b] Relative potencies are given in parentheses. These values were derived by dividing the cell or enzyme EC_{50} for each drug into the cell or enzyme EC_{50} of the drug having the lowest potency.

[c] The growth medium contained 10 μM folic acid.

[d] Resistance refers to the EC_{50} of PD-2-resistant cells divided by the EC_{50} of sensitive cells.

[e] Assayed in the presence of 0.45 mM *dl*-H_4-folate, 10 mM HCHO, and 0.04 mM dUMP.

[f] Enzyme extracted from dichloroamethopterin-resistant *Lactobacillus casei* (13).

[g] Assayed in the presence of 10 μM H_2-folate and 80 μM NADPH.

crude PD-2, chromatographically purified PD-2, and DAQ are almost identical. However, PD-2-resistant cells are only 3.6 times more resistant to AHQ than sensitive cells.

Concentrations of PD-2, DAQ, and AHQ causing 50% inhibition of thymidylate synthetase and dihydrofolate reductase from sensitive and PD-2-resistant neuroblastoma cells. The original crude sample of 2-amino-4-hydroxyquinazoline (PD-2) received from Parke, Davis inhibited thymidylate synthetase from *Escherichia coli* at micromolar concentrations, in confirmation of the results of Bird *et al.* (7). After partial purification by paper chromatography (see MATERIALS AND METHODS) PD-2 caused 50% inhibition of thymidylate synthetase from neuroblastoma at 0.18 μM whereas the diamino analogue DAQ was approximately 11 times less inhibitory, with an EC_{50} of 2 μM (Table 1). AHQ synthesized by a method which precluded the presence of contaminating DAQ showed an EC_{50} of 0.14 μM. Since the concentration of *dl*-H_4-folate in the thymidylate synthetase assay mixture was 0.46 mM, the ratio of active H_4-folate diastereoisomer to AHQ for 50% inhibition was approximately 1643:1, compared with a ratio of 140:1 reported by Bird *et al.* (7) for thymidylate synthetase from *E. coli*. Thus the 2-amino-4-hydroxyquinazoline analogue is a remarkably potent inhibitor of neuroblastoma thymidylate synthetase.

H_2-folate reductase from neuroblastoma was inhibited 50% by approximately 0.002 μM DAQ and methotrexate; both analogues were twice as potent as methasquin. Most of the inhibitory action of PD-2 with an EC_{50} of 0.02 μM is attributed to contaminating DAQ, since AHQ, the major constituent of PD-2, showed an EC_{50} enzyme value of 0.18 μM, compared with a value of 0.002 μM for DAQ.

We are well aware of the difficulties of comparing percentage inhibitions by diaminofolate analogues that have such a high affinity for H_2-folate reductase (14, 15). Even with highly purified H_2-folate reductase, the kinetics is complex and not amenable to simple interpretation (16, 17). Nevertheless, since we were testing a series of chemically related analogues and conditions for the assays were uniform throughout (the same amount of enzyme was used for all assays and H_2-folate was always added last), the values for 50% inhibition are informative, providing an approximation of potency. As can be seen from the relative potency data presented in Table 1, the correlations between enzyme inhibition and growth inhibition were reasonably good.

Since the H_2-folate reductase activity of the sensitive line of neuroblastoma was quite low, preliminary kinetic inhibition studies were carried out with the PD-2-resistant line, which exhibited 6.5 times the H_2-folate reductase activity of PD-2-sensitive cells. Although double-reciprocal plots (in which both dihydrofolate and quinazoline concentrations were varied) resembled theoretical plots for reversible competitive inhibition, it was impossible to obtain consistent values for K_i with the crude extracts employed in our studies. As emphasized by Morrison (14), accurate estimates of dissociation constants can be made only if equilibria among enzyme, substrates, and inhibitor are attained at a sufficiently rapid rate to attain steady-state initial velocities. Until large-scale tissue culture of neuroblastoma cells can make available enough material for preparation of purified H_2-folate reductase, precise estimates of affinity constants are not possible.

Thymidylate synthetase and H_2-folate reductase levels in PD-2-resistant neuroblastoma cells. Cultures containing 2×10^6 sensitive or PD-2-resistant cells were begun in 150-mm Petri dishes containing Dulbecco's modified Eagle's medium with 20% fetal calf serum. PD-2-resistant cells were also grown in dishes containing control medium plus 2.2 μM PD-2. After 4 days in culture, duplicate cultures were assayed for thymidylate synthetase and dihydrofolate reductase activities. Thymidylate synthetase activities were about the same in the three series: 8.6 ± 2.9 units/mg of protein for sensitive cells grown in control medium, 8.8 ± 3.6 units/mg for resistant cells grown in PD-2-containing medium, and 8.0 ± 3.2 units/mg for resistant cells grown in control medium. Dihydrofolate reductase activity was 6.5 times greater in the resistant cells grown in control medium (840 ± 113 units/mg of

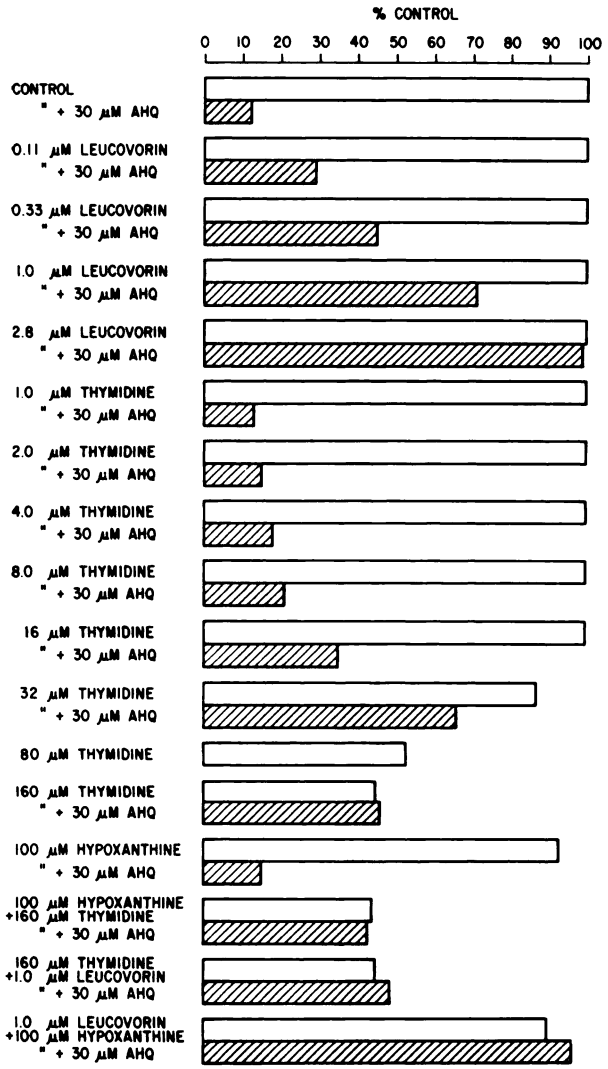

FIG. 1. *Leucovorin and thymidine protection against toxic effects of AHQ*

Sensitive C46 neuroblastoma cells (1.7×10^5) were plated in 35-mm tissue culture dishes containing 1.5 ml of medium with 10% fetal calf serum and 200 μg/ml of kanamycin, 125 μg/ml of spectinomycin, and 2.5 μg/ml of amphotericin B, supplemented as indicated. Four dishes were plated with each combination of drugs, and the cells were grown for 3 days in culture with medium changes on days 1 and 2. Cell numbers were determined on day 3 by dissociating the adherent cells for 10 min with 0.25% trypsin in medium (without serum) as described under MATERIALS AND METHODS and counting the dissociated cells in a Coulter model Z_{BI} electronic cell counter. The four plates for each combination were pooled prior to counting. The results are expressed as a percentage of the control with no hypoxanthine, leucovorin, or thymidine.

protein) as in sensitive cells grown in control medium (128 ± 49 units/mg).

Leucovorin and thymidine protection against toxic effects of AHQ. As shown in Fig. 1, both leucovorin and thymidine protect sensitive neuroblastoma cells against the lethal effects of AHQ at a concentration (30 μM) that normally inhibits growth by 88% in a typical 3-day assay. Hypoxanthine, however, does not afford any protection. Leucovorin gives partial protection at 0.11 μM and gives complete protection at

2.8 μM. The protection by thymidine is complicated by the finding that thymidine seems to be toxic for neuroblastoma above 20 μM; the EC_{50} for recrystallized thymidine is 93 μM. Thymidine does not begin to give significant protection until 16 μM. At 32 μM there is even greater protection, although at this concentration it manifests some toxicity. At 160 μM thymidine there is no difference between the control and thymidine plus 30 μM AHQ, but thymidine alone causes 55% inhibition of growth. The inhibition by 160 μM thymidine is not changed by either leucovorin or hypoxanthine.

DISCUSSION

The report of Bird *et al.* (7) that a quinazoline analogue of folic acid (PD-2) is a potent inhibitor of thymidylate synthetase from *E. coli* prompted us to investigate its effects as well as those of AHQ and DAQ on growth, thymidylate synthetase, and H_2-folate reductase of mouse neuroblastoma. Our studies confirm the finding of Bird *et al.* (7) that PD-2 is a potent inhibitor of thymidylate synthetase in addition to inhibiting H_2-folate reductase. Moreover, it effectively inhibits the growth of C1300 mouse neuroblastoma cells in culture.

Our study initially was based on the concept that PD-2 inhibited the growth of neuroblastoma cells because it inhibited thymidylate synthetase. The development of a PD-2-resistant line was carried out with this in mind. It soon became apparent that the action of PD-2 was more consistent with inhibition of H_2-folate reductase than of thymidylate synthetase; this made us suspect that PD-2 might be contaminated with DAQ. The PD-2-resistant line of neuroblastoma derived in this study is 7.5-fold resistant to PD-2 (Table 1) and has a 6.5-fold elevated level of H_2-folate reductase, but its thymidylate synthetase activity is the same as that of sensitive cells. Moreover, PD-2-resistant cells are cross-resistant to other 2,4-diaminoquinazolinyl and pteridinyl antifolates such as DAQ (7.3-fold), methasquin (20-fold), and methotrexate (11-fold); in fact, the cells probably became resistant to the contaminating DAQ present in PD-2. Our data with neuroblastoma confirm the general pattern seen with mouse leukemias and Chinese hamster cells (18, 19), in that there is a general correlation between inhibition of growth and of H_2-folate reductase, although this correlation is not perfect. The data for PD-2, DAQ, methasquin, and methotrexate are best explained in terms of inhibition of H_2-folate reductase. However, the data for AHQ can be explained only partially on the basis of H_2-folate reductase inhibition. If the cross-resistance of PD-2R cells to AHQ were due entirely to H_2-folate reductase elevation, one would expect PD-2-resistant neuroblastoma cells to have the same degree of resistance to AHQ as they do to PD-2 and DAQ. Since the resistant cells are only half as resistant to AHQ (3.6-fold resistant) as they are to PD-2 and DAQ (7.5-fold resistant), it is possible that the inhibition of neuroblastoma growth by AHQ may be due in part to the inhibition of thymidylate synthetase (50% inhibition at 0.14 μM; Table 1).

The observation that both leucovorin and thymidine can protect neuroblastoma against the lethal effects of AHQ (Fig. 1) is consistent with the view that AHQ owes its lethal effects to interference with folic acid metabolism in neuroblastoma cells. Demonstration of effects of AHQ *in vitro* on H_2-folate reductase and thymidylate synthetase obviously focuses attention on these two enzymes as possible targets of AHQ action. The observed apparent lack of protection by hypoxanthine, coupled with the considerable protection by thymidine, supports the view that both these enzymes may be involved in the action of AHQ on neuroblastoma growth. Based only on the inhibition of H_2-folate reductase, some protection by hypoxanthine would be expected. Also, one would not expect thymidine to protect neuroblastoma against AHQ as well as it does, since thymidine synthesis is only one of several key reactions involving reduced folate.

Because of the inhibition of neuroblastoma cell growth by thymidine alone at concentrations above 20 μM, it is not possible to state unequivocally that thymidine completely protects the cells against AHQ. With 160 μM thymidine the number of cells surviving after 3 days is the same with or without the addition of 30 μM AHQ, but it is

only 45% of the control with no thymidine. Thymidine at 16 μM does not slow growth and provides 34% protection against 30 μM AHQ, while 32 μM thymidine only slightly arrests growth (14%) and provides 61% protection (calculated as a percentage of the difference between the control with no AHQ or thymidine and that with AHQ alone). High concentrations of thymidine have been shown to arrest the growth of many cells in tissue culture.[4] This phenomenon has been used to obtain synchronous cultures of mammalian cells (20, 21), although generally at higher thymidine concentrations (2 mM).

The quantitative difference between the concentrations of leucovorin (0.56 μM) and thymidine (21 μM) required to achieve 50% protection against 30 μM AHQ can best be explained by noting that leucovorin probably protects by providing a source of H_4-folate cofactor and hence is only needed in catalytic amounts, whereas thymidine protects merely by providing an alternative source of TMP for DNA synthesis.

AHQ is a very potent inhibitor of thymidylate synthetase, inhibiting the enzyme from sensitive neuroblastoma cells by 50% at a concentration of 0.14 μM. Since it contains 93% AHQ, PD-2 is also a potent inhibitor of thymidylate synthetase. In neuroblastoma cells thymidylate synthetase inhibition by PD-2 is overshadowed by the stronger H_2-folate reductase inhibition produced by the 7% DAQ contaminant. On the other hand, Bird *et al.* (7) reported that PD-2 and DAQ are almost equally effective in inhibiting growth, even though DAQ is 500 times more effective as an H_2-folate reductase inhibitor. Here thymidylate synthetase inhibition by PD-2 seems to contribute significantly to the inhibition of growth. These results can be reconciled with ours by noting that with the bacterial enzymes the concentrations of PD-2 needed to cause 50% inhibition of both thymidylate synthetase and H_2-folate reductase are of the same order of magnitude. With the neuroblastoma enzymes the concentration of PD-2 causing 50% inhibition of H_2-folate reductase is 10 times lower than that required to inhibit thymidylate synthetase to the same extent. AHQ, however, inhibited neuroblastoma thymidylate synthetase and H_2-folate reductase equally well. Thus it appears that for the inhibition of thymidylate synthetase by an antifolate to be significant in the inhibition of cell growth, the affinity of the drug for thymidylate synthetase must be at least of the same order of magnitude as its affinity for H_2-folate reductase.

This agrees with the hypothesis of Borsa and Whitmore (22), who suggested that inhibition of H_2-folate reductase interferes with expression of the deleterious effects of thymidylate synthetase inhibition by preventing the conditions necessary for thymineless death (namely, unaltered protein and RNA synthesis). According to Borsa and Whitmore, an inhibitor that does not require an enzyme for activation (as does fluorodeoxyuridine) and that blocks thymidylate synthetase without also blocking H_2-folate reductase (and hence purine and RNA synthesis) would be ideal for producing thymineless death in cancer cells. To our knowledge, AHQ presently comes the closest to this ideal, since it does not require an enzyme for activation to the inhibitory form and has a relatively high potency toward thymidylate synthetase combined with a relatively low potency toward H_2-folate reductase.

Because of its unique potency toward thymidylate synthetase and its ability to inhibit the growth of neuroblastoma cells in decimicromolar amounts, we plan to extend our study of AHQ to experimental animals. Although AHQ is less effective than the diaminoquinazolines as an inhibitor of neuroblastoma cell growth in culture, its solubility in organic solvents suggests greater solubility in lipid membranes. This may lead to patterns of body distribution and toxicity that differ markedly from the diamines.

[4] J. Lever and J. E. Seegmiller, unpublished results.

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