Biochemical Pharmacology, Vol. 37, No. 6, pp. 997–1001, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

UPTAKE AND METABOLISM OF 5,8-DIDEAZAISOFOLIC ACID IN HUMAN COLON CARCINOMA CELLS*

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(*Received* 31 *March* 1987; *accepted* 27 *July* 1987)

Abstract—The uptake and metabolism of radiolabeled 5,8-dideazaisofolic acid (IAHQ) (*N*-[*p*-{[(2-amino-4-hydroxy-6-quinazolinyl)amino]methyl}benzoyl]-L-glutamic acid), a new antifol targeted to thymidylate synthase, has been investigated in the human colon adenocarcinoma cell line HCT-8. [^{3}H]IAHQ uptake was very slow, requiring days to achieve the intracellular level achieved in minutes by [^{3}H]methotrexate. This slow transport of IAHQ was consistent with the long exposures required to achieve cytotoxicity. Intracellular [^{3}H]IAHQ was converted in a concentration-dependent manner to polygamma-glutamate derivatives containing between two and four additional glutamate residues. These results are consistent with our hypothesis that IAHQ is a "pro-drug" which must be converted to polyglutamate derivatives before it is a sufficiently potent inhibitor of thymidylate synthase to induce a pyrimidineless state and cell death.

In some experimental tumor systems, the methotrexate (MTX)§ induced purineless state contributes to toxicity but not to tumor cell kill [1–3]. Specific thymidylate synthase inhibitors should cause only a pyrimidineless state and thus may have a greater therapeutic index than MTX.

A series of 2-amino-4-hydroxyquinazolines (5,8-dideazafolates) directed toward inhibition of thymidylate synthase was synthesized and screened for activity in experimental tumor systems [4–6]. One of these drugs, 5,8-dideazaisofolic acid (IAHQ; H-338) was chosen for detailed study of its mechanism of action [4, 7]. This derivative has an N-9, C-10 in the "bridge" connecting the heterocycle and benzoylglutamate (an "iso" linkage) rather than the normal C-9, N-10. Based on cytotoxicity protection experiments (with leucovorin, folic acid, hypoxanthine and/or thymidine) and studies with partially purified folylpolyglutamate synthetase and thymidylate synthase from HCT-8 cells, we have postulated that IAHQ is converted intracellularly to polyglutamate derivatives which specifically and potently inhibit thymidylate synthase via IAHQ polyglutamate–deoxyuridylate–enzyme ternary complex formation [4, 7]. The preparation of ^{3}H-labeled IAHQ has enabled us to study the uptake and metabolism of this promising compound in order to test this hypothesis.

MATERIALS AND METHODS

Chemicals. MTX was supplied by the Division of Cancer Treatment, National Cancer Institute. LV was a gift of Lederle Laboratories. [3′,5′,9-^{3}H]MTX was purchased from the Amersham Searle Corp. (Arlington Heights, IL) and was greater than 95% pure as measured by HPLC. Chemically synthesized MTX polyglutamates containing one and two additional glutamates were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). The derivatives containing three and four additional glutamates was purchased from the South Alabama Medical Sciences Foundation, University of South Alabama, Mobile, AL. IAHQ and its polyglutamate derivatives containing three and four additional glutamates were thesized as previously described [6].

IAHQ was purified by DEAE-cellulose chromatography. IAHQ was added to 20 mM NH_4CO_3 (pH 8.5), the pH was readjusted to 8.5, and insoluble material (up to approximately 5%) was removed by centrifugation. The sample was applied to a DEAE-cellulose column equilibrated with the same buffer. Washing with this buffer was continued until a slowly migrating component had eluted (> 5 column volumes). The long wash was essential to obtaining pure compound. IAHQ was eluted in a linear gradient from 20 to 500 mM NH_4HCO_3 (pH 8.5). The

* This work was supported in part by USPHS Grants CA-08010 and CA-24887 awarded by the National Cancer Institute as part of the National Large Bowel Cancer Project.

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§ The abbreviations used are: MTX, methotrexate (2,4-diamino-10-methylpteroylglutamate); IAHQ, 5,8-dideazaisofolic acid (H-338); LV, leucovorin ((6*R*,*S*)-5-formyltetrahydrofolate); poly-gamma-glutamate derivatives are indicated by a Glu (equals G) with a subscript appended to the parent compound. The subscript indicates the number of additional glutamates added in gamma-linkage to the Glu already present in the parent.

appropriate fractions were identified by their UV spectra, pooled, and lyophilized. The fluffy yellow solid was stored at −90°. The re-purified material was stable, readily soluble in neutral aqueous buffers, and co-chromatographed on HPLC with the major peak of the unpurified material.

Radiolabeled IAHQ was obtained by exposing chromatographically repurified IAHQ to tritium gas at slightly elevated pH (Moravek Biochemical, Inc.). High pH caused extensive breakdown of the unlabeled material (J. McGuire, unpublished observation). The crude material was purified by preparative TLC to yield 11 mCi at 25 Ci/mmol. The chemical and radiochemical purities of this material were carefully checked by a number of independent means. The radiolabeled material co-chromatographed with repurified IAHQ on analytical TLC, DEAE-cellulose chromatography (pH 8.5), and HPLC (strong anion exchanger, pH 3.3). Also the radiolabeled material served as a substrate for partially purified rat liver folylpolyglutamate synthetase and was nearly quantitatively converted to product(s). This enzyme is known to require an intact pteridine-like heterocycle coupled with a terminal benzoylglutamic acid. Since purified IAHQ was used as the starting material for radiolabeling, these results indicated that the label was in an intact quinazoline and that it was intact IAHQ. The nearly quantitative conversion indicated that very little degraded material was present. Some loss in purity of this material (assessed by HPLC) was observed on storage at −20°, presumably by radiolysis of this high specific radioactivity solid material. The degradation rate was retarded significantly by addition of unlabeled re-purified IAHQ (final specific radioactivity 2.2 Ci/mmol). When necessary, [^{3}H]IAHQ was re-purified by DEAE-cellulose chromatography (see above). Solutions of [^{3}H]IAHQ at pH 8.5 (NH_4HCO_3) were stable for weeks at −20°, but freshly prepared solutions were used for the experiments reported. No degradation was detected when [^{3}H]IAHQ was incubated at 37° for 72 hr in tissue culture medium. Re-injection of [^{3}H]IAHQ collected in the HPLC effluent (pH 3.3; 45°) demonstrated that there was no breakdown of [^{3}H]IAHQ on the column under HPLC conditions.

Tissue culture cells. The human colon adenocarcinoma line HCT-8 [8] was tested for *Mycoplasma* contamination every 6 months and was consistently found to be negative. Cultures were maintained in 25 cm^2 sterile plastic flasks (Costar, Cambridge, MA) as a monolayer in RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% horse serum and subcultured weekly after 5-min trypsinization. Under these conditions, the doubling time was 18 hr.

Uptake of MTX and IAHQ by HCT-8 cells. Uptake of radiolabeled drugs was measured in monolayer cultures of HCT-8 cells using the technique of Galivan [9]. In brief, growth medium (15 ml) was removed, and drug-containing medium (5 ml) was added to individual plates (100 mm Petri Dishes, Costar) containing 1 to 1.4 × 10^7 HCT-8 cells (>80% confluent) and incubated at 37°. To terminate uptake, duplicate plates were rapidly cooled to 0°, the radiolabeled medium was aspirated, and the plates were rapidly washed three times with 20 ml of ice-cold phosphate-buffered saline. The third wash contained approximately background radioactivity. Cells were solubilized with 2 ml of 1 N NaOH and incubated for > 30 min at 37°. The radioactivity was measured in 15 ml of Hydrofluor (New England Nuclear) after neutralization with an equal volume of 1 N HCl. Cell number per plate was determined from triplicate cultures that were collected by 30-min trypsinization, dispersed by passing the trypsinized cells through a 25 gauge needle, and appropriate dilutions of the cell suspensions counted using a Coulter Counter model ZB1 (Hialeah, FL). Results were normalized to drug/10^6 cells.

Isolation and identification of MTX and IAHQ polyglutamates from HCT-8 cells. Cells were preincubated in 100 mm Petri dishes with radiolabeled drug at the concentrations and times indicated (Table 1). The cultures were chilled, the medium was removed, the plates were washed three times with 20 ml of ice-cold saline, and the cells were collected with a rubber policeman in 2 ml of either 50 mM (MTX) or 20 mM (IAHQ) sodium phosphate (pH 5.5). Samples were then boiled for 5 min and immediately cooled in ice. Cellular debris was removed by centrifugation at 1400 *g* for 5 min at room temperature. The supernatant fraction was stored at −20° until analysis by HPLC. Both [^{3}H]MTX and [^{3}H]IAHQ were stable to this extraction (data not shown). Of the total extractable [^{3}H]MTX radioactivity, > 95% was present in this supernatant, and ≤ 2% remained in a fully extracted cell pellet. For [^{3}H]IAHQ, 86% of the total extractable radioactivity was present in this supernatant and 13% remained in a fully extracted pellet. HPLC analyses of cell extracts were performed using an anion exchange method [10], except that for MTX the initial buffer concentration was reduced to 50 mM to allow greater resolution of MTX and MTX(G_1) and the initial buffer was 20 mM for IAHQ. Cell extracts were adjusted to pH 3.3 with H_3PO_4 and clarified by centrifugation through an MPS-1 (Amicon Corp., Danvers, MA) filtration system. Prior to chromatography, sufficient chemically synthesized MTX polyglutamates were added to the clarified extracts from [^{3}H]MTX-treated cells to allow unambiguous assignment of their elution positions based on absorbance at 280 nm. Synthetic standards up to IAHQ(G_2) were similarly included in extracts from [^{3}H]IAHQ-treated cells. Longer standards were unavailable, and assignment of radioactivity eluting later than IAHQ(G_2) was based on several criteria. First, on the anion exchange column employed, polyglutamates elute in order of increasing chain length [10, 11]. Second, synthetic IAHQ derivatives containing 0–2 additional gamma-glutamates eluted just prior to the corresponding MTX derivative. Late eluting [^{3}H] in IAHQ-treated cells eluted in discrete peaks at retention times just before synthetic MTX(G_3) and MTX(G_4). Third, when [^{3}H]IAHQ was used as a substrate for purified rat liver folylpolyglutamate synthetase [11], the products eluted at the same retention times as the synthetic IAHQ(G_{1-2}) and the late eluting [^{3}H] from cell extracts. These results indicate that the late eluting peaks (retention time = 49 and 58 min respectively)

represent IAHQ(G_3) and IAHQ(G_4) respectively. Further evidence that these later peaks were [^{3}H]IAHQ polyglutamates was obtained by hydrolysis of the products with purified chicken pancreas gamma-glutamyl hydrolase as previously described [10]. This enzyme hydrolyzes only polyglutamates longer than diglutamate species, yielding a diglutamate product. The radioactivity eluting at positions assigned to longer derivatives in buffer control incubations all eluted with the diglutamate in the hydrolase-treated samples. This is consistent with their being IAHQ poly-gamma-glutamates.

Estimation of protein binding by dialysis. [^{3}H]IAHQ-containing complete medium (RPMI 1640 containing 10% horse serum) from the 96-hr uptake experiment (Fig. 2) was placed in a cellulose dialysis sac (Union Carbide) and dialyzed at 4° against phosphate-buffered saline. The appearance of radioactivity in the external fluid was measured. [^{3}H]IAHQ was added at the same final concentration and level of radioactivity to an identical volume of RPMI 1640 lacking horse serum. Dialysis was carried out as described above.

RESULTS AND DISCUSSION

Modifications of the substituents of the pyrimidine nucleus in analogues of folic acid change the relative inhibitory potency of these compounds toward dihydrofolate reductase and thymidylate synthase [12]. IAHQ is a 2-amino-4-hydroxyquinazoline folate analog that specifically inhibits intracellular thymidylate synthase with little, if any, effect on dihydrofolate reductase. In addition, IAHQ is very active *in vitro* against both MTX and 5-fluorodeoxyuridine-resistant cells [7, 13]. The present study has focused on uptake and metabolism of IAHQ in HCT-8 cells, and these have been compared with MTX to strengthen the conclusion that these drugs have different mechanisms of action. These studies also tested our hypothesis that polyglutamates of IAHQ are essential to its cytotoxic mechanism.

Uptake of [^{3}H]*MTX and* [^{3}H]*IAHQ.* Uptake of 1 μM [^{3}H]MTX proceeded linearly for 10 min and then gradually declined in rate reaching a plateau in about 45 min (Fig. 1). Essentially all of the intracellular label at the 45-min point co-eluted with MTX on HPLC analysis, indicating that there was no degradation and little or no polyglutamate formation. The level of [^{3}H]MTX accumulation at 45 min was reduced up to 85% if incubation was at 0°, indicating that this was a mediated process. Simultaneous inclusion of 10 μM IAHQ did not affect [^{3}H]MTX transport (data not shown) and 100 μM IAHQ only reduced the initial rate by 34% and the plateau level by 32%, indicating either that these two drugs do not share the same uptake mechanism or that the K_t for IAHQ is extremely high. Interestingly, leucovorin ([*dl*]-5-formyltetrahydrofolate), which shares a high affinity transport system with MTX in most cell lines [14], at 200 μM only inhibited the initial velocity and plateau level of uptake of 1 μM [^{3}H]MTX by 60%. Since leucovorin at low concentrations (0.5 μM) protects this line against MTX at an ED_{95} level [7], it must be taken up and may utilize a separate transport system from MTX in HCT-8 cells. Similar results with leucovorin inhibition of [^{3}H]MTX uptake have been obtained in two other colon carcinoma cell lines: the well-differentiated LoVo [15] and the poorly-differentiated SW 620 [16], where it was most striking. It is possible that the low competition for the reduced folate carrier between MTX and LV is a peculiar phenomenon of colonic cells which might be exploited to therapeutic advantage.

Preliminary studies indicated a much slower time course for 1 μM [^{3}H]IAHQ uptake, so the course was extended to 96 hr (Fig. 2). The initial sharp rise in counts may represent surface adsorption and not actual uptake. Consistent with this hypothesis is the observation (see Materials and Methods) that a fraction of the cell-associated radioactivity could not be extracted. At 96 hr the cells contained 1.3 pmol drug/10^6 cells compared with MTX uptake of 2.1 pmol/10^6 cells in 45 min and 5.9 pmol/10^6 cells of MTX accumulated at 4 hr (Table 1B). This extremely slow uptake of IAHQ may explain the strong dependence of cytotoxicity on length of drug exposure described

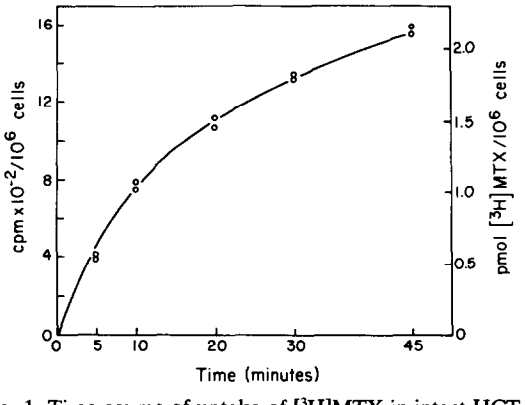

Fig. 1. Time course of uptake of [^{3}H]MTX in intact HCT-8 cells. Cells were incubated with 1 μM (1 × 10^6 cpm/nmol) [^{3}H]MTX and assayed for uptake as described in Materials and Methods. This experiment was repeated at least five times with similar results.

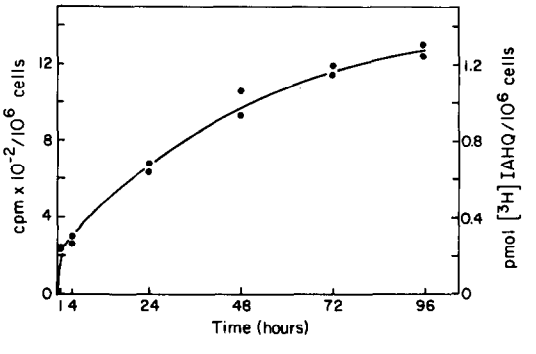

Fig. 2. Time course of uptake of [^{3}H]IAHQ in intact HCT-8 cells. Cells were incubated with 1 μM [^{3}H]IAHQ (1.4 × 10^6 cpm/nmol) and assayed for uptake as described in Materials and Methods. This experiment was performed up to 96 hr only once in order to conserve radioactive material. The results of preliminary experiments looking at shorter times of uptake as well as experiments examining metabolism of [^{3}H]IAHQ as a function of time (described in Results) were consistent with this time course of uptake.

Table 1. Formation of IAHQ and MTX polyglutamates in intact HCT-8 cells

A. Synthesis of IAHQ polyglutamates in HCT-8 cells after a 48-hr exposure to [^{3}H]IAHQ

Medium concn. (μM)	Intracellular drug						
	IAHQ	IAHQ(G_1)	IAHQ(G_2)	IAHQ(G_3)	IAHQ(G_4)	Total IAHQ(G_n)	IAHQ + IAHQ(G_n)
1	2.15	0.149	0.091	0.054	0.019	0.313	2.46
10	22.35	1.35	0.81	0.54	0.25	2.95	25.3

B. Synthesis of MTX polyglutamates in HCT-8 cells after a 4-hr exposure to [^{3}H]MTX

Medium concn. (μM)	Intracellular drug						
	MTX	MTX(G_1)	MTX(G_2)	MTX(G_3)	MTX(G_4)	Total MTX(G_n)	MTX + MTX(G_n)
1	2.99	2.15	0.49	0.23	0	2.87	5.86

Procedures are described in Materials and Methods. [^{3}H]IAHQ was present at specific radioactivities of 7.3×10^6 cpm/nmol (1 μM) or 7.4×10^5 cpm/nmol (10 μM). [^{3}H]MTX was present at 7.25×10^5 cpm/nmol. Each experiment was repeated twice with similar results. All values are given as pmol/10^6 cells.

previously [7]. An attempt was made to increase the uptake of IAHQ by synthesizing the di-*tert*-butyl ester. This compound was not cytotoxic to HCT-8 cells even at 20 μM (data not shown). A similar lack of potency of this di-ester was observed with other human gastrointestinal adenocarcinoma cell lines [6]. Competition for [^{3}H]IAHQ uptake by leucovorin and MTX was not measured because their own rapid uptake and subsequent metabolism would not allow unambiguous conclusions to be drawn.

An alternative explanation for the low uptake was that IAHQ was tightly bound to serum proteins in the medium and thus unavailable for transport. The medium removed from the 96-hr uptake experimental point was dialyzed against phosphate-buffered saline. Radioactivity appeared in the dialysate with the same kinetics as those observed when [^{3}H]IAHQ was dialyzed out of medium lacking serum. The slow uptake was thus not a result of tight binding to serum proteins.

Metabolism of [^{3}H]*MTX and* [^{3}H]*IAHQ*. A major part of our hypothesis concerning IAHQ cytotoxicity is that polyglutamate derivatives of IAHQ are synthesized intracellularly. Synthesis of IAHQ polyglutamates was examined in HCT-8 cells following a 48-hr exposure to either 1 or 10 μM [^{3}H]IAHQ (Table 1). Total intracellular drug was proportional to the extracellular drug concentration. At both concentrations the same percentage of labeled drug (12%) was converted to polyglutamates; however, the absolute levels of polyglutamates were 10-fold higher at 10 μM. Polyglutamates containing up to four additional glutamates were detected at both concentrations. In a separate, single experiment (data not shown), dependence of accumulation and polyglutamate formation on the medium concentration of IAHQ was verified. In addition, time dependence of both accumulation and polyglutamate synthesis at a fixed concentration of IAHQ (10 μM) in the incubation medium was observed. Finally, when [^{3}H]IAHQ was removed from the extracellular medium following a 48-hr incubation at 10 μM, there was extensive loss of unmetabolized IAHQ in 1.5 hr, but the polyglutamates of IAHQ, in particular IAHQ(G_3) and IAHQ(G_4), were retained and their concentration declined only slightly over 19 hr. This finding is similar to that seen with retention of MTX polyglutamates [17]. It should be noted that a 48-hr exposure to 1 μM IAHQ is non-toxic while at 10 μM approximately 40% cell kill is observed in a clonogenic assay with HCT-8 cells [7]. Thus, synthesis of larger amounts of longer IAHQ polyglutamates is associated with increased cytotoxicity.

Preliminary studies of the metabolism to polyglutamates in mice of a related TS inhibitor, 10-propargyl-5,8-dideazafolic acid (CB3717), have been reported recently [18]. Longer polyglutamate species of CB3717 were also noted, but toxicity or cytotoxicity was not assessed.

MTX polyglutamate synthesis in HCT-8 cells was examined after a 4-hr exposure to 1 μM [^{3}H]MTX (Table 1). This exposure gives cell kill (20–40%) in the clonogenic assay which is comparable to that seen with the 48-hr exposure to 10 μM IAHQ [7]. The total MTX accumulated at 4 hr was less than that seen at 48 hr with IAHQ, but MTX polyglutamates were present at about the same absolute level as with IAHQ. In the 4-hr exposure, MTX polyglutamates containing up to three additional glutamates were observed. These results indicate that HCT-8 cells are efficient at glutamylation, in general. Since we have shown previously that IAHQ is a better substrate than MTX for HCT-8 cell folylpolyglutamate synthetase [7], the lower uptake of IAHQ was probably responsible for its lower conversion to polyglutamates.

These results can be related to the cytotoxic mechanism of IAHQ. There are positive correlations between the level of total drug, parent IAHQ, or IAHQ polyglutamates (Table 1) and the degree of cytotoxicity [7]. Since unmetabolized IAHQ is only a weak inhibitor of its target enzyme thymidylate synthase [4, 7], accumulation of IAHQ itself (or as part of total drug) is unlikely to be significant in

terms of cytotoxicity. However, since IAHQ polyglutamates are much more potent inhibitors of thymidylate synthase [4, 7], their accumulation, particularly of long chain lengths, could provide the necessary enzyme blockade to achieve cytotoxicity. Thus, cytotoxicity seems to correlate best with the synthesis and accumulation of IAHQ polyglutamates. These results support our hypothesis that IAHQ is a prodrug which must undergo "lethal synthesis" to its polyglutamate derivatives before it can exert cytotoxic inhibition at its target enzyme, thymidylate synthase.

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