Cancer Chemother Pharmacol (1986) 18: 231–234

Cancer Chemotherapy and Pharmacology

Effects of 5,8-dideazaisopteroylglutamate (IAHQ) on L1210 leukemia in mice when given alone and in combination with methotrexate, probenecid, or verapamil*

John B. Hynes[1], Alayne B. Smith[2], and Glen R. Gale[2, 3]

[1] Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC 29425, USA
[2] Veterans Administration Medical Center, Charleston, SC 29403, USA
[3] Department of Pharmacology, Medical University of South Carolina, Charleston, SC 29425, USA

Summary. The folate analogue 5,8-dideazaisopteroylglutamate (IAHQ; NSC-289517) inhibits the growth of a variety of human tumor cells in vitro such as colon, breast and osteosarcoma. Since IAHQ has only modest activity against L1210 leukemia in mice, it was tested in combination with methotrexate (MTX), probenecid, or verapamil in an effort to enhance efficacy. Single drug or drug combinations were administered every other day 3 or 5 times beginning on day 1 following the administration of 10^6 L1210 cells per animal. The combination of IAHQ (100 mg/kg) plus MTX (10 mg/kg) produced a decrease in mean survival time compared to that of MTX alone, regardless of whether the drugs were initiated on the same day or whether either one was started 2 days prior to the other. IAHQ (150 mg/kg) plus verapamil (5, 10, or 20 mg/kg) did not alter significantly the results produced by IAHQ alone. However, the combination of IAHQ (150 mg/kg) plus probenecid (250 mg/kg) augmented the increase in mean survival time above that produced by IAHQ alone by 82% ($p = < 0.001$). The results suggest that probenecid could be used to enhance the effectiveness of IAHQ against solid tumors such as colon adenocarcinoma.

Introduction

The folate analogue 5,8-dideazaisopteroylglutamate (IAHQ) was first described in 1975 as part of an ongoing synthetic program concerned with quinazoline analogues of folic acid as potential chemotherapeutic agents [6]. However, its potential value as an antitumor agent was not realized until later, when it was found to inhibit the growth of human colon adenocarcinoma cells (HCT-8) in vitro [2]. When evaluated against colon tumor 38 in mice, IAHQ delayed tumor growth, and six of 20 animals survived over 90 days and were found to be tumor-free [2]. Methotrexate (MTX) was not effective in this model [4]. It was also found that IAHQ protected newborn hamsters from mortality due to transplantable human osteosarcoma cells, whereas MTX was without effect at its maximal tolerated dose [17]. An improved method of synthesis of IAHQ and its 9-methyl (9-CH_3-IAHQ) and 9-formyl (9-CHO-IAHQ) derivatives was recently described [8]. This report showed that IAHQ and 9-CH_3-IAHQ have similar levels of inhibitory potency against the growth of four human gastrointestinal adenocarcinoma cell lines in vitro, while 9-CHO-IAHQ is far less active [8].

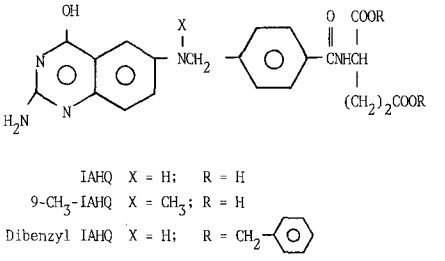

Fig. 1. Structures of IAHQ and its derivatives

Several years ago, IAHQ was selected for antitumor evaluation in the tumor panel by the National Cancer Institute. In addition to retarding the growth of the human CX-1 colon tumor xenograph in the nude mouse, IAHQ also displayed marginal activity toward both P388 and L1210 leukemias in mice at doses of 200, 100, and 50 mg/kg respectively. IAHQ was not active in the other six standard evaluation systems of the panel according to the criteria established by the National Cancer Institute. This study describes efforts to enhance the therapeutic efficacy of IAHQ against L1210 leukemia in mice by administering it in combination with the established folate antagonist MTX; the uricosuric agent probenecid; or the calcium channel blocker verapamil. In addition, 9-CH_3-IAHQ and the newly synthesized dibenzyl ester of IAHQ were evaluated in the L1210 model. Structures of IAHQ and its derivatives which were studied are shown in Figure 1.

Methods

Probenecid was obtained from Sigma Chemical Co., St. Louis, MO., while verapamil hydrochloride was obtained

* Supported by Public Health Service Grant CA-25014 (National Cancer Institute) from the National Institutes of Health, Department of Health and Human Services, by the Veterans Administration, and by VA-NCI Interagency Agreement IGA V101 (134A) P-77014. Presented in part at the Annual Meeting of the Southeastern Section, Society for Experimental Biology and Medicine, Charleston, SC, November 14–15, 1985.

Offprint requests to: John B. Hynes, Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC 29425, USA

from Knoll Pharmaceutical Co., Whippany, NJ. MTX was a gift from Dr. J. A. R. Mead, National Cancer Institute, Bethesda, MD. IAHQ and 9-CH_3-IAHQ were resynthesized as described previously [8]. Dibenzyl 5,8-dideazaisopteroyl-*L*-glutamate (dibenzyl IAHQ) was prepared as follows: A mixture of dibenzyl *L*-glutamate *p*-toluenesulfonate [1] (0.51 g, 1 mmol), 5,8-dideazaisopteroic acid [7] (0.36 g, 1 mmol), triethylamine (0.50 g, 5 mmol), and diethyl phosphorocyanidate (0.48 g, 3 mmol) in 45 ml dimethylformamide was stirred at 50 °–55 °C for 18 h. After removal of the solvent at reduced pressure, the resulting oil was triturated with Et_2O and then H_2O. The solid was separated by filtration, washed with H_2O, $CHCl_3/Et_2O$ (1:1), and then Me_2CO/Et_2O (1:1). After drying in vacuo at 60 °C, 0.40 g (66% yield) yellow solid, mp 150 °–155 °C, was obtained. ^1H NMR (dimethyl sulfoxide-d_6; Varian EM-390 Spectrometer): δ 8.50–5.40 (*m*, 22, aromatic + NH_2 + 3NH), 5.10 (*s*, 2, $CH_2C_6H_5$), 5.03 (*s*, 2, $CH_2C_6H_5$), 4.70–4.20 (*m*, 3, NCH_2 + NCH), 2.40–1.83 [*m*, 4, $(CH_2)_2$]. *Anal:* ($C_{35}H_{33}N_5O_6 \cdot 2.5\ H_2O$). *Calcd:* C, 63.24; H, 5.76; N, 10.53. *Found:* C, 63.08; H, 5.31; N, 10.16.

The L1210 subline was obtained from the Mammalian Genetics and Animal Production Section, NCI, Frederick, MD. It was serially propagated in DBA/2 mice (Simonsen Laboratories, Gilroy, Calif), and testing was done in BDF_1 male mice (Simonsen). Compounds were administered intraperitoneally on the days indicated following intraperitoneal implantation of 10^6 cells on day 0. MTX and IAHQ were dissolved in 0.1 *N* NaOH immediately prior to administration and diluted with 0.9% NaCl to achieve the appropriate concentrations for injection. Probenecid was dissolved in 1.0 *N* NaOH and diluted with phosphate buffer (pH 7.4), while verapamil hydrochloride was dissolved in 0.9% NaCl. Dimethylacetamide was employed as the vehicle for 9-CH_3-IAHQ and dibenzyl IAHQ. In a separate experiment (data not shown), the increase in mean survival time (ILS) obtained for MTX (10 mg/kg) dissolved in dimethylacetamide was 128%. This value is not significantly different from that obtained when MTX was administered dissolved in dilute base (*cf.* Table 1). Statistical evaluation of data was done by analysis of variance using a microcomputer.

Results

Initial studies were concerned with the effects of IAHQ administered Q2DX3 against L1210 leukemia in mice as shown in Table 1. The alternating day approach has been shown to produce optimum results with MTX [15]. Higher doses of IAHQ (250 and 300 mg/kg, Q2DX5) produced reduced ILS values, although no early deaths indicative of toxicity were observed (data not shown). In addition, treatment Q2DX5 with 150 mg/kg IAHQ (Table 2) was no more effective than administration of the same dose on days 1, 3, and 5 only. Cotreatment with MTX (10 mg/kg) and IAHQ (100 mg/kg) was less effective than MTX alone (10 mg/kg). Therefore, higher doses of IAHQ in combination with MTX were not evaluated.

Results obtained using IAHQ in combination with probenecid are presented in Table 2. The dosages of probenecid employed were based upon earlier studies of the effects of this agent in combination with MTX in the treatment of L1210 leukemia and Sarcoma 180 ascites in mice [15]. While probenecid at 125 mg/kg did not alter significantly the effectiveness of IAHQ (150 mg/kg), at 250 mg/kg probenecid improved the ILS to 69% ($p = < 0.001$). As shown in Table 3, cotreatment with verapamil did not cause a statistically significant increase in mean survival time over that produced by IAHQ alone (150 mg/kg) at any of the verapamil doses evaluated. 9-CH_3-IAHQ dissolved in dimethylacetamide showed evidence of toxicity at 200 mg/kg when given Q2DX5. At lower doses (5, 10,

Table 1. Effects of IAHQ alone and in combination with MTX against L1210 leukemia in mice

IAHQ (mg/kg)	MTX (mg/kg)	Treatment (days)	MST (days)	ILS (%)
Control	–	–	7.2	–
100	–	1,3,5	9.1	28
150	–	1,3,5	10.0	39
200	–	1,3,5	10.8	51
–	10	1,3,5	16.9	136
100	10	1,3,5	12.3	72*
100	10	3,5 (IAHQ) 1,3,5 (MTX)	12.4[a]	73*
100	10	1,3,5 (IAHQ) 3,5 (MTX)	11.0	54*

MST, mean survival time; ILS, increase in mean survival time
[a] One animal died on day 5 and was included in the ILS calculation for this group
* $P < 0.001$ with respect to MTX alone

Table 2. Antitumor effects of IAHQ in combination with probenecid against L1210 leukemia in mice

IAHQ (mg/kg)	Probenecid[a] (mg/kg)	Treatment (days)	MST (days)	ILS (%)
Control	–	–	7.7	–
150	–	1,3,5,7,9	10.6	38
150	125	1,3,5,7,9	10.2	32
150	250	1,3,5,7,9	13.0	69*

Abbreviations as in Table 1
[a] Earlier studies conducted in these laboratories have shown that probenecid is without effect against this tumor model [13]
* $P < 0.001$ with respect to IAHQ alone

Table 3. Antitumor effects of IAHQ in combination with verapamil against L1210 leukemia in mice

Experiment	IAHQ (mg/kg)	Verapamil (mg/kg)	Treatment (days)	MST (days)	ILS (%)
A	Control	–	–	7.5	–
	–	5	1,3,5,7,9	7.9	5
	150	–	1,3,5,7,9	10.5	40
	150	5	1,3,5,7,9	11.3	51*
B	Control	–	–	7.4	–
	–	10	1,3,5,7,9	7.7	4
	–	20	1,3,5,7,9	7.3	–
	150	–	1,3,5,7,9	11.1	50
	150	10	1,3,5,7,9	10.3	39
	150	20	1,3,5,7,9	12.0	62*

Abbreviations as in Table 1
* $P < 0.05$ with respect to IAHQ alone

25, and 100 mg/kg) there was no evidence of antitumor activity (data not shown). Dibenzyl IAHQ was dissolved in dimethylacetamide and evaluated using the same schedule at doses of 150, 300, and 450 mg/kg. This derivative of IAHQ was also devoid of antitumor activity at each of these levels (data not shown).

Discussion

The existing evidence suggests that the cytotoxic action of IAHQ is due to its intracellular conversion to polyglutamyl metabolites which are superior inhibitors of thymidylate synthase, particularly in the presence of elevated levels of dUMP [2], which result from this inhibition. Since the primary mode of action of MTX is inhibition of the enzyme dihydrofolate reductase (DHFR), it was of interest to evaluate the effects of MTX and IAHQ in combination. Two agents, each of which is capable of reducing levels of thymidylate via different mechanisms, could potentially achieve greater cell-killing action in combination than either compound employed alone.

The results in Table 1 show that the combination of IAHQ (100 mg/kg) and MTX (10 mg/kg) produces antagonistic effects compared to those due to MTX alone, regardless of whether they are initiated simultaneously or whether either agent is initiated 2 days prior to the other. It has been suggested that intracellular conversion of MTX to polyglutamate metabolites is an important determinant in maintaining MTX concentrations above those of the target enzyme DHFR [11]. A reduction in MTX polyglutamate formation would lead to more rapid efflux of MTX from cells, thereby permitting a resumption of DNA synthesis [3]. Since IAHQ has been shown to be superior to MTX as a substrate for folylpolyglutamate synthetase from HCT-8 cells [10], its presence at levels higher than MTX may result in reduction of the polyglutamylation of MTX.

Preliminary studies using ^{3}H-IAHQ showed that its rate of uptake into HCT-8 cells is much slower than that of ^{3}H-MTX [16]. Therefore, 9-CH_3-IAHQ was evaluated in the hope that this lipophilic analogue could more readily gain entry into tumor cells. While this compound had a level of inhibitory activity against the growth of human gastrointestinal adenocarcinoma cells similar to that of IAHQ in vitro [8], it was found to be devoid of activity against L1210 leukemia in vivo. The potential prodrug form of IAHQ, dibenzyl IAHQ, was prepared according to the method of Rosowsky and Yu [12]. Several MTX esters of this type were found to be more active than MTX against L1210 leukemia in mice [12]. When dibenzyl IAHQ was administered dissolved in dimethylacetamide, no increase in mean survival time or evidence of toxicity was observed, even at doses as high as 450 mg/kg. It is likely that this compound forms a precipitate in body fluids which is extremely insoluble and incapable of reaching and/or entering cells. Alternatively, it may not be converted to the active antimetabolite by intracellular esterase activity.

Next, the effect of the uricosuric agent probenecid upon the activity of IAHQ against L1210 leukemia in mice was evaluated. A less than optimal dose level of IAHQ was selected in order to avoid potential toxicity. This approach was patterned after studies with MTX in combination with probenecid, where enhanced activity against both L1210 leukemia and Sarcoma 180 ascites tumor in mice was observed [15]. In this study, the simultaneous administration of probenecid at 125 mg/kg and various doses of MTX produced substantial increases in ILS, while probenecid alone had no antitumor effect [15]. Combinations of probenecid (250 mg/kg) with MTX at dosages above 6 mg/kg were toxic, while with lower dosages of MTX the ILS values did not exceed those produced by MTX alone [15]. From the results presented in Table 2, it will be seen that the addition of 250 mg/kg probenecid yielded an ILS 82% higher than that produced by IAHQ (150 mg/kg) alone. However, at 125 mg/kg probenecid failed to augment the antitumor effectiveness of IAHQ. In vitro studies have shown that probenecid inhibites efflux of ^{3}H-MTX from L1210 cells more effectively than its influx, thus elevating the intracellular concentration of MTX [5]. However, additional studies using ^{3}H-IAHQ will be required in order to determine whether the positive effect of probenecid upon the activity of IAHQ is mediated at the membrane level or is due to inhibition of its renal clearance, which would prolong its biological half-life.

The calcium channel blocker verapamil has been shown to enhance the cytotoxicity of vincristine and adriamycin toward P388 leukemia cells as well as P388 sublines resistant to these agents [19]. The increased cellular accumulation of drug due to verapamil has been attributed to inhibition of the drug efflux mechanism, which can become more efficient in resistant cell populations. Verapamil was also shown to enhance the efficacy of vincristine against drug-sensitive P388 leukemia in $CD2F_1$ mice, and in two treatment schedules cures were obtained [18]. The apparent drawback in this study was the potentially toxic dose of verapamil (75 mg/kg) which was required to produce these results [18]. More recently, the efficacy of etoposide (VP-16) against both L1210 leukemia and P388 leukemia was markedly enhanced by the addition of verapamil (25 mg/kg) to the therapeutic regimen [14]. The effects of IAHQ in combination with verapamil against L1210 leukemia in mice are shown in Table 3. We found that a single 100 mg/kg injection of verapamil to BDF_1 mice was rapidly lethal. Therefore, doses of 5, 10, and 20 mg/kg verapamil with IAHQ (150 mg/kg) on a Q2DX5 schedule were evaluated. Using 5 or 20 mg/kg of verapamil, the ILS values were enhanced with respect to IAHQ alone, but the increases were not significant. The use of a less toxic calcium channel blocker [9] might improve these results.

These studies suggest that the antitumor potential of IAHQ can be enhanced through the use of pharmacologic modulating agents. However, refinements in the schedules and doses employed will be necessary. The combination of probenecid and IAHQ appears promising and should be evaluated against slower-growing tumors, particularly in models of colon adenocarcinoma.

References

1. Bose AK, Strube RE (1963) Some derivatives of aspartic and glutamic acids. J Pharm Sci 52: 847
2. Fernandes DJ, Bertino JR, Hynes JB (1983) Biochemical effects of 5,8-dideazaisopteroylglutamate, a unique quinazoline inhibitor of thymidylate synthase. Cancer Res 43: 1117
3. Galivan J (1980) Evidence for the cytotoxic activity of polyglutamate derivatives of methotrexate. Mol Pharmacol 17: 105
4. Goldin A, Venditti JM, Mac Donald JS, Muggia JS, Henney JE, DeVita VT (1981) Current results of the screening pro-

gram at the Division of Cancer Treatment, National Cancer Institute. Eur J Cancer 17: 129
5. Henderson GB, Zevely EM (1985) Inhibitory effects of probenecid on the individual transport routes which mediate the influx and efflux of methotrexate in L1210 cells. Biochem Pharmacol 34: 1725
6. Hynes JB, Garrett CM (1975) Synthesis of quinazoline analogs of isofolic acid. J Med Chem 18: 632
7. Hynes JB, Buck JM, D'Souza L, Freisheim JH (1975) Quinazolines as inhibitors of dihydrofolate reductase. 3. Analogs of pteroic and isopteroic acids. J Med Chem 18: 1191
8. Hynes JB, Yang YCS, McGill JE, Harmon SJ, Washtien WL (1984) Improved synthesis and antitumor evaluation of 5,8-dideazaisofolic acid and closely related analogues. J Med Chem 27: 232
9. Kessel D, Wilberding C (1985) Promotion of daunorubicin uptake and toxicity by the calcium antagonist tiapamil and its analogs. Cancer Treat Rep 69: 673
10. McGuire JJ, Mini E, Hsieh P, Bertino JR (1983) Folylpolyglutamate synthetase: relation to methotrexate action and as a target for new drug development. In: Chene Y-C, Goz B, Minkoff M (eds) Progress in cancer research and therapy. 28. Development of target-oriented anticancer drugs. Raven, New York, p 97
11. Rosenblatt DS, Whitehead VM, Vera N, Pottier A, Dupont MM, Vuchich MJ (1978) Prolonged inhibition of DNA synthesis associated with the accumulation of methotrexate polyglutamates by cultured human cells. Mol Pharmacol 14: 1143
12. Rosowsky A, Yu C-S (1983) Methotrexate analogues. 18. Enhancement of the antitumor effect of methotrexate and 3′, 5′-dichloromethotrexate by the use of lipid-soluble diesters. J Med Chem 26: 1448
13. Ross DA, Gale GR (1979) Reduction of the renal toxicity of cis-dichlorodiammine platinum (II) by probenecid. Cancer Treat Rep 63: 781
14. Siater LM, Murry SL, Wetzel MW, Sweet P, Stupecky M (1986) Verapamil potentiation of VP-16-213 in acute lymphatic leukemia and reversal of pleiotropic drug resistance. Cancer Chemother Pharmacol 16: 50
15. Sirotnak FM, Moccio DM, Hancock DH, Young CW (1981) Improved methotrexate therapy of murine tumors obtained by probenecid mediated pharmacological modulation at the level of membrane transport. Cancer Res 41: 3944
16. Sobrero AF, McGuire JJ, Fernandes DJ, Hynes JB, Bertino JR (1983) Biochemical and antitumor effects of 5,8-dideazaisopteroylglutamate. In: A decade of achievements and challenges in large bowel cancer research 1972–1982. Workshop, Houston, June 23–26, abstracts, p 249
17. Tsang K-Y, Hynes JB, Fudenberg HH (1982) Effects of 5,8-dideazaisofolic acid (IAHQ) on human tumor cells in culture and on normal and tumor-bearing hamsters. Chemotherapy 28: 276
18. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y (1985) Cure of mice bearing P388 leukemia by vincristine in combination with a calcium channel blocker. Cancer Treat Rep 69: 523
19. Tsuruo T, Iida H, Yamashiro M, Tsukagoshi S, Sakurai Y (1982) Enhancement of vincristine- and adriamycin-induced cytotoxicity by verapamil in P388 leukemia and its sublines resistant to vincristine and adriamycin. Biochem Pharmacol 31: 3138

Received April 24, 1986/Accepted August 22, 1986