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STRUCTURE–ACTIVITY RELATIONSHIP OF QUINOLINE CARBOXYLIC ACIDS

A NEW CLASS OF INHIBITORS OF DIHYDROOROTATE DEHYDROGENASE

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Abstract—The novel anticancer drug candidate brequinar sodium [DuP 785, NSC 368390, 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt] inhibits dihydroorotate dehydrogenase, the fourth enzyme in the *de novo* pyrimidine biosynthetic pathway leading to the formation of UMP. Sixty-nine quinoline 4-carboxylic acid analogs were analyzed as inhibitors of L1210 dihydroorotate dehydrogenase. This structure–activity relationship study identified three critical regions of brequinar sodium and its analogs, where specific substitutions are required for the inhibition of the activity of dihydroorotate dehydrogenase. The three principal regions are: (i) the C(2) position where bulky hydrophobic substituents are necessary, (ii) the C(4) position which has a strict requirement for the carboxylic acid and its corresponding salts, and (iii) the benzo portion of the quinoline ring with appropriate substitutions. These results will be useful in the elucidation of the precise nature of the interaction between brequinar sodium and dihydroorotate dehydrogenase.

The novel anticancer drug candidate brequinar sodium [DuP 785, NSC 368390, 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt, Fig. 1] inhibits the growth of a broad spectrum of human solid tumors implanted in nude mice [1]. Because of its activity against experimental tumors, brequinar sodium was selected for further development, and the drug candidate is now in Phase 2 clinical trials. It has been shown previously that brequinar sodium exerts its tumoricidal activity by inhibiting the activity of dihydroorotate dehydrogenase, the fourth enzyme in the *de novo* pyrimidine biosynthetic pathway [2, 3]. The structure of brequinar sodium does not resemble that of the substrate or cofactor required in this enzymatic reaction (Fig. 2), and brequinar sodium inhibits the activity of dihydroorotate dehydrogenase non-competitively with respect to either the substrate (dihydroorotate) or the cofactor (ubiquinone) [4]. Therefore, studies were undertaken to determine the structure–activity relationship of this series of quinoline carboxylic acids to understand the nature of the drug–enzyme interaction and the role of various pharmacophores on the activity of the compound. Our results demonstrate several important regions of the molecule that are essential for inhibiting the enzyme activity. Portions of this work have been presented in a preliminary form [5].

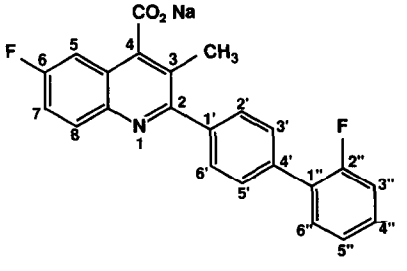

Fig. 1. Chemical structure and numbering system of brequinar sodium.

MATERIALS AND METHODS

Materials and chemicals. All of the quinoline carboxylic acid analogs including brequinar sodium were synthesized by the Medicinal Chemistry Section, Pharmaceuticals and Biotechnology Research and Development Division, Du Pont Medical Products Department [6]. L-[*carboxyl*-^{14}C]Dihydroorotate was synthesized enzymatically from [*carboxyl*-^{14}C]orotate and purified as previously described [2]. Ubiquinone (Q_{30}) was purchased from the Sigma Chemical Co. (St. Louis, MO). [*carboxyl*-^{14}C]Orotic acid was obtained from New England Nuclear Research Products (Boston, MA).

Enzyme assay. Dihydroorotate dehydrogenase was partially purified from murine leukemia L1210 mitochondria [2]. The activity of dihydroorotate dehydrogenase was determined by the direct conversion of L-[*carboxyl*-^{14}C]dihydroorotate to

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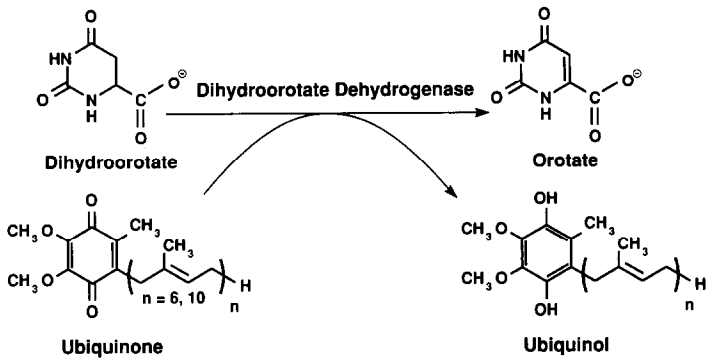


Fig. 2. Reaction catalyzed by L1210 dihydroorotate dehydrogenase.

[*carboxyl*-^{14}C]orotate. The reaction mixture containing 67 mM Tris–HCl buffer (pH 7.4), 5 mM KCN, 600 μM Q_{30}, and 4 μg of Lubrol PX-solubilized L1210 mitochondria (1 mg protein : 0.3 mg Lubrol PX) with or without test analogs in a total volume of 160 μL was incubated at room temperature for 30 min. After adding 40 μL of L-[*carboxyl*-^{14}C]dihydroorotate (final concentration 10 μM), a sample (20 μL) of the reaction mixture was removed every minute (1–8 min) and spotted on Whatman DE-81 chromatographic paper containing 50 pmol of nonradiolabeled carrier orotate at the origin. The chromatograms were developed with 0.4 N formic acid for 5 hr. The spots containing [*carboxyl*-^{14}C]orotate were detected by irradiation with 254 nm ultraviolet light and cut out; the amounts of radioactivity were determined in Biofluor using a Packard Tricarb scintillation counter.

Inhibition constant (apparent K_i*) determination.* The initial velocity of orotate formation in the presence of various inhibitor concentrations was determined at a fixed concentration of dihydroorotate (10 μM). A Dixon plot (the plot of 1/rate of orotate formation versus test analog concentration) was constructed from the data; the intersection point with the x-axis allowed estimation by linear regression of the apparent K_i value. The ratio obtained by dividing the apparent K_i value of the test analog by that of the brequinar sodium (Compound 1, apparent K_i = 25 nM) was used as an index to determine whether a particular modification of the molecule had any effect on inhibitory activity.

RESULTS

The enzymatic inhibitory activities of sixty-nine quinoline carboxylic acids and their corresponding salts were determined. In all analogs tested, the free carboxylic acid and its corresponding salts equally inhibited dihydroorotate dehydrogenase (data not shown). Therefore, the data presented below are independent of whether the free acid or its salt was used in the study.

Quinoline carboxylic acid analogs were tested as inhibitors of L1210 dihydroorotate dehydrogenase. The K_m of the substrate, dihydroorotate, was 1.5 μM for the L1210 enzyme. The inhibitory activities were evaluated by determining the apparent K_i values and by calculating the ratios between the apparent K_i of the test analogs and that of brequinar sodium. Those analogs with apparent K_i lower than 15 nM were better inhibitors than brequinar sodium. The analogs with apparent K_i = 15–50 nM (ratio 0.6 to 2) were considered to be as potent as brequinar sodium. The analogs with apparent K_i > 250 nM (ratio > 10) were significantly less active than brequinar sodium. The analogs with apparent K_i > 10 μM (ratio > 400) were considered inactive compounds.

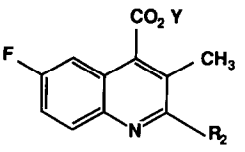
Table 1 lists a series of analogs with C(2) substitutions. The substitution on the second phenyl ring generally did not affect the binding affinity toward dihydroorotate dehydrogenase (compounds 1–11). The biphenyl group could be replaced with an appropriate bulky hydrophobic group, e.g. 4-cyclohexylphenyl (compound 14) or 4-*t*-butylphenyl (compound 23). However, the analogs with only one ring substituted at the C(2) position, e.g. phenyl (compound 22), or 2-furfuryl (compound 39), were inactive compounds. Inserting one or two atoms between the biphenyl rings also had no effect on the inhibitory activity (compounds 26–28, 31, and 32). Replacing the biphenyl ring with a fused ring was also acceptable (compounds 33–35). However, the 2-naphthyl substitution (compound 34) was preferable compared to the 1-naphthyl substitution (compound 35).

The analogs with C(3) substitutions are listed in Table 2. The compounds with the methyl substitution were the best inhibitors (compounds 14 and 41). When the methyl group was replaced by a hydrogen or ethyl group, the inhibitory activity was reduced; substitution of a propyl group resulted in an even greater loss of activity.

Various C(4) substituents were also examined. Selective examples are listed in Table 3. Only the carboxylic acid and its corresponding salts (Na, K) were acceptable substituents on the C(4) position (compounds 14, 41, and 44). The compounds with an ester or an amide group substituted at the C(4) position were inactive. Reducing the carboxylic acid to carboxaldehyde or hydroxylmethyl also resulted in a substantial loss of the inhibitory acivity.

The C(6) substituents were also modified, and the results are presented in Table 4. Appropriate halogen substitutions (F, Cl) at the 6 position gave good inhibitory activity, and the activity decreased

Table 1. Effect of R_2 substitution of 6-fluoro-3-methyl-4-quinoline carboxylic acids/salts on the inhibition of dihydroorotate dehydrogenase activity



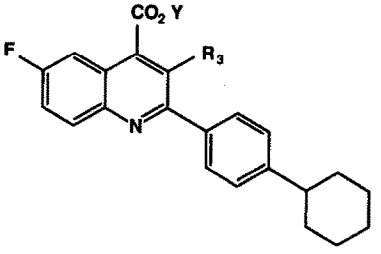
Compound	R_2	Y	appK_i (nM)	Ratio
1	4—(2—F—C_6H_4)C_6H_4	Na	25.0 ± 2.6	1.0
2	4—(3—F—C_6H_4)C_6H_4	H	52.2 ± 8.0	2.1
3	4—(4—F—C_6H_4)C_6H_4	H	58.7 ± 7.4	2.3
4	4—(4—CH_3—C_6H_4)C_6H_4	H	2.1 ± 1.7	0.1
5	4—(4—OH—C_6H_4)C_6H_4	H	39.3 ± 2.8	1.3
6	4—(4—C_2H_5—C_6H_5)C_6H_4	Na	18.3 ± 10.7	0.7
7	4—(C_6H_5)C_6H_4	H	23.5 ± 2.9	0.9
8	4—(2,4—F_2—C_6H_3)C_6H_4	Na	52.0 ± 3.9	2.1
9	4—(4—Br—C_6H_4)C_6H_4	H	112 ± 11.4	4.5
10	4—(3,4—$(CH_3)_2$—C_6H_3)C_6H_4	Na	45.3 ± 3.9	1.8
11	4—(3—Cl—4—CH_3—C_6H_3)C_6H_4	Na	49.7 ± 7.3	2.0
12	4—(3,4—Cl_2—C_6H_3)C_6H_4	Na	3,530 ± 2,560	141
13	4—(2—thenyl)C_6H_4	Na	50.3 ± 6.2	2.0
14	4—(*c*—C_6H_{11})C_6H_4	H	27.5 ± 1.6	1.1
15	4—(*c*—C_6H_9)C_6H_4	H	892 ± 2,740	36.0
16	4—(piperidine)C_6H_4	H	3,690 ± 2,710	147
17	4—(*n*—C_6H_{13})C_6H_4	H	46.3 ± 13.6	1.9
18	4—(*n*—C_4H_9O)C_6H_4	H	121 ± 17.8	4.8
19	4—(CH_3O)C_6H_4	H	3,710 ± 880	148
20	4—(C_2H_5)C_6H_4	H	93.5 ± 6.9	3.7
21	4—(Br)C_6H_4	H	3,030 ± 760	121
22	C_6H_5	H	40,300 ± 600	1,600
23	4—(*t*-butyl)C_6H_4	H	7.8 ± 1.2	0.3
24	4—($(CH_3)_2CHS$)C_6H_4	H	36.8 ± 3.2	1.5
25	4—($(CH_3)_2CHSO_2$)C_6H_4	H	6,490 ± 860	260
26	4—(C_6H_5O)C_6H_4	H	25.7 ± 5.1	1.0
27	4—($C_6H_5CH_2$)C_6H_4	H	41.7 ± 17.1	1.7
28	4—(C_6H_5S)C_6H_4	H	36.1 ± 3.7	1.4
29	4—($C_6H_5SO_2$)C_6H_4	H	1,900 ± 2,900	76
30	4—(C_6H_5SO)C_6H_4	H	4,300 ± 770	170
31	4—($C_6H_5CH_2S$)C_6H_4	H	8.9 ± 5.3	0.4
32	4—($C_6H_5CH_2CH_2$)C_6H_4	H	105 ± 9.0	4.2
33	5,6,7,8—H_4-naphthalene-2-yl	H	21.4 ± 2.0	0.9
34	Naphthalene-2-yl	Na	62.8 ± 6.4	2.5
35	Naphthalene-1-yl	Na	437 ± 56.9	17.5
36	4—(C_6H_5)C_6H_4—CH=CH—	H	20,500 ± 4,440	820
37	2—(CH_3)—4—(C_6H_5)C_6H_3	Na	73.2 ± 6.8	2.9
38	3—(C_6H_5)—4—(CH_3O)C_6H_3	H	80.6 ± 7.8	3.9
39	Furfur-2-yl	H	261,000 ± 98,500	10,500

The inhibition constant (apparent K_i ± standard deviation calculated by linear regression from the slope of the Dixon plot) was determined as described in Materials and Methods, and the ratio was calculated by dividing the apparent K_i value of the test analogs by that of brequinar sodium (compound 1 apparent K_i = 25 nM).

as the electronegativity decreased (F > Cl > Br > I). The electron-withdrawing trifluoromethyl group (compound 55) was a good substituent. However, other electron withdrawing groups (NO_2, SO_2CH_3, COONa and $COOCH_3$) substituted at the C(6) position resulted in a less active or inactive analog. Electron donating groups (methyl, ethyl, amino, hydroxy, methoxy and methylthio groups) substituted at the C(6) position also reduced the enzyme inhibitory activity.

The effect of halogen (F or Cl) substitution at the C(5), C(7), and C(8) positions on dihydroorotate dehydrogenase activity was then investigated (Table 5). The inhibitory activity was retained with the chloro substitution at the C(5) position; however, the inhibitory activity was greatly reduced with the chloro substitution at the C(7) and C(8) positions. It is of interest to note that the inhibitory activity was also reduced when chlorine was substituted on both the C(5) and C(7) positions. When the quinoline ring was replaced by a pyridine ring, that compound did not inhibit the dihydroorotate dehydrogenase activity (apparent K_i > 85,000 nM). These findings suggest that it is necessary to have an intact quinoline

Table 2. Effects of R_3 substitution of 6-fluoro-2-(cyclohexylphenyl-4-yl)-4-quinoline carboxylic acids/salts on the inhibition of dihydroorotate dehydrogenase activity



Compound	R_3	Y	appK_i (nM)	Ratio
40	H	H	122.0 ± 19.6	4.9
14	CH_3	H	27.5 ± 1.6	1.1
41	CH_3	Na	25.5 ± 5.1	1.0
42	C_2H_5	H	111 ± 62.9	4.4
43	C_3H_7	H	2870 ± 260	115

The inhibition constant (apparent K_i ± standard deviation calculated by linear regression from the slope of the Dixon plot) was determined as described in Materials and Methods, and the ratio was calculated by dividing the apparent K_i value of the test analogs by that of brequinar sodium (compound 1 apparent K_i = 25 nM).

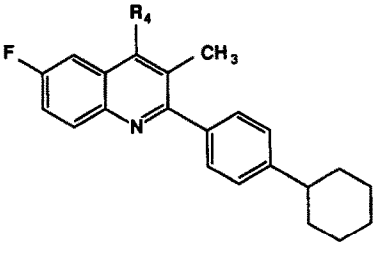
moiety with appropriate substitutions in these compounds in order to have any enzyme inhibitory activity.

DISCUSSION

We have shown previously [2], and Peters *et al.* have confirmed [3], that brequinar sodium exerts its antitumor effect by inhibiting the activity of dihydroorotate dehydrogenase, the fourth enzyme in the *de novo* pyrimidine biosynthetic pathway leading to the formation of UMP. Using partially purified dihydroorotate dehydrogenase isolated from L1210 mitochondria, brequinar sodium was found to be a potent inhibitor of this enzyme with an apparent K_i value of 25 nM. The binding affinity of brequinar sodium was approximately 100- to 1000-fold greater than that of the substrate or cofactor required in the dihydroorotate dehydrogenase catalyzed reaction. Recently, DeFrees *et al.* [7] reported several pyrimidine analogs of dihydroorotate and orotate as inhibitors of rat liver dihydroorotate dehydrogenase. The binding affinities of these analogs are >360-fold lower than the binding affinity of brequinar sodium. As might be expected, these analogs were shown to be competitive inhibitors with respect to the substrate (dihydroorotate). Our finding that brequinar sodium inhibited dihydroorotate dehydrogenase with high affinity was somewhat surprising, since its structure does not resemble that of either the substrate (dihydroorotate) or the cofactor (ubiquinone) required in the enzymatic reaction. Moreover, we have also found that brequinar sodium is a noncompetitive inhibitor of dihydroorotate dehydrogenase with respect to either dihydroorotate or ubiquinone [4]. To characterize the drug–enzyme interaction, we therefore initiated a detailed study on the structure–activity relationship in the brequinar series.

Our study identified three critical regions of brequinar sodium, where specific substitutions are required for the inhibition of the activity of dihydroorotate dehydrogenase, the cellular target of

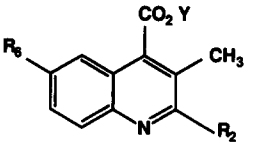
Table 3. Effects of R_4 substitution of 6-fluoro-2-(cyclohexylphenyl-4-yl)-3-methylquinolines on the inhibition of dihydroorotate dehydrogenase activity



Compound	R_4	appK_i (nM)	Ratio
14	COOH	27.4 ± 1.6	1.1
41	COONa	25.5 ± 5.1	1.0
44	$COO^-NH_3^+(CH_2)_4CH(NH_3^+)COO^-$	25.4 ± 4.7	1.0
45	$COOC_2H_5$	74,800 ± 10,800	2,990
46	$CONHC_3H_7$	79,600 ± 27,000	3,180
47	$CON(C_2H_5)_2$	13,300 ± 2,340	533
48	$CON(CH_2CH_2)_2NCH_3$	3,250 ± 528	130
49	CHO	3,900 ± 510	156
50	CH_2OH	3,130 ± 818	125

The inhibition constant (apparent K_i ± standard deviation calculated by linear regression from the slope of the Dixon plot) was determined as described in Materials and Methods, and the ratio was calculated by dividing the apparent K_i value of the test analogs by that of brequinar sodium (compound 1 apparent K_i = 25 nM).

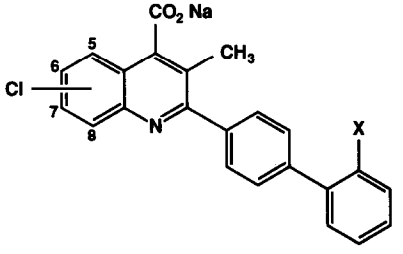
Table 4. Effects of R_6 substitution of 3-methyl-4-quinoline carboxylic acids/salts on the inhibition of dihydroorotate dehydrogenase activity



Compound	R_2	Y	R_6	appK_i (nM)	Ratio
51	4—(C_6H_5)C_6H_4	Na	H	136 ± 6.0	5.4
1	4—(2—F—C_6H_4)C_6H_4	Na	F	25.0 ± 2.6	1.0
52	4—(2—F—C_6H_4)C_6H_4	H	Cl	36.6 ± 7.7	1.5
53	4—(C_6H_5)C_6H_4	H	Br	77.3 ± 5.3	3.1
54	4—(2—I—C_6H_4)C_6H_4	H	I	242 ± 44.1	9.7
55	4—(C_6H_5)C_6H_4	Na	CF_3	9.2 ± 5.1	0.4
56	4—(*c*—C_6H_{11})C_6H_4	H	CH_3	442 ± 81.5	17.7
57	4—(*c*—C_6H_{11})C_6H_4	Na	C_2H_5	171.8 ± 5.7	6.9
58	4—(*c*—C_6H_{11})C_6H_4	H	NO_2	190.3 ± 16.0	7.6
59	4—(*c*—C_6H_{11})C_6H_4	H	NH_2	15,700 ± 730	626
60	4—(*c*—C_6H_{11})C_6H_4	H	OH	6,230 ± 60.1	249
61	4—(*c*—C_6H_{11})C_6H_4	H	OCH_3	4,670 ± 1,450	187
62	4—(C_6H_5)C_6H_4	Na	SCH_3	676 ± 122	27
63	4—(*c*—C_6H_{11})C_6H_4	Na	SO_2CH_3	30,200 ± 3,100	1,200
64	4—(C_6H_5)C_6H_4	Na	COONa	311,000 ± 156,000	12,400
65	4—(*c*—C_6H_{11})C_6H_4	H	$COOCH_3$	30,400 ± 1,690	1,220

The inhibition constant (apparent K_i ± standard deviation calculated by linear regression from the slope of the Dixon plot) was determined as described in Materials and Methods, and the ratio was calculated by dividing the apparent K_i value of the test analogs by that of brequinar sodium (compound 1 apparent K_i = 25 nM).

Table 5. Effects of chloro substitution of 2-(biphenyl)-3-methyl-4-quinoline carboxylic acid sodium salt on the inhibition of dihydroorotate dehydrogenase activity



Compound	X	Position of Cl	appK_i (nM)	Ratio
66	H	5	65.8 ± 8.9	2.6
52	F	6	36.6 ± 7.7	1.6
67	H	7	4750 ± 519	190
68	H	8	2580 ± 412	130
69	H	5,7	4280 ± 540	171

The inhibition constant (apparent K_i ± standard deviation calculated by linear regression from the slope of the Dixon plot) was determined as described in Materials and Methods, and the ratio was calculated by dividing the apparent K_i value of the test analogs by that of brequinar sodium (compound 1 apparent K_i = 25 nM).

this drug candidate. Other portions of the molecules were sensitive to chemical modification for activity as well. The three principal regions are: (i) the C(2) position where bulky hydrophobic substituents are necessary, (ii) the C(4) carboxylic acid, and (iii) the benzo portion of the quinoline ring with appropriate substitutions.

The carboxylic acid was found to be the only acceptable substituent at the C(4) position; the apparent K_i values were essentially identical for the acid and its salts. Other substitutions at the C(4) position drastically reduced or totally abolished the inhibitory activity. These data suggest that an important ionic interaction exists between the carboxylate group of the brequinar sodium analogs and a positively charged group of dihydroorotate dehydrogenase.

When the quinoline-4-carboxylic acid was substituted with a small group at the C(2) position (e.g. a phenyl group of compound 22, or a furfur-2-yl group of compound 39), these analogs were essentially inactive. However, when the phenyl group of compound 22 was replaced with a 4-(ethyl)phenyl group (compound 20) or a 4-(*n*-hexyl)phenyl group (compound 17), the activity increased 400- to 850-fold respectively. These data suggest that adding one phenyl group at the C(2) position of quinoline-4-carboxylic acid allows the compound to interact with the enzymes very weakly, if at all. Only when additional hydrophobic groups were attached to the first phenyl group did the interaction between the compound and the enzyme become significantly enhanced, with apparent K_i values in the 100 nM range. These data suggest that there is a strong hydrophobic interaction between the enzyme and brequinar sodium. To substantiate this hypothesis, many analogs were tested, and we found that those

analogs with a bulky hydrophobic group were excellent inhibitors. These substituents were: substituted biphenyl (compounds 1–11); cyclohexyl phenyl (compound 14); 4-(*t*-butyl)phenyl (compound 23); and naphthalene-2-yl (compound 34). Inserting one or two atoms (oxygen, sulfur or methylene) between the biphenyl rings did not alter the inhibitory activity (compounds 26–28, 31, and 32). However, when the sulfur atom was oxidized to sulfone or sulfoxide (compounds 29, 30, and 25), the inhibitory activities were greatly reduced. It is also of interest to note that although the compound with a cyclohexyl phenyl group (compound 14) was as good an inhibitor as brequinar sodium, introducing a double bond to form the cyclohexenyl group (compound 15) greatly reduced the inhibitory activity. Several analogs (compounds 4, 23 and 31) were better inhibitors than brequinar sodium. These analogs contained a bulky hydrophobic group at the C(2) position but lacked an electron withdrawing group such as fluorine on the biphenyl substituent of brequinar sodium. These observations suggest that bulky hydrophobic groups without an electron withdrawing group on the biphenyl moiety are preferred substituents at the C(2) position. However, these more potent inhibitors are also significantly less water soluble than brequinar sodium.

The methyl group at the C(3) position was the best substituent. The inhibitory activity decreased when the chain length decreased or increased. The activity decreased substantially when the ethyl group (compound 42) was replaced by the propyl group (compound 43). This suggests that the C(3) substituent may interfere sterically with the hydrophobic interaction of the C(2) group with the enzyme.

Since the benzo portion of the quinoline ring is necessary for the biological activity, we then investigated whether the substitutions at the C(5) to C(8) position affected the inhibitory activity. The compounds with a chlorine substitution at the C(5) or C(6) position retained the inhibitory activity. However, the compounds with a chlorine substitution at the C(7) or C(8) position had drastically reduced inhibitory activity. It is of interest to note that the compound with a chloro group substituted at both the C(5) and the C(7) positions (compound 69) was also not active. These data suggest that the substituents at the C(7) and C(8) position hinder the binding of the compound to the enzyme.

The compounds described in this report were also tested against L1210 cells grown in culture and against mice bearing L1210 leukemia. Preliminary data indicate that there is a good correlation between the enzyme inhibitors and the growth inhibition of cultured L1210 cells (unpublished results). Because these compounds inhibit the enzyme activity in a cell free system, no enzymatic activation is required. Unless there is a differential transport of these compounds into cells, it is reasonable to postulate that a better enzyme inhibitor would be a better cytotoxic agent. The good correlation between the cell-free enzymatic inhibitory activity and tumor cell growth inhibitory activity is quite consistent with our earlier report [2] that the mechanism of action of brequinar sodium is due to the inhibition of dihydroorotate dehydrogenase.

In summary, we have identified several key structural features of the novel anticancer drug candidate brequinar sodium. The structure–activity relationship data presented here, together with enzyme kinetics and modeling studies, will hopefully lead to a more precise elucidation of the brequinar sodium–enzyme interaction. These studies are currently in progress in our laboratory.

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