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IMMUNOSUPPRESSIVE STRUCTURE-ACTIVITY RELATIONSHIPS OF BREQUINAR AND RELATED CINCHONINIC ACID DERIVATIVES

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Abstract. The immunosuppressive structure-activity relationships of substituted cinchoninic acid derivatives related to Brequinar were explored. Activities as inhibitors of dihydroorotate dehydrogenase and the mixed lymphocyte reaction were related to benzo-ring substitution, replacement of the benzo-ring by heterocycles, and variation in the 2-biphenyl, 3-methyl, and 4-carboxy groups.

Introduction. Immunosuppressive drugs have assumed a critical role in the suppression of tissue rejection reactions, allowing life-saving organ transplantation to become almost routine.[1] Cyclosporine is the mainstay of transplant rejection therapy but suffers from severe side effects, notably nephrotoxicity. More recent entrants to the field include FK-506 (Tacrolimus) and mycophenolate mofetil. The search for safer immunosuppressive drugs continues; other potential applications include diseases having an autoimmune component such as rheumatoid arthritis and psoriasis.

Brequinar sodium, originally discovered as an anticancer agent,[2] has more recently been found to display potent, selective immunosuppressive activity, and is undergoing clinical trials for the prevention of organ transplant rejection.[3] This drug is a potent inhibitor (IC_{50} of 12 nM) of dihydroorotate dehydrogenase (DHODase), which is the controlling step in de novo pyrimidine nucleotide biosynthesis.[4] This activity is manifested in the selective inhibition of activated lymphocyte function, leading to suppression of the cellular immune response.

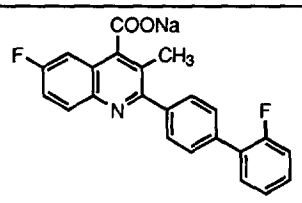
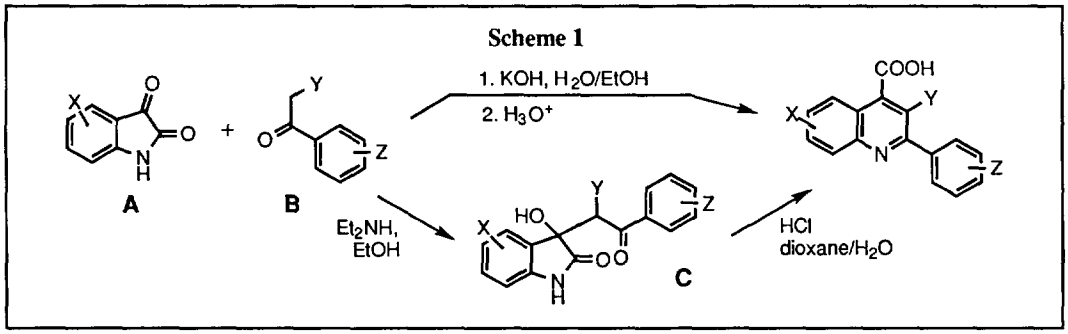


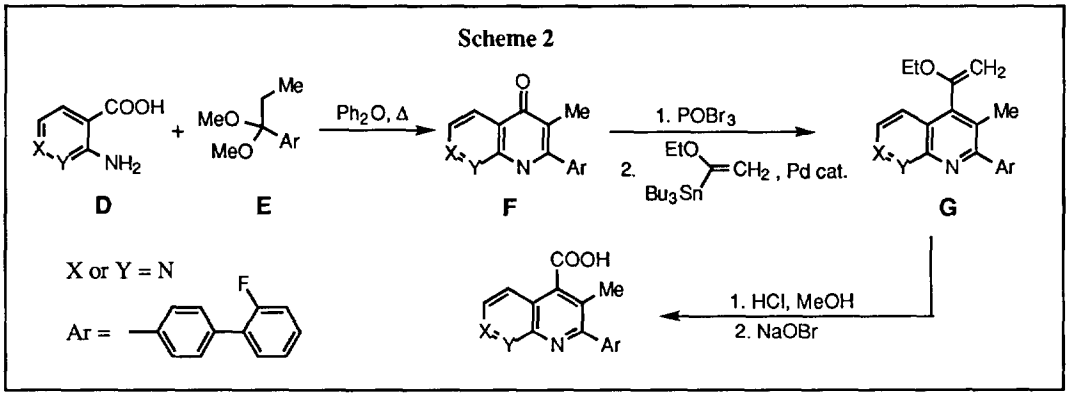
Fig. 1. Brequinar sodium

In our attempts to determine the structural features required for good activity and an optimal pharmacokinetic profile, we evaluated a number of analogs with a variety of substituents and variants on the basic cinchoninic acid core of Brequinar. Substitution on the benzo-ring, replacement of the benzo ring by a

heterocyclic ring, and variation of the substituents on the pyridine ring and of the 2-biphenyl group were explored with respect to both enzymatic and cellular immunosuppressive activity.[5]

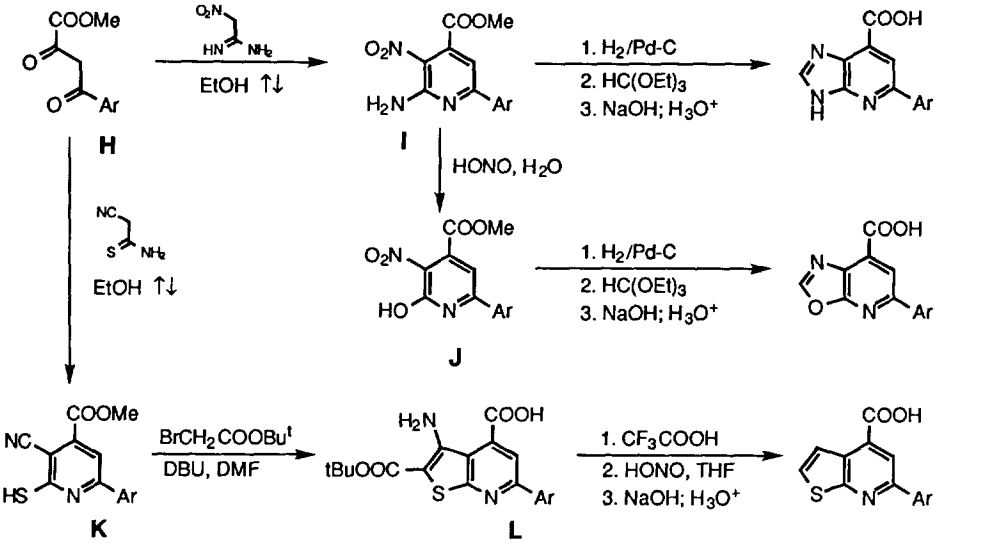
Chemistry. Brequinar and many of its analogs were prepared by the Pfitzinger condensation of appropriately substituted isatins **A** and phenyl ketones **B** (Scheme 1).[6] This could usually be accomplished in a single step using the classical conditions, with yields generally greater than 60%. In some cases it was preferable to use a milder base to perform the initial aldol condensation, followed by treatment of the isolated intermediate **C** with acid. Compounds with various substitutions in the 2 and 3 positions of the quinoline ring, as well as in the benzo-fused ring, could be easily prepared in this way. 4-Substituted variants were generally prepared by simple functional group manipulations of the acid; the hydroxyquinoline (quinolone) **26** was prepared by the ketal route shown in Scheme 2. The tetrazole **25** was prepared from the nitrile (derived[7] from acid **1**) by a literature method.[8]





5- and 6-aza analogs **13** and **14** could be prepared using the method of Scheme 1, but the 7- and 8-aza analogs **15** and **16** were more conveniently prepared by an alternative route (Scheme 2) based on the von Niementovski condensation.[9] In this case, thermal condensation of the appropriate aza-anthranilic acid **D** with a propiophenone dimethyl ketal **E** provided variable yields (30-80%) of the quinolones **F**. Treatment with phosphorus oxybromide yielded the 4-bromoquinoline analogs, which could be coupled with tributyl 1-ethoxyvinyl stannane to provide **G** in 90% yield. Hydrolysis and oxidation with sodium hypobromite provided the desired compounds in 60% yield.

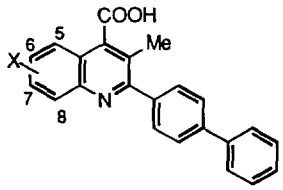
Scheme 3



Compounds with an electron-rich heterocyclic ring fused to the pyridine ring were prepared using a different strategy (Scheme 3).[10] Guareschi condensation of nitroacetamidine with the appropriate acylpyruvate **H** gave the pyridine intermediates **I** in 90% yield. Methylation of the amino group, for preparation of **18**, could be achieved at this stage in 60% yield using sodium hydride and methyl iodide. Reduction of the nitro group and condensation of the resulting diamine with ethyl orthoformate, followed by ester saponification, provided the imidazoles **17** and **18** in 70% overall yield. Diazotization of the amine of **I** provided the pyridone **J** in quantitative yield after in situ hydrolysis; reduction, condensation with ethyl orthoformate and saponification as above provided the oxazole **19** in 35% yield. The thiophene analog **20** was also prepared from a pyridine precursor, which arose from the Guareschi condensation of the acylpyruvate with cyanothioacetamide (90%).[11] Alkylation of the thiol of **K** followed by in situ cyclization afforded the substituted ring system **L** in 50% yield. Protonolysis of the tert-butyl ester occurred with concomitant decarboxylation; reductive diazotization of the resulting aminothienopyridine followed by saponification provided **20** in 12% yield.

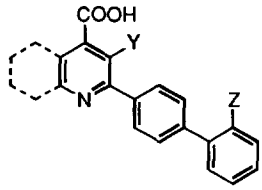
Biology. Two primary assays were utilized to study the analogs of Brequinar. The first, an enzyme assay (DHOD) using partially purified DHODase isolated from human liver, measured the inhibition of the formation of orotate from radiolabelled dihydroorotate;[12] the results are given as K_i values in nM. The second test used was the human mixed lymphocyte reaction (MLR), a standard model of cell-based immunity indicative of potential allograft rejection.[13] Here, the compounds were evaluated for their ability to inhibit proliferation in a mixture of human lymphocytes from two unrelated donors. The results for this test are given as IC_{50} values (nM).

Table 1.



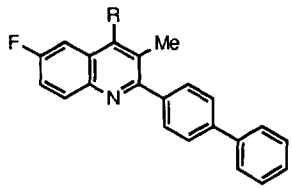
Cmpd.	X	DHOD	MLR
1	6-F	12	15
2	H	1.7	180
3	5-Cl	25	150
4	6-Cl	20	35
5	7-Cl	220	>500
6	8-Cl	150	>500
7	6-CF_3	8.4	7
8	6-Me	10	380
9	6-SMe	270	>500
10	6-OH	>500	>500
11	6-NHAc	>500	>500
12	6-COOMe	>500	>500

Table 2.



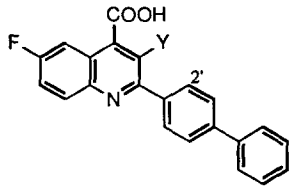
Cmpd	Ring	Y	Z	DHOD	MLR
13		Me	F	500	>500
14		Me	F	420	>500
15		Me	F	48	290
16		Me	F	210	>500
17		Me	F	>500	>500
18		H	H	>500	>500
19		H	H	>500	>500
20		H	H	210	>500

Table 3.



Cmpd.	R	DHOD	MLR
1	COOH	12	15
21	COOMe	400	>500
22	$CONH_2$	>500	>500
23	CH_2OH	>500	>500
24	CH_2OAc	>500	>500
25	tetrazole	>500	>500
26	OH	>500	>500

Table 4.



Cmpd.	Y	DHOD	MLR
1	Me	12	15
27	H	10	150
28	Et	30	210
29	OH	40	>500
30	OMe	13	250
31	SMe	13	18
32	CH_2CH_2*	17	14

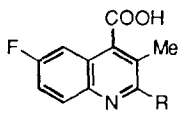
*ethylene bridge to 2' position

Discussion and Conclusions. Compounds in which the substitution of the benzo-fused ring was varied are shown in Table 1. Removal of Brequinar's 6-fluoro substituent (**2**) somewhat enhanced the DHOD activity, but the potency in the cellular assay was reduced by an order of magnitude. From the other examples shown it is apparent that, for optimal activity, substitution on this ring must be limited to electron-withdrawing, lipophilic groups. Substitution at the 6 position appears to be optimal; 5-substitution (**3**) caused retention of enzyme

activity but reduced the cellular activity significantly. Substitution at the 7 or 8 positions (**5, 6**) caused a dramatic loss of activity, as did insertion of a large or polar group at the 6 position (**9 - 12**). Table 2, which illustrates heterocyclic replacements for this ring, also supports the detrimental effect of polar and electron-rich rings. Thus, all of the heterocycles were less potent than Brequinar, especially in the cellular assay. These results suggest that cell penetration is hindered by polarity in this ring.

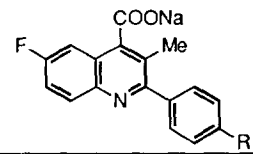
The results in Table 3 show the effect of varying the carboxylic acid moiety. Any change of this functional group led to complete loss of activity, except for the methyl ester which retained a slight amount of activity (which may be due to partial hydrolysis in the assay medium). Neither similarly shaped hydrogen-bonding groups (**22, 23**) nor other acidic groups (**25**) showed any activity at all. Table 4 lists compounds in which the 3-methyl of Brequinar has been changed. Varying this group seemed to have relatively little effect on the enzyme activity, but much greater effects were seen on the activity in cells. This again may be due to reduced cell penetration, although it is difficult to identify trends in these data. Interestingly, bridging the 3-position to the ortho position of the 2-phenyl substituent (**32**) gives a compound which is almost equipotent with Brequinar. Further studies on this structural feature will be published elsewhere.[14]

Table 5.



Cmpd.	R	DHOD	MLR
1	4-(2-F-Ph)-Ph-	12	15
33	Ph-	>500	>500
34	Ph(CH2)4-	>500	>500
35	1-naphthyl	>500	>500
36	2-naphthyl	300	>500
37	4-(n-hexyl)-Ph-	60	>500
38	4-(t-butyl)-Ph-	40	400
39	3-Ph-Ph-	>500	>500
40	4-cyclohexyl-Ph-	28	25
41	4-PhO-Ph-	29	500
42	3-PhO-Ph-	63	>500
43	5-Ph-2-thienyl	390	>500

Table 6.



Cmpd.	R	DHOD	MLR
1	2-F-Ph-	12	15
44	3-F-Ph	50	20
45	4-F-Ph-	210	260
46	Ph-	8	31
47	4-Cl-Ph-	140	>500
48	4-Br-Ph-	38	>500
49	4-CF3-Ph-	230	>500
50	4-Me-Ph-	24	150
51	2-F-4-OH-Ph-	11	300
52	2-thienyl	56	350
53	3-pyridyl	200	>500
54	1-imidazolyl	>500	>500

Variation of the 2-substituent is shown in Tables 5 and 6. The biphenyl moiety or a similar group was required for good potency; removal of the distal ring (**33**) caused complete loss of activity. This ring could be replaced with another lipophilic group such as alkyl (**37, 38**) or cyclohexyl (**40**) with only a slight loss in enzyme potency, although cellular activity was lost. Simply extending the phenyl group from the quinoline core (**34**) was not sufficient to regain activity, nor was benzo fusion to give naphthyl analogs (**35** or **36**). However, the 2-naphthyl group showed a small degree of enzyme activity, suggesting a shape specificity in this portion of

the molecule. This is supported by the fact that the meta-biphenyl **39** was also inactive; however the enzyme activity of the phenoxy compounds **41** and **42** runs counter to this argument.

Only limited substitution was allowed on the para-biphenyl group, as shown in Table 6. The ortho-fluoro was not important for potency (see **46**), and moving it to the meta-position was tolerated (**44**), but para-substituted biphenyls showed much reduced activity, especially in the cellular assay. As was the case in the benzo-fused ring, electron-poor rings appeared to be optimal, since replacement of the terminal phenyl by a thiophene (**52**) caused loss of activity. Polarity was also not tolerated (**53** and **54**). The para-hydroxy compound **51**, which was found to be a metabolite of Brequinar in mice and humans,[15] interestingly retained enzyme activity, but showed a major loss of potency in the cellular assay, presumably due to reduced cell penetration.

In summary, a number of analogs of Brequinar were evaluated in both the DHODase enzyme assay and the human mixed lymphocyte reaction to determine the structural requirements for immunosuppressive activity. The cinchoninic acid core was required, with only small, lipophilic, electron-deficient substitution allowed on the benzo-ring. Methyl was optimal at the 3-position, although bridging to the 2-biphenyl retained potency. The two substituent must be para-biphenyl or a similarly-shaped group, with little substitution allowed. The immunosuppressive results essentially mirror those reported earlier for suppression of cancer cell growth,[16] indicating that both effects are probably a direct result of DHODase inhibition. It seems clear that DHODase inhibition is necessary (although clearly not sufficient) for immunosuppressive action by Brequinar and its analogs in a whole cell assay.

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