Antiplasmodial and Cytotoxic Activity of Natural Bisbenzylisoquinoline Alkaloids

Cindy K. Angerhofer,[†] Hélène Guinaudeau,*[,‡] Varima Wongpanich,[†] John M. Pezzuto,[†] and Geoffrey A. Cordell[†]

Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, College of Pharmacy, Chicago, Illinois, and Department of Pharmacognosy, Faculty of Pharmacy, University of Angers, France

Received April 13, 1998

As part of an ongoing collaborative effort to discover new antimalarial agents from natural sources, we have tested 53 bisbenzylisoquinoline alkaloids for cytotoxicity against cultured mammalian cells and for antiplasmodial activity against chloroquine-sensitive and chloroquine-resistant clones of *Plasmodium falciparum*. The isolates from *Cyclea barbata*, *Stephania pierrei*, *Stephania erecta*, *Pachygone dasycarpa*, *Cyclea atjehensis*, *Hernandia peltata*, *Curare candicans*, *Albertisia papuana*, and *Berberis valdiviana* exhibited a wide range of biological potencies in antiplasmodial assays, and the majority exhibited some degree of cytotoxicity against human KB cells. More than half of the compounds tested, however, showed selective antiplasmodial activity, with >100-fold greater toxicity toward one or both of the *P. falciparum* clones, relative to cultured mammalian cells. The most selective alkaloids were (−)-cycleanine (**40**), (+)-cycleatjehine (**50**), (+)-cycleatjehenine (**49**), (+)-malekulatine (**3**), (−)-repandine (**13**), and (+)-temuconine (**2**). As a result of these studies, relationships between the structures, the stereochemistry, and the substitution patterns of these alkaloids and their in vitro antiplasmodial and cytotoxic activities are beginning to emerge.

Bisbenzylisoquinolines are a large and diverse group of natural alkaloids that occur in many plant species, particularly in members of the Menispermaceae, Berberidaceae, Ranunculaceae, Annonaceae, and Monimiaceae.[1−4] Many of the plants that contain these compounds enjoy a folkloric reputation as medicinals in various cultures.[1−4] Recently, bisbenzylisoquinoline alkaloids have been widely demonstrated to possess a number of interesting and potent biological activities, including cytotoxicity and/or antiplasmodial activity.[1−4] Classically, these dimers can be divided into three categories: biscoclaurines, coclaurine−reticulines, and bisreticulines. The two moieties are usually bound by one diaryl ether bridge or more, although carbon−carbon bridges or a methylene−oxy bridge may be present. The bisbenzylisoquinoline alkaloids are classified according to the nature, the number, and the attachment point of the bridges. In each subgroup, the alkaloids differ by the nature of their oxygenated substituents, the degree of unsaturation of the heterocyclic rings, and the stereochemistry of their two chiral centers, C-1 and C-1′. The diversity of pharmacological effects observed within this group of molecules is obviously a function of differences in chemical structures; however, convincing structure−activity relationships had not been developed previously for the bisbenzylisoquinoline alkaloids.

Over the past several years, we have explored this class of complex alkaloids in considerable spectroscopic detail, as well as evaluating their biologic potential to serve as new antimalarial agents. Through bioassay-directed fractionation, we have isolated a variety of known and novel bisbenzylisoquinoline alkaloids from several plants in the Menispermaceae, including *Stephania erecta*,[5] *Stephania pierrei*,[6] *Cyclea barbata*,[7−9] and *Pachygone dasycarpa*.[10] In addition, a number of bisbenzylisoquinoline alkaloids that had been isolated in the course of phytochemical studies of other species, such as *Cyclea atjehensis*[11,12] (Guinaudeau and Ovono, unpublished result), *Curare candicans*,[13] *Cocculus pendulus* (Menispermaceae),[14] *Hernandia peltata* (Hernandiaceae),[15] and *Berberis valdiviana* (Berberidaceae)[16] were available for investigation. Applying identical methodologies, these compounds have been analyzed for cytotoxicity toward mammalian cells, as well as for antiplasmodial activity with chloroquine-sensitive and chloroquine-resistant, mefloquine-sensitive clones of *Plasmodium falciparum*. This approach is used to determine antimalarial potency as well as selectivity. Compilation of the results obtained with 53 bisbenzylisoquinoline alkaloids has facilitated the analysis of structure−activity relationships wherein the goal is to define the structural features that might be responsible for selective antiplasmodial activity.

Results and Discussion

Of the bisbenzylisoquinoline alkaloids examined in this study (**1**−**53**), only three, (−)-isocuricycleatjine (**45**), (−)-dehydroisocuricycleatjenine (**47**), and (+)-tubocurarine chloride (**48**) failed to show significant in vitro antiplasmodial activity against either of the *P. falciparum* clones tested (Table 1). Seven additional compounds (**15**, **19**, **38**, **43**, **44**, **46**, **53**) exhibited weak activity, with antiplasmodial IC_{50} values of 1000−2400 nM in at least one of the clones. The remaining 43 compounds were determined to have IC_{50} values of <1000 nM against both D6 and W2 clones, and of these, 27 demonstrated potent activity of <200 nM with at least one of the clones. To further analyze the antimalarial potential of these bisbenzylisoquinoline alkaloids, all compounds were evaluated for cytotoxicity with human epidermoid carcinoma (KB) cells.

Many compounds have been reported in the literature as "antimalarials" on the basis of in vitro data against malarial parasites. Although completely valid, these data

* To whom correspondence should be addressed: Department of Pharmacognosy, Faculty of Pharmacy, University of Angers, 16 boulevard Danviers, 49045 Angers Cedex, France. Tel.: 33(0) 2 41 22 66 63. Fax: 33-(0) 2 41 48 67 33.
[†] University of Illinois at Chicago.
[‡] University of Angers, France.

10.1021/np980144f CCC: $18.00
Published on Web 10/31/1998

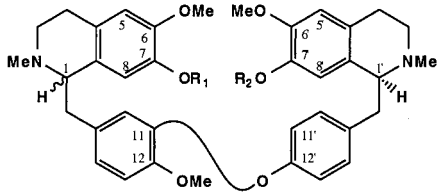
Table 1. Cytotoxic and Antiplasmodial Activity of Selected Bisbenzylisoquinolines

compound no.	bisbenzylisoquinoline name/code	KB ED_{50}[a]	D6 IC_{50}[a]	D6 SI[b]	W2 IC_{50}[a]	W2 SI[b]
1	(+)-neothalibrine	10 100	47	215	135	75
2	(+)-temuconine	>32 100	213	>150	227	>140
3	(+)-malekulatine	>29 900	61	>490	164	>180
4	(−)-cycleapeltine[d]	3620	48	76	67	54
5	(+)-limacusine	4110	64	64	192	21
6	(+)-homoaromoline	10 400	173	60	474	22
7	(+)-obaberine[c]	13 800	371	37	347	40
8	(+)-stephibaberine[c]	10 500	214	49	510	21
9	(+)-daphnandrine[c]	9800	106	92	375	26
10	(+)-2-norobaberine[c]	9900	75	130	154	64
11	(+)-2-norcepharanthine[c]	6600	79	84	219	30
12	(+)-cepharanthine[c]	9700	231	42	478	20
13	(−)-repandine	15 000	42	350	67	220
14	(+)-candicusine	8600	29	300	105	82
15	(+)-aromoline	14 800	446	33	1400	11
16	(+)-tetrandrine[d]	5800	288	20	257	22
17	(−)-pheanthine	10 800	305	35	498	22
18	(+)-isotetrandrine[c]	10 600	260	40	88	120
19	(+)-tetrandrine 2′-*N*-oxide	>31 300	1980	>16	948	>34
20	(+)-fangchinoline	9210	179	51	306	30
21	(−)-limacine[d]	16 100	86	186	263	61
22	(+)-thalrugosine[c]	10 700	199	54	378	28
23	(+)-berbamine	9210	128	72	313	29
24	(+)-atherospermoline	3700	232	16	623	6
25	(+)-obamegine	14 100	354	40	825	17
26	(+)-*N*-methyl-7-*O*-desmethylpeinamine	17 500	134	130	609	29
27	(+)-2-norberbamine	7100	47	149	193	36
28	(+)-2-norisotetrandrine[c]	10 100	106	95	728	140
29	(+)-2-northalrugosine[c]	10 400	115	92	210	50
30	(−)-2′-norlimacine	2700	118	23	218	12
31	(+)-tricordatine	4900	90	55	259	19
32	(+)-isotrilobine	24 000	388	62	788	30
33	(+)-12-*O*-methyltricordatine	6000	30	200	112	54
34	(+)-cocsuline	10 000	87	110	502	7
35	(+)-2′-norcocsuline	3800	48	79	281	14
36	(+)-*N*-methyltelobine[c]	8300	169	49	451	19
37	(+)-1,2-dehydrotelobine[c]	5000	554	11	464	11
38	(−)-6,12-*O*-desmethylthalmine	17 800	883	20	1590	11
39	(+)-thalmirabine	8400	224	37.7	110	76.4
40	(−)-cycleanine	>33 700	73	>460	247	>140
41	(−)-curine	7700	128	60	387	20
42	(+)-2′-norcuricycline	14 400	530	27.1	676	21.2
43	(+)-2′-norisocuricycline	8800	1270	7.5	662	13.3
44	(−)-curicycleatjenine	8360	2320	3.6	1200	7.0
45	(−)-isocuricycleatjine	8870	6890	1.3	4030	2.2
46	(−)-isocuricycleatjenine	13 600	1910	1.6	1050	13
47	(−)-isocuricycleatjehimine	>33 900	4980	>6.8	2100	>16.1
48	(+)-tubocurarine chloride	>32 800	15300	>2.1	>16 400	ca. 2
49	(+)-cycleatjehenine	14 700	115	130	66	220
50	(+)-cycleatjehine	>33 900	110	>310	59	>580
51	(+)-3′,4′-dihydrocycleatjehenine	>33 800	346	>98	169	>200
52	(+)-2′-noratjecycline	8580	348	24.6	199	43.2
53	(−)-*N*-acetyl-2′-noratjecycline	20 600	1030	20	605	34
	antimalarial standard drugs ($n = 12$)	KB ED_{50}[a]	D6 IC_{50}[a]	D6 SI[b]	W2 IC_{50}[a]	W2 SI[b]
	chloroquine	33700	6.18 ± 0.71	5460	135 + 10	250
	quinine	>55 400	23.38 ± 7.04	>285	250 + 16.2	>222
	mefloquine	8430	18.12 ± 1.52	465	7.78 + 0.51	1080
	artemisinin	>70 900	15.14 ± 0.26	>4680	14.60 + 2.02	>4860

[a] IC_{50} and ED_{50}values are expressed in nM. [b] Selectivity index (SI) is defined as the ratio of cytotoxicity over antiplasmodial activity. [c] Data previously published in Likhitwitayawuid et al.[5] [d] Data previously published in Lin et al.[7]

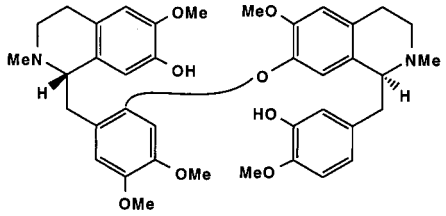
can be somewhat misleading. Most general cytotoxins can be legitimately called "antiplasmodial" under conditions of in vitro testing. As an attempt to establish a working model system to estimate the potential of test compounds such as bisbenzylisoquinolines for inhibiting the growth of an intra-erythrocytic malaria parasite without host toxicity, we have defined the in vitro selectivity index (SI) of a test substance as ED_{50} (in KB cells) /IC_{50} (in *P. falciparum*).[8] KB cells (human oral epidermoid carcinoma) were selected in part by precedent[17] and also because, in our experience, this line exhibits an intermediate sensitivity to a large number of cytotoxic agents when compared to cell lines derived from a variety of other human tumors (unpublished observations). Because the in vivo therapeutic index of a drug encompasses a much broader spectrum of toxic effects than would be reflected in the SI, it would be inappropriate

Chart 1

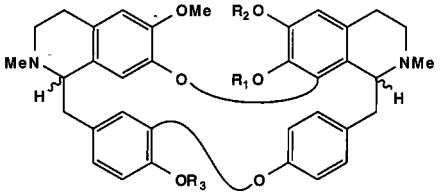


1 : 1*S* , 1'*S*; R_1=H; R_2=Me

2 : 1*R* , 1'*S*; R_1=Me; R_2=H



3



4 : 1*S* , 1'*S*; R_1=H; R_2=Me; R_3=Me

5 : 1*R* , 1'*R*; R_1=H; R_2=Me; R_3=Me

6 : 1*R* , 1'*S*; R_1=H; R_2=Me; R_3=Me

7 : 1*R* , 1'*S*; R_1=Me; R_2=Me; R_3=Me

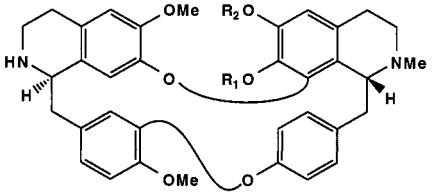
8 : 1*R* , 1'*S*; R_1=Me; R_2=H; R_3=Me

12 : 1*R* , 1'*S*; R_1-R_2=CH_2; R_3=Me

13 : 1*S* , 1'*S*; R_1=Me; R_2=Me; R_3=H

14 : 1*R* , 1'*R*; R_1=H; R_2=Me; R_3=H

15: 1*R* , 1'*S*; R_1=H; R_2=Me; R_3=H



9 : R_1=H; R_2=Me

10 : R_1=Me; R_2=Me

11 : R_1-R_2=CH_2

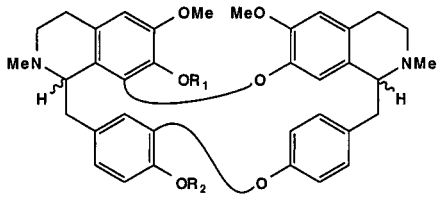
to speculate on the clinical significance of SI values. Nonetheless, this does not diminish the utility of the SI parameter in guiding bioactivity-directed fractionation and prioritizing the further evaluation of compounds/extracts exhibiting in vitro antiplasmodial activity. The SIs of a number of the bisbenzylisoquinolines reported here compare favorably to those of quinine, chloroquine, and mefloquine, which have been included as controls.

Among the 53 alkaloids tested for toxicity toward cultured *P. falciparum* and mammalian KB cells, **1**−**3** contain only one etheroxy bridge (Chart 1). Each exhibited an impressive SI of well over 100, with the exception of (+)-neothalibrine (**1**) in the W2 clone. The structural differences between (1*S*,1′*S*)-(+)-neothalibrine and (1*R*,1′*S*)-(+)-temuconine (**2**) are the different substituents at C-7 and C-7′ and that the configuration at C-1 is *S* for the former alkaloid and *R* for the latter. The effect of these differences on the activity can be related to the toxicity; the threefold enhancement of activity observed for the *S* enantiomer is parallel to the change in cytotoxicity, with the result that the SI values of (+)-neothalibrine (**1**) are not notably different from those for (+)-temuconine (**2**). The lack of detectable toxicity in (+)-temuconine (**2**) is a rare characteristic among the bisbenzylisoquinoline alkaloids that we[5,7] and others[1−4] have assayed. Lack of toxicity is also observed with (+)-malekulatine (**3**). It is interesting to note that (+)-malekulatine (**3**) belongs to a different class of bisbenzylisoquinoline, being a bisreticuline derivative and not a biscoclaurine, as are (+)-temuconine (**2**) and all of the other compounds in Table 1. Moreover, (+)-malekulatine (**3**) is a head-to-tail dimer, whereas (+)-neothalibrine (**1**) and (+)-temuconine (**2**) are linked tail-to-tail, although in each case, the two benzylisoquinoline moieties are linked by only one etheroxy bridge. This structural characteristic allows these three compounds to assume a relatively linear conformation, as shown by NOE experiments.[15,16]

Twenty-seven dimers, half of the compounds we have subjected to bioassay evaluation, belong to the two most prevalent subgroups of bisbenzylisoquinoline alkaloids, in which the two benzylisoquinoline moieties are linked by two etheroxy bridges either between C-7,C-8′ and C-11,C-12′, or between C-8,C-7′ and C-11,C-12′. Twelve alkaloids possessing the former type of linkage were evaluated, of which only three presented appreciable selectivity for malarial parasites: (−)-repandine (**13**), (+)-candicusine (**14**), and (+)-2-norobaberine (**10**).

(1*S*,1′*S*)-(−)-Cycleapeltine (**4**) and (1*R*,1′*R*)-(+)-limacusine (**5**), mirror images of each other, exhibit good antiplasmodial activity against both clones of *Plasmodium*, but their substantial cytotoxicity yields a low SI. (1*R*,1′*S*)-(+)-Homoaromoline (**6**), which presents the same substitution pattern as (+)-limacusine (**5**) and (−)-cycleapeltine (**4**) but with a different absolute configuration, is not as cytotoxic, and this difference is reflected in reduced activity against both clones. *O*-Methylation at C-7′ of (+)-homoaromoline (**6**), as in (+)-obaberine (**7**), leads to a slight decrease in cytotoxicity, while the antiplasmodial IC_{50} increases approximately twofold for both clones. No change in activity is observed when the hydroxyl group is at C-6′, as in (+)-stephibaberine (**8**), instead of C-7′, as in (+)-homoaromoline (**6**). Demethylation at N-2 in (+)-homoaromoline (**6**) to give (1*R*,1′*S*)-(+)-daphnandrine (**9**) does not significantly alter the antiplasmodial activity against the D6 and W2 clones, or the KB cytotoxicity. It is interesting to note that, of the 53 compounds tested so far, this is the

Chart 2



16 : 1*S*, 1'*S*; R_1=Me; R_2=Me

17 : 1*R*, 1'*R*; R_1=Me; R_2=Me

18 : 1*R*, 1'*S*; R_1=Me; R_2=Me

20 : 1*S*, 1'*S*; R_1=H; R_2=Me

21 : 1*R*, 1'*R*; R_1=H; R_2=Me

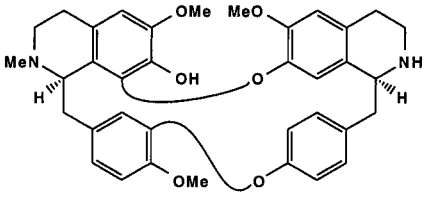
22 : 1*R*, 1'*S*; R_1=H; R_2=Me

23 : 1*R*, 1'*S*; R_1=Me; R_2=H

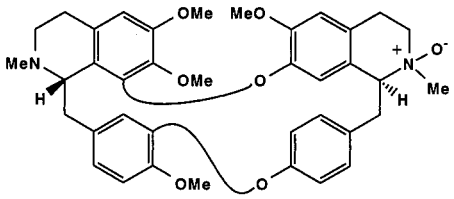
24 : 1*S*, 1'*S*; R_1=H; R_2=H

25 : 1*R*, 1'*S*; R_1=H; R_2=H

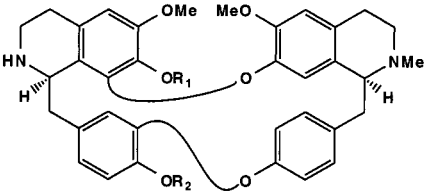
26 : 1*S*, 1'*R*; R_1=H; R_2=H



30

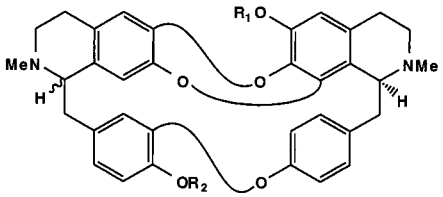


19



27 : R_1=Me; R_2=H **29** : R_1=H; R_2=Me

28 : R_1=Me; R_2=Me



31 : 1*S*; R_1=H; R_2=H **34** : 1*S*; R_1=Me; R_2=H

32: 1*S*; R_1=Me; R_2=H **36** : 1*S*; R_1=Me; R_2=Me

33 : 1*S*; R_1=H; R_2=Me

only case in which no change in bioactivity was found when a hydrogen atom replaced the methyl group at N-2.

The two other 2-nor compounds of this subgroup are (1*R*,1′*S*)-(+)-2-norobaberine (**10**) and (1*R*,1′*S*)-(+)-2-norcepharanthine (**11**). Both compounds are between twofold and fivefold more active against the W2 and D6 clones than the corresponding *N*-methylated compounds. Despite a slight increase in mammalian cytotoxicity, the alkaloids bearing a secondary amine at N-2 exhibit a slight, but consistent, increase in selectivity.

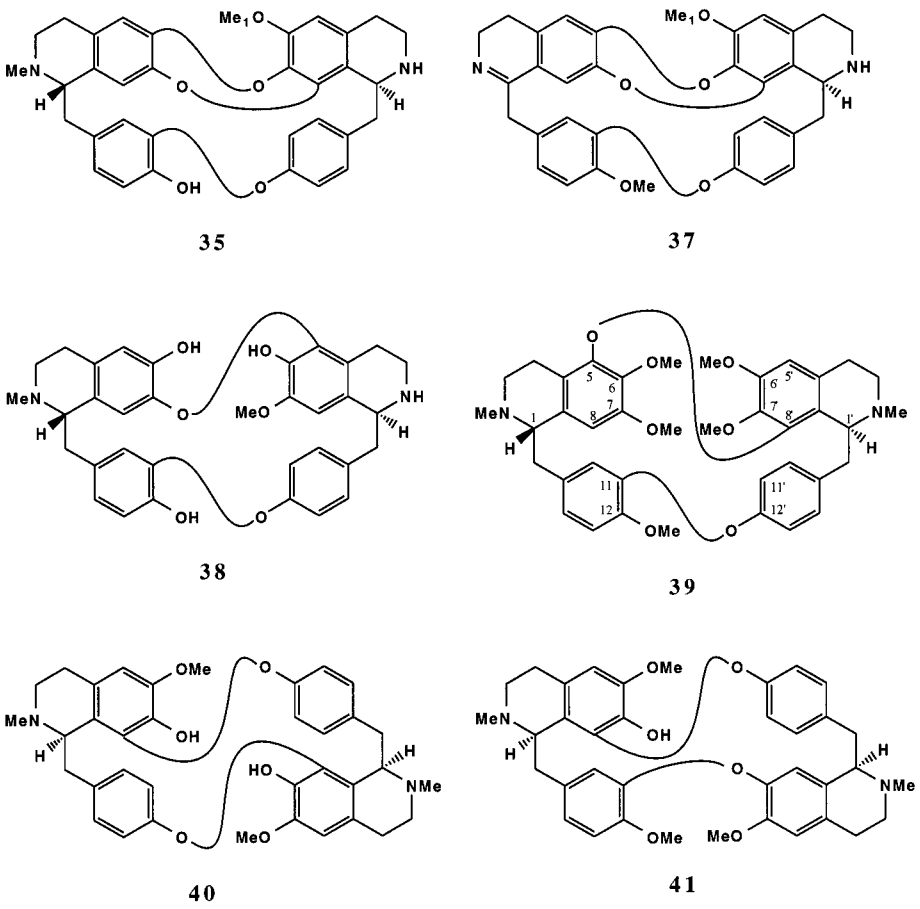
In the group of 12 alkaloids considered thus far, (1*S*,1′*S*)-(−)-repandine (**13**) is the most interesting, with an SI of 350 for the D6 clone and 220 for the W2 clone. (1*R*,1′*R*)-(+)-Candicusine (**14**) displays antiplasmodial IC_{50} values similar to (+)-repandine (**13**), but increased cytotoxicity results in lower selectivity. The *O*-methylation of (+)-candicusine (**14**) at C-12 leads to (1*R*,1′*R*)-(+)-limacusine (**5**). This change of substitution increases the toxicity and decreases the antiplasmodial activity for both clones by about twofold; thus, a fourfold loss in selectivity is observed for (+)-limacusine (**5**). The change of configuration at C-1′ in (1*R*,1′*R*)-(+)-candicusine (**14**), as in (1*R*,1′*S*)-(+)-aromoline (**15**), leads to a slight decrease in the cytotoxicity, but activity against *P. falciparum* diminishes more than tenfold.

Fifteen of the bisbenzylisoquinoline alkaloids tested possess an 8,7′/11,12′ linkage. (1*S*,1′*S*)-(+)-Tetrandrine (**16**), (1*R*,1′*R*)-(−)-phaeanthine (**17**), (1*R*,1′*S*)-(+)-isotetrandrine (**18**), and (1*R*,1′*S*)-(+)-tetrandrine 2′-*β*-*N*-oxide (**19**) present the same substitution pattern with four methoxyl groups at C-6, C-7, C-6′, and C-12 (Chart 2). (+)-Tetrandrine (**16**) is two times more cytotoxic than (−)-phaeanthine (**17**) and (+)-isotetrandrine (**18**), while activity against the D6 clone is very similar for these three bisbenzylisoquinoline alkaloids. Against the W2 clone, (+)-isotetrandrine (**18**) is six times more active than (−)-phaeanthine (**17**) and four times more active than (+)-tetrandine (**16**). The presence of an *N*-oxide function at N-2 dramatically decreases the cytotoxicity as well as the antiplasmodial activity.

(1*S*,1′*S*)-(+)-Fangchinoline (**20**), (1*R*,1′*R*)-(−)-limacine (**21**), and (1*R*,1′*S*)-(+)-thalrugosine (**22**) bear a hydroxyl at C-7, while C-6, C-6′, and C-12 are substituted by a methoxyl group. As observed for (1*S*,1′*S*)-(+)-tetrandrine (**16**), (+)-fangchinoline (**20**), also having the (1*S*,1′*S*)-configuration, is the most cytotoxic. (−)-Limacine (**21**), the mirror image of (+)-fangchinoline, is less toxic and about twice as active as the two other alkaloids against the D6 clone. This difference leads to a better SI for (−)-limacine (**21**) against the D6 clone. The demethylation of (+)-isotetrandrine (**18**) at C-12, as seen in (1*R*,1′*S*)-(+)-berbamine (**23**), tends to increase the toxicity against the D6 clone, but decreases activity against W2 almost fourfold.

(1*S*,1′*S*)-(+)-Atherospermoline (**24**), (1*R*,1′*S*)-(+)-obamegine (**25**), and (1*S*,1′*R*)-(−)-*N*-methyl-7-*O*-demethylpeinamine (**26**) differ in configuration but have the same general substitution pattern (methoxyl groups at C-6 and C-6′ and hydroxyl groups at C-7 and C-12). The change of configuration at C-1′ from 1′*S* to 1′*R* or the change of configuration from 1*S* to 1*R* leads to a notable decrease in cytotoxicity (3−4-fold), while the effect on antiplasmodial

Chart 3



IC_{50} values is minor and inconclusive. None of these compounds shows impressive selectivity against *P. falciparum*.

The demethylation of N-2 in (+)-berbamine to yield **27**, in (+)-isotetrandrine to yield **28**, and in (+)-thalrugosine to yield **29** does not affect cytotoxicity, although the antiplasmodial IC_{50} values showed a consistent decrease. In contrast, the demethylation of N-2′, as in 2′-*N*-norlimacine (**30**), increases cytotoxicity by more than fivefold, while antiplasmodial activity is almost unchanged.

Seven of the test compounds represent the bisbenzylisoquinoline subgroup containing three etheroxy bridges. (1*S*,1′*S*)-(+)-Tricordatine (**31**), (1*S*,1′*S*)-(+)-isotrilobine (**32**), (1*S*,1′*S*)-(+)-12-*O*-methyltricordatine (**33**), and (1*S*,1′*S*)-(+)-cocsuline (**34**) differ only in the nature of the substituents at C-12 and C-6′. When the two hydroxyl groups of (+)-tricordatine (**31**) are methylated, as in (+)-isotrilobine (**32**), the cytotoxicity decreases by approximately fourfold, with a proportionate decrease in antiplasmodial activity. The consequence is that the SI is in the same range for both alkaloids.

If only one of the hydroxyl groups is methylated, as in (+)-12-*O*-methyltricordatine (**33**) and (+)-cocsuline (**34**), the cytotoxicity is intermediate between the toxicity of (+)-isotrilobine (**32**) and of (+)-tricordatine (**31**). When only C-6′ bears a methoxyl group as in (+)-cocsuline (**34**), the activity against KB and W2 decreases compared to that of (+)-tricordatine (**31**), although activity against the D6 clone appears unchanged. In the case of a methoxyl at C-12, that is, (+)-12-*O*-methyltricordatine (**33**), the toxicity is similar to that observed for (+)-tricordatine (**31**); however, the antiplasmodial activity is increased more than twofold against both clones of *Plasmodium*. Comparing the activities of (+)-cocsuline (**34**) and (+)-2′-norcocsuline (**35**, Chart 3), it may be noted that the replacement of a methyl group at N-2′ by a hydrogen atom leads to a twofold increase in cytotoxicity, which is paralleled by a twofold increase in antiplasmodial activity, which results in SIs that are essentially unchanged.

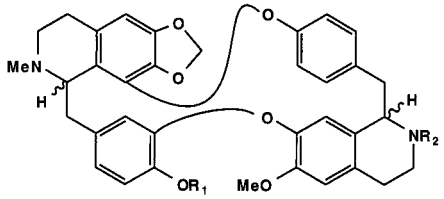
In the same manner, the observed increase in the antiplasmodial activity from (1*S*,1′*S*)-(+)-isotrilobine (**32**) and (1*R*,1′*S*)-(+)-*N*-methyltelobine (**36**), respectively, correlated with an increase in cytotoxicity, and this may be related to the change of configuration at C-1. Oxidation of the secondary amine at N-2′, as in (1′*S*)-(+)-1,2-dehydrotelobine (**37**), decreased the activity against the D6 clone, while the cytotoxicity increased, thereby reducing the SI.

(1*S*,1′*S*)-(−)-6,12-*O*-Demethylthalmine (**38**) is the only representative of its subgroup with a C-7,C-5′ and a C-11,C-12′ linkage. Due to the relatively high IC_{50} values observed with both clones of *Plasmodium* and appreciable cytotoxicity, it did not yield a promising SI.

(1*S*,1′*S*)-(+)Thalmirabine (**39**) belongs to a rare subgroup of bisbenzylisoquinoline alkaloids possessing two etheroxy bridges at C-8,C-5′ and C-11,C-12′. IC_{50} values against both clones of *Plasmodium* indicate good antiplasmodial activity, but the high cytotoxicity results in only modest selectivity.

The remaining compounds, summarized in Table 1, are head-to-tail dimers and belong to three different groups. (1*R*,1′*R*)-(−)-Cycleanine (**40**) was the only available compound representing its subgroup (two etheroxy bridges at C-8,C-12′ and C-12,C-8′). It is important to note that the substitution pattern is identical in each benzylisoquinoline moiety, and the same configuration at C-1 and C-1′ lends

Chart 4



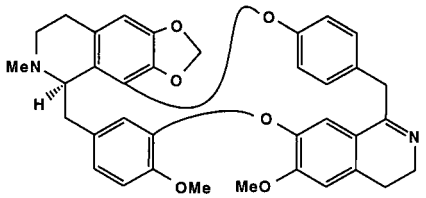
42 : 1*R*, 1'*S*; R_1=Me R_2=H

43 : 1*R*, 1'*R*; R_1=Me; R_2=H

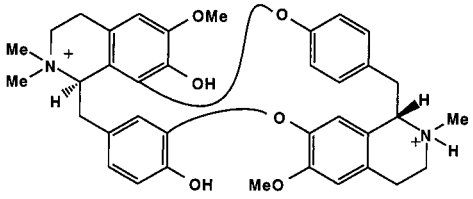
44 : 1*S* 1'*R*; R_1=Me; R_2=COMe

45 : 1*R* 1'*R*; R_1=H; R_2=COMe

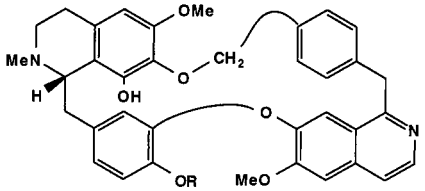
46 : 1*R* 1'*R*; R_1=Me; R_2=COMe



47

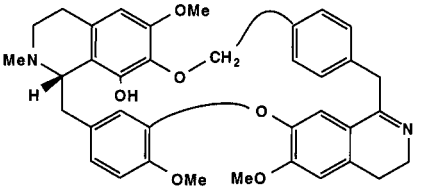


48

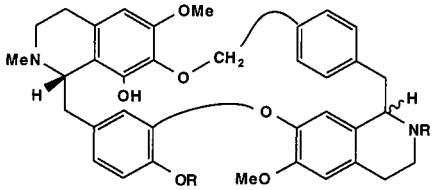


49 : R=Me

50 : R=H



51



52 : R=H

53: R=COMe

a perfect symmetry to the molecule, which is reflected in the ^{1}H NMR spectrum.[2] It is therefore a true dimer. As with (+)-temuconine (**2**), the lack of cytotoxicity observed with (+)-cycleanine (**40**) is remarkable among the bisbenzylisoquinoline alkaloids tested to date. Moreover, relatively potent antiplasmodial activity was observed, leading to impressive SIs of >460 against D6 and >140 against the W2 clone.

Eight of the bisbenzylisoquinoline alkaloids evaluated belonged to the (−)-curine (**41**) group with two etheroxy bridges at C-8, C12′ and C-11,C-7′. None of these eight alkaloids showed appreciable antiplasmodial selectivity. The 2′-*N*-acetylation seen in (−)-curicycleatjenine (**44**), (−)-isocuricycleatjine (**45**), and (−)-isocuricycleatjenine (**46**) greatly decreased their activity against both *P. falciparum* clones, while the cytotoxicity was unchanged or slightly lower. The presence of two quaternary nitrogen atoms, as in (−)-tubocurarine (**48**), leads to a lack of toxicity against KB cells, and of activity against both *Plasmodium* clones. (Chart 4).

The last five compounds tested belong to a subgroup of bisbenzylisoquinoline alkaloids that, thus far, have been isolated only from *Cyclea atjehensis*. The two benzylisoquinoline moieties are linked by an etheroxy bridge at C-11,C-7′ and by a methylenoxy bridge at C-7,C-12′. (+)-Cycleatjehenine (**49**) and (+)-cycleatjehine (**50**) possess a pyridine ring. (+)-Cycleatjehenine (**49**) exhibits an ED_{50} against KB cells of 14 700 nM, with a much lower IC_{50} against both clones of *Plasmodium*. The result is an SI of >100. Demethylation at C-12, as in (+)-cycleatjehine (**50**), does not interfere with the antiplasmodial activity, but decreases KB cytotoxicity twofold, thereby increasing the selectivity. The partial reduction of the pyridine ring, as in 1′,2′-dihydrocycleatjehenine (**51**), does not obviously alter the cytotoxicity (limited by the screening concentration used for the assay) but decreases the antiplasmodial activity threefold. When ring B′ is further reduced, as in (+)-2′-noratjecycline (**52**), no change is observed in the antiplasmodial activity, but the cytotoxicity increases about fourfold. The presence of a 2′-*N*-acetyl group (**53**) gives slightly lower toxicity and an approximately threefold decrease of the antiplasmodial activity.

Within each subgroup of bisbenzylisoquinolines analyzed, we have shown that a change of configuration of the chiral center, as well as modification of substituents, may lead to independent changes in cytotoxicity and antiplasmodial activity. However, except for the three one-bridged compounds, (+)-neothalibrine (**1**), (+)-temuconine (**2**), and (+)-malekulatine (**3**), which show low toxicity and appreciable antiplasmodial activity, the current results do not reveal any clear structure−activity relationship between subgroups of bisbenzylisoquinoline alkaloids. With the

exception of the one-bridged bisbenzylisoquinolines, all possess a large heterocycle of 18 to 20 atoms, which confers flexibility to the molecule. A study of the conformations assumed by compounds of the same subgroup (i.e., modification of conformation with the change of configuration at C-1 and C-1′) should give more information about the structure−activity relationship.

Within the limits of our data, several remarks may be made. The quaternarization of one or two nitrogen atoms, as in compound **48**, leads to a loss of toxicity and antimalarial activity. The same effect is observed by *N*-oxide formation in compound **19**. The presence of an acetyl function at N-2′, as in compounds **44**, **45**, **46**, and **53**, also results in a decrease in the cytotoxicity and antimalarial activity observed. The decrease in lipophilicity (membrane permeability) of all of these alkaloids probably contributes to the lower toxicity observed.

Nonetheless, it must be stressed that this group of natural compounds exhibits selective toxicity toward cultured malarial parasites. In terms of SI, some compounds compare favorably with known antimalarial agents (Table 1). Based on these data, it may be suggested that certain bisbenzylisoquinolines are worth considering as potential antimalarial agents. In this endeavor, the most promising compounds will need to be tested with in vivo models to define more clearly the structure−activity relationships and true efficacy. Compounds with favorable SI values represent reasonable starting materials, but based on resulting in vivo data, the compounds could be synthetically modified to yield drugs with higher selectivity. These studies may also provide valuable insight into new mechanisms of antiplasmodial action or structure−activity relationships.

Experimental Section

All compounds have been isolated by the authors or their research groups as referenced above. Immediately prior to biological testing, all compounds were purified by preparative TLC on Si gel. Greater than 95% purity, as verified by ^{1}H NMR spectroscopy, was considered acceptable.

Plasmodial Culture System. Cultures of *Plasmodium falciparum* (chloroquine-sensitive clone D6 derived from CDC Sierra Leone, and chloroquine-resistant clone W2 derived from CDC Indochina III) were maintained in human erythrocytes according to established methods.[18] Parasites were inoculated into type A+ human erythrocytes at a hematocrit of 6% in RPMI-1640 culture medium (GIBCO Laboratories, Grand Island, NY) supplemented with 32 mM $NaHCO_3$ (GIBCO), 25 mmol HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, Sigma Chemical Co., St. Louis, MO), and 10% heat-inactivated human plasma type A+. Parasitemia was maintained below 4% under an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 in 25 cm^2 culture flasks at 37 °C.

Antiplasmodial Bioassay. The antiplasmodial activity of test compounds was assessed with an in vitro radioisotope-incorporation method.[19] A suspension (200 μL) of *P. falciparum*-infected red blood cells (0.5−1.0% parasitemia, 1.0% cell hematocrit) was added to wells of a standard 96-well tissue culture plate containing 25 μL of substance to be tested. Each test compound was assayed in duplicate over a seven-point concentration range. In addition, the known antimalarial drugs quinine, chloroquine, mefloquine, and artemisinin were tested in each experiment over a seven-point concentration range. Microtiter plates were incubated for 24 h at 37 °C in a sealed chamber under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. After this incubation period, 0.5 μCi of ^{3}H(G)-hypoxanthine (New England Nuclear Research Products, Boston, MA, NET 177) was added to each well (25 μL of 20 μCi/mL), and the microtiter plate was returned to the sealed chamber at 37 °C for an additional 18 h of incubation. The assay was terminated by harvesting the contents of each microtiter plate onto a glass fiber filter using a Tomtec Mach III automatic cell harvester. Filters were dried and placed in polyethylene bags with 3.5 mL of biologically safe scintillation cocktail. Radioactivity was determined with a Wallac Microbeta liquid scintillation counter. Concentrations of both test compounds and positive controls that inhibited parasite-specific incorporation of [^{3}H]hypoxanthine by 50% (IC_{50}) were determined by nonlinear regression analysis. Zero-drug controls defined 100% incorporation. Limitations of the radioisotope-incorporation protocol include the acknowledgment that some antimalarials acting at the vector or exoerythrocytic stages of the life cycle or as gametocidal agents may be missed by an assay that depends on erythrocytic schizogony. Other limitations of this assay include the reliance upon some undefined media: the blood cells are obtained from different human donors at frequent intervals and thus introduce a degree of unavoidable variability. This was compensated for by including the IC_{50} data of the known antimalarial standard drugs with the report of each assay (mean ± standard error is shown in Table 1 for all standard drugs). Sample data were rejected unless the chloroquine-sensitive, quinine-sensitive, mefloquine-resistant phenotype of clone D6 and the chloroquine-resistant, quinine-resistant, mefloquine-sensitive phenotype of clone W2 showed appropriate responses.

Cytotoxicity Screening and ED_{50} Determination. KB-3 cells were cultured in Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and penicillin−streptomycin−fungizone (GIBCO) at 37 °C at 100% relative humidity with 5% CO_2 in air.

Evaluation of Cytotoxic Potential.[5,17] Cells were typically grown to 60%−70% confluence; the medium was then changed, and the cells were used for test procedures 1 day later. In each case, 96-well tissue culture plates were used. Test samples were initially dissolved in DMSO, and then diluted tenfold with H_2O. Serial dilutions were then performed using 10% aqueous DMSO as the solvent; 5×10^4 cells (in 190 mL of media) were then added to the 96-well plates and incubations performed for 72 h. All incubations were performed at 37 °C in a CO_2 incubator with the plates covered by vented plastic lids.

After the incubation period, cells were fixed to the plastic substratum by the addition of 50 μL of cold 50% aqueous trichloroacetic acid. The trichloroacetic acid-fixed cells were then stained by the addition of 0.4% sulforhodamine B (w/v) dissolved in 1% HOAc (30 min) and washed with 1% aqueous HOAc (4 ×). The bound dye was solubilized by the addition of 10 mmol unbuffered Tris base, pH 10 (200 μL). The absorption was determined at 515 nm using an ELISA plate reader. In each case, a zero-day control was performed by adding an equivalent number of cells to several wells of the 96-well plates and incubating at 37 °C for 10 min. The cells were then fixed with trichloroacetic acid and processed as described above.

Finally, the absorption values obtained with each of the treatment procedures were averaged, and the average value obtained with the zero-day control was subtracted. These values were then expressed as a percentage, relative to the solvent-treated control incubations, and ED_{50} values were calculated using nonlinear regression analysis (percent survival versus concentration). These experimental conditions were established in preliminary studies wherein it was shown (a) there is at least a sevenfold increase in cell number relative to the number of cells added to the plates at time zero; (b) the resulting absorption values are in an appropriate range to ensure reading accuracy (i.e., >1.4 A_{515} units); and (c) the cell number attained during the incubation period did not reach a plateau region on the growth curve.

Acknowledgment. The authors gratefully acknowledge the excellent technical assistance of Dr. Min You in the cytotoxicity bioassays. We also thank the Parasitology Laboratory at Walter Reed Army Institute of Research for generously providing the *Plasmodium falciparum* clones D6 and W2. This

work has been supported in part by grant #R29 AI34408 awarded by the National Institutes of Allergy and Infectious Diseases.

References and Notes

(1) Guha, K. P.; Mukherjee, B.; Mukherjee, R. *J. Nat. Prod.* **1979**, *42*, 1−84.
(2) Schiff, P. L., Jr. *J. Nat. Prod.* **1983**, *46*, 1−43.
(3) Schiff, P. L., Jr. *J. Nat. Prod.* **1987**, *50*, 529−599.
(4) Schiff, P. L., Jr. *J. Nat. Prod.* **1991**, *54*, 645−749.
(5) Likhitwitayawuid, K.; Angerhofer, C. K.; Ruangrungsi, N.; Cordell, G. A.; Pezzuto, J. M. *J. Nat. Prod.* **1993**, *56*, 30−38.
(6) Tantisewie, B.; Amurrio, S.; Guinaudeau, H.; Shamma, M. *J. Nat. Prod.* **1989**, *52*, 846−851.
(7) Lin, L.-Z.; Xue, L.; Shieh, H.-L.; Angerhofer, C. K.; Ruangrungsi, N.; Pezzuto, J. M.; Johnson, M. E.; Cordell, G. A. *J. Nat. Prod.* **1993**, *56*, 22−29.
(8) Angerhofer, C. K.; König, G. M.; Wright, A. D.; Sticher, O.; Milhous, W. K.; Cordell, G. A.; Farnsworth, N. R.; Pezzuto, J. M. In *Advances in Natural Product Chemistry*; Atta-ur-Rahman, Ed., Harwood Academic: Chur, Switzerland, 1992; pp 311−329.
(9) Guinaudeau, H.; Lin, L.-Z.; Ruangrungsi, N.; Cordell, G. A. *J. Nat. Prod.* **1993**, *56*, 1989−1992.
(10) Guinaudeau, H.; Böhlke, M.; Lin, L.-Z.; Angerhofer, C. K.; Cordell, G. A.; Ruangrungsi, N. *J. Nat. Prod.* **1997**, *60(3)*, 258−260.
(11) Tantisewie, B.; Pharadai, T.; Freyer, A. J.; Guinaudeau, H.; Shamma, M. *J. Nat. Prod.* **1990**, *53*, 553−558.
(12) Tantisewie, B.; Pharadai, K.; Amnauypol, S.; Freyer, A. J.; Guinaudeau, H.; Shamma, M. *Tetrahedron* **1990**, *46*, 325−330.
(13) Lavault, M.; Fournet, A.; Guinaudeau, H.; Bruneton, J. *J. Chem. Research* **1985**, *(S)*, 248−249.
(14) Guinaudeau, H.; Leet, J. E.; Freyer, A. J.; Shamma, M. *Tetrahedron* **1984**, *40*, 2513−2517.
(15) Bruneton, J.; Shamma, M.; Minard, R. D.; Freyer, A. J.; Guinaudeau, H. *J. Org. Chem.* **1983**, *48*, 3957−3960.
(16) Guinaudeau, H.; Cassels, B. K.; Shamma, M. *Heterocycles* **1982**, *19*, 1009.
(17) Partridge, S. J.; Russell, P. F.; Anderson, M. M.; Wright, C. W.; Phillipson, J. D.; Kirby, G. C.; Warhurst, D. C.; Schiff, P. L., Jr. *J. Pharm. Pharmacol.* **1990**, *42*(suppl), 97.
(18) Milhous, W. K.; Weatherly, N. F.; Bowdre, J. H.; Desjardins, R. E. *Antimicrob. Agents Chemother.* **1995**, *27*, 525−530.
(19) Desjardins, R. E.; Canfield, C. J.; Haynes, D. M.; Chulay, J. D. *Antimicrob. Agents Chemother.* **1979**, *16*, 710−718.

NP980144F