Effect of Plasma Protein Binding on in Vivo Activity and Brain Penetration of Glycine/NMDA Receptor Antagonists†

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A major issue in designing drugs as antagonists at the glycine site of the NMDA receptor has been to achieve good in vivo activity. A series of 4-hydroxyquinolone glycine antagonists was found to be active in the DBA/2 mouse anticonvulsant assay, but improvements in in vitro affinity were not mirrored by corresponding increases in anticonvulsant activity. Here we show that binding of the compounds to plasma protein limits their brain penetration. Relative binding to the major plasma protein, albumin, was measured in two different ways: by a radioligand binding experiment or using an HPLC assay, for a wide structural range of glycine/NMDA site ligands. These measures of plasma protein binding correlate well ($r = 0.84$), and the HPLC assay has been used extensively to quantify plasma protein binding. For the 4-hydroxyquinolone series, binding to plasma protein correlates ($r = 0.92$) with log *P* (octanol/pH 7.4 buffer) over a range of log *P* values from 0 to 5. The anticonvulsant activity increases with in vitro affinity, but the slope of a plot of pED_{50} versus pIC_{50} is low (0.40); taking plasma protein binding into account in this plot increases the slope to 0.60. This shows that binding to albumin in plasma reduces the amount of compound free to diffuse across the blood−brain barrier. Further evidence comes from three other experiments: (a) Direct measurements of brain/blood ratios for three compounds (**2**, **16**, **26**) show the ratio decreases with increasing log *P*. (b) Warfarin, which competes for albumin binding sites dose-dependently, decreased the ED_{50} of **26** for protection against seizures induced by NMDLA. (c) Direct measurements of brain penetration using an in situ brain perfusion model in rat to measure the amount of drug crossing the blood−brain barrier showed that compounds **2**, **26**, and **32** penetrate the brain well in the absence of plasma protein, but this is greatly reduced when the drug is delivered in plasma. In the 4-hydroxyquinolones glycine site binding affinity increases with lipophilicity of the 3-substituent up to a maximum at a log *P* around 3, then does not improve further. When combined with increasing protein binding, this gives a parabolic relationship between predicted in vivo activity and log *P*, with a maximum log *P* value of 2.39. Finally, the plasma protein binding studies have been extended to other series of glycine site antagonists, and it is shown that for a given log *P* these have similar protein binding to the 4-hydroxyquinolones, except for compounds that are not acidic. The results have implications for the design of novel glycine site antagonists, and it is suggested that it is necessary to either keep log *P* low or pK_a high to obtain good central nervous system activity.

Introduction

The factors that affect the penetration of a drug from the systemic circulation into the central nervous system (CNS) are complicated. Several models for the physical properties that affect a compound's ability to cross the blood−brain barrier have been proposed. The simplest, and one which has wide currency in medicinal chemistry folklore, is that there is an optimal octanol/water partition (log *P*) for brain penetration. This is based on observations[1−3] that for neutral molecules a parabolic relationship exists between central nervous system activity and log *P* with a maximum at a log *P* value around 2. Hansch[1] discusses this optimal log *P* with reference to increases in receptor binding with increased lipophilicity and concomitant increases in metabolism and simple, nonspecific binding by proteins.

There is some disagreement in the literature on the relevance of plasma protein binding to the clinical efficacy of drugs.[4−6] The major plasma protein, albumin, is present in amounts around 40 g/L (or about 600 μM) in the blood.[7] Albumin is responsible for the binding of acidic molecules, while the globulin α_1-acidic glycoprotein is important for the binding of basic and neutral compounds. There are believed to be a number of different binding sites on albumin for different types of compound, and association constants for natural ligands vary from 10^3 L/mol for compounds such as steroids to 10^8 L/mol for long-chain fatty acids. Association constants for drugs rarely exceed 10^6 L/mol.

Traditionally it has been felt that binding to plasma protein can reduce brain penetration, because for compounds that enter the central nervous system by a process of passive diffusion it is only the free drug in plasma that is important for the concentration gradient across the blood−brain barrier. However, an examination[8] of the relationship between percentage plasma protein binding and the ratio of the concentrations in CSF to that in plasma for a range of drugs showed that the relationship is not simple. Generally speaking, however, the compounds that were highly plasma

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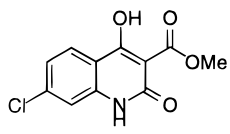
protein-bound did show poor CSF/plasma ratios. A study has been made of the effects of lipophilicity on the brain uptake of benzodiazepines.[9] An attempt was made to fit the extraction of the drugs into the brain with the concentration of free drug in plasma estimated from in vitro measurements of binding to plasma proteins; however, extraction into the brain was considerably higher than that expected on the basis of the in vitro measurements of the amount of free drug available in the presence of plasma protein.

It has been suggested that an important property in a compound for limiting its ability to cross the blood−brain barrier is the ability to form hydrogen bonds.[10] This can be calculated either by observation using Stein's[11] figures for the number of hydrogen bonds a particular functional group can form or by measurement of the Δlog *P* parameter,[12] defined as the difference between the log *P* values for the octanol/water and cyclohexane/water partitions. Δlog *P* is seen as a measure of a compound's ability to either donate or accept hydrogen bonds and correlates negatively with brain penetration. In an excellent study, Young et al.[13] showed that for a structurally diverse group of compounds designed as central H_2 receptor antagonists there is not a significant correlation between the logarithm of the brain/blood concentration ratio and the octanol/water partition coefficient, log P_{oct}. However if Δlog *P* was used, then a significant correlation was found with brain penetration. Young et al. make passing comment at the end of their paper that the log P_{oct} term in Δlog *P* might account for protein binding in peripheral blood, therefore limiting drug free to penetrate the brain.

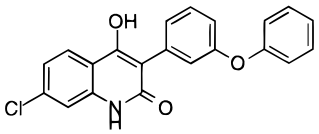
van der Waterbreemd[14] has suggested that the blood/brain concentration ratio for Young's set of compounds can be calculated from purely theoretical descriptors, the molecular volume and surface area of the hydrophilic portion of the molecule. Commenting on this work, Ganellin[15] found that when extrapolating either Young's or van der Waterbreemd's correlations to histaminergic agonists, they both overestimated brain penetration, with the latter behaving less well than the former. The agonists used to test the correlations were all smaller than the training set, and it is suggested that part of the problem may lie in the lack of sufficient experimental data relating to brain penetration, in particular the extent of binding to plasma protein in peripheral blood. Abraham[16] has commented that Young's correlation is specific to his data set, and he attempted to describe brain penetration more generally in terms of a number of descriptor variables for compounds, including excess molar refraction, polarizability, volume, and hydrogen-bonding terms. While the ability of a compound to bind to plasma protein in the blood might well be associated with some of these variables, this problem is not explicitly discussed in relation to brain penetration.

A model to predict whether a compound can cross the blood−brain barrier has been proposed based on the measurement of its surface activity.[17] In this model, compounds are divided into those that are CNS-active and those that are not. If one then plots a graph of the critical micelle concentration (CMC) against the lowest concentration for the onset of surface activity (C_0), then this divides into three regions: very hydrophobic compounds (low CMC and C_0), very hydrophilic compounds (high CMC and C_0), both of which do not penetrate the blood−brain barrier, and an intermediate region where they do. This model is predictive but is purely qualitative and does little to help explain the factors underlying brain penetration.

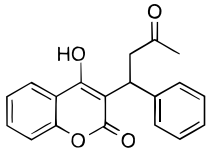
Chart 1. 4-Hydroxyquinolone Glycine/NMDA Antagonists and Warfarin



2



26



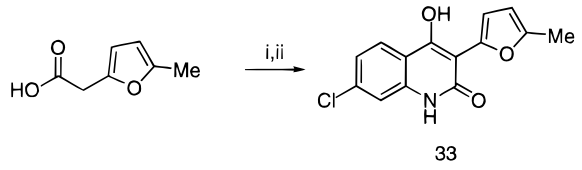
Warfarin

For some time we have been interested in antagonists at the glycine site of the NMDA receptor for possible use in stroke and schizophrenia.[18] An early problem was designing compounds that penetrated into the CNS. While high binding affinity could be achieved in kynurenic acid derivatives[19] and tetrahydroquinolines,[20] both these series contain carboxylic acids which, due to the highly polar nature of the charged carboxylate, limit their brain penetration. We set out to devise a carboxylic acid bioisostere that allowed brain penetration and succeeded with the design of 4-hydroxy-2-quinolones,[21,22] which for the first time gave consistent CNS activity in a series of full antagonists at this receptor. The prototype compound in this series, the methyl ester **2** (L-695,902; Chart 1), has moderate affinity (IC_{50} 6.4 μM) and activity in an anticonvulsant model in DBA/2 mouse (ED_{50} 52 μmol/kg). Manipulation of the substituent at the 3-position of the quinolone, leading to **26** (L-701, 324; Chart 1), gave a 3000-fold increase in binding affinity but only 20-fold improvement in in vivo activity (IC_{50} 2 nM, ED_{50} 2.4 μmol/kg). In trying to understand why this was the case, we considered the possibility that these compounds bind to plasma protein in the systemic circulation, therefore lowering the amount of drug free in the aqueous phase of the blood to penetrate into the brain. Compounds such as **26** resemble the structure of warfarin (Chart 1), an anticoagulant that binds tightly to a site on albumin defined as the warfarin binding site.[23] This paper discusses the results of experiments performed to determine the extent of plasma protein binding with 4-hydroxy-2-quinolones and the effect of that binding on CNS-mediated in vivo activity in an anticonvulsant screen. It then goes on to investigate whether other structural classes of glycine antagonists fit the trends found for the quinolone series.

Chemistry

Compounds **1**−**4**, **6**, **8**, **9**, **11**, and **15** are described in ref 21. **26**, **31**, **35**, **39**, and **48** are described in ref 22, and full characterization is given in the Experimental Section. Compounds **17**, **29**, **32**, **33**, and **59** were made by the general route shown in Scheme 1. Thus, coupling of 5-methylfuran-2-acetic acid[24] to the methyl ester of 4-chloroanthranilic acid followed by cyclization with potassium hexamethyldisilazide gave **33**. Compounds

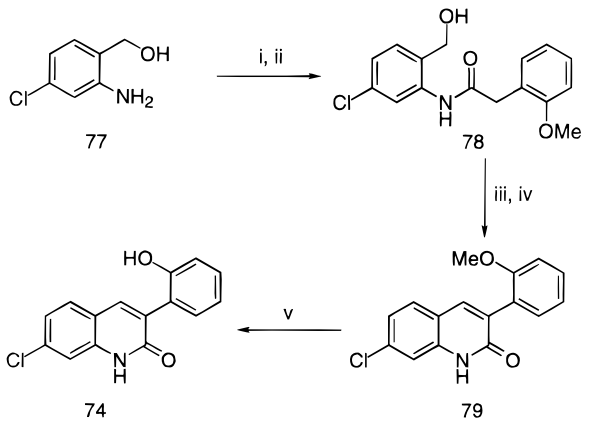
Scheme 1[a]



33

[a] Reagents: (i) methyl 4-chloroanthranilate, BOP-Cl, $ClCH_2CH_2Cl$, room temperature; (ii) KHMDS, THF, room temperature.

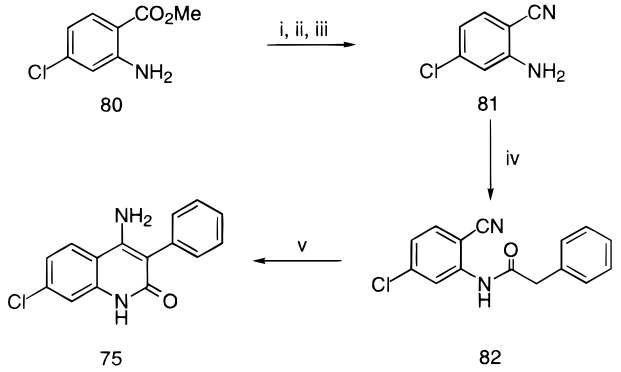
Scheme 2[a]



77 78 74 79

[a] Reagents: (i) 2-methoxyphenylacetyl chloride, dichloroethane, reflux, 50%; (ii) K_2CO_3, MeOH, 75%; (iii) PCC, CH_2Cl_2; (iv) NaOMe, MeOH, room temperature, 68% (two steps); (v) BBr_3, CH_2Cl_2, 0 °C, 83%.

Scheme 3[a]



80 81 75 82

[a] Reagents: (i) NH_3, MeOH, 150 °C, 61%; (ii) $(CF_3CO)_2O$, Et_3N, THF, 0 °C; (iii) K_2CO_3, $MeOH/H_2O$, 70 °C, 100%; (iv) phenylacetyl chloride, dichloroethane, reflux, 44%; (v) NaH, DMF, 100 °C, 33%.

16,[25] **60** and **61**,[26] **62**,[27] **63** and **64**,[19] **65**,[20] **66**,[28] **67** and **68**,[29] **69**,[30] **70**,[31] **71** (MNQX),[32] **72** (ACEA 1021),[33] **73**,[34] and **76**[35] are described in the literature. Esters **5** and **7** and ketones **10** and **12**−**14** were made using general procedures in ref 21.

The quinolone **74** was made from 2-amino-4-chlorobenzyl alcohol (**77**; Scheme 2). Coupling with the acid chloride derived from 2-methoxyphenylacetic acid gave the ester amide, in which the ester could be selectively hydrolyzed with potassium carbonate in methanol to give alcohol **78**. Oxidation followed by cyclization to **79** and removal of the methyl group from the anisole gave **74**. Conversion of the anthranilic ester **80** (Scheme 3) to the nitrile **81** followed by coupling to phenylacetyl chloride gave amide **82**, which was cyclized with sodium hydride to give the 4-aminoquinolone **75**.

Biology

Affinities of test compounds for the glycine site of the NMDA receptor were determined[36] by displacement of the glycine site antagonist [^{3}H]L-689,560 ([^{3}H]-**65**). IC_{50} values (concentration of test compound required to inhibit 50% of the specific binding) were evaluated via construction of five-point inhibition curves. IC_{50} values are given as the geometric mean of at least three experiments. The maximum standard error calculated from the pIC_{50} values was always less than 5% of the mean. Functional antagonism was measured[37] by the ability of test compounds to cause a rightward shift in the dose−response curve for NMDA-induced polarizations in rat cortical slices (K_b values). Results are the mean of at least three experiments, and errors are given as standard deviations in parentheses after the result. To test the effect of albumin on the functional antagonism, compounds were tested in the same way but including 2% (w/v) bovine serum albumin (Sigma) in the assay. The anticonvulsant activity of compounds was measured by their ability to protect against audiogenic seizure in DBA/2 mice when dosed intraperitoneally 30 min prior to seizure induction (either ED_{50} values or quoted as number protected/number tested at a particular dose).[38]

Protein Binding Assays. The affinity of **26** for human serum albumin (HSA) has been measured by the equilibrium dialysis method[39] and shown to have a K_a of 5×10^5 L/mol, a high binding affinity by comparison to many drug molecules. This means that when plasma concentrations of **26** are well below the concentration of albumin (i.e., $\ll$600 μM), which is the case in all the experiments described herein, then the drug is >99% bound to plasma protein. However, to measure relative binding to plasma protein for a large number of compounds, more accessible screens were developed.

Preliminary experiments were performed in the functional assay, where apparent K_b values were measured in the presence and absence of 2% (w/v) bovine serum albumin. It was found that the K_b values for **2** and **26** were reduced from 23.0 (±1.6) and 0.028 (±0.006) μM, respectively, when the assay was run under normal conditions to 598 (±198) and 5.9 (±1.0) μM, respectively, in the presence of bovine serum albumin. Therefore the reduction in potency of **2** is 25-fold and that of **26** is 200-fold when the plasma protein is present. This assay provided the first indication that the response of receptors to the 4-hydroxyquinolone glycine site antagonists was affected by plasma protein, but it is not suitable for screening large numbers of compounds due to the time required for each experiment.

The binding affinity of a compound for the glycine site of the NMDA receptor in the presence and absence of 0.2% (w/v) HSA was then investigated. Since compounds under test bind to the NMDA receptor and albumin, the affinity of a compound for the glycine site of the NMDA receptor is reduced in the presence of HSA in proportion to its binding to HSA. It is not possible to use [^{3}H]L-689,560 ([^{3}H]-**65**) in such an assay, because the radioligand itself binds to albumin (see Tables 1 and 2). However [^{3}H]glycine does not bind to HSA, so its affinity for the glycine site is not affected by HSA (Figure 1a). It has been shown[37] that affinities of ligands for the glycine site are the same when measured using either [^{3}H]L-689,560 or [^{3}H]glycine, and so the latter radioligand can be used to measure the binding of compounds in the presence and absence of HSA. The results of one such experiment are illustrated in Figure 1b, for compound **26**. As can be seen, the dose−response

Table 1. 4-Hydroxyquinolones

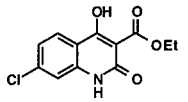
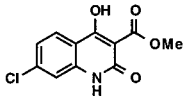
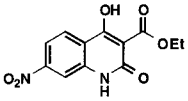
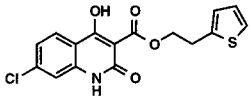
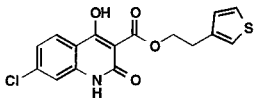
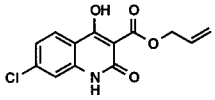
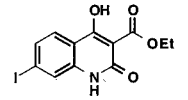
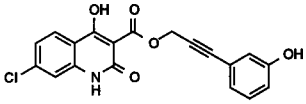
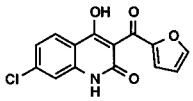
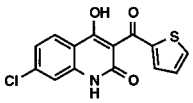
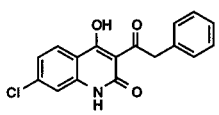
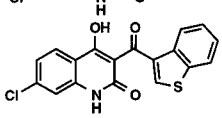
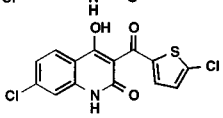
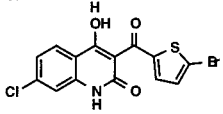
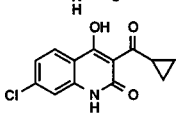
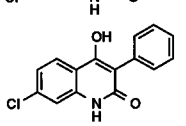
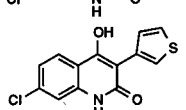
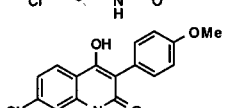
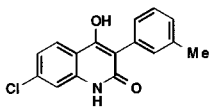
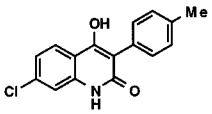
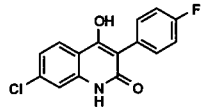
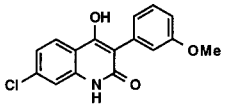
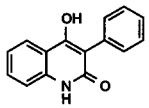
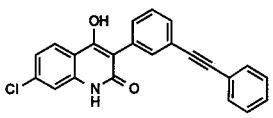
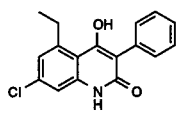
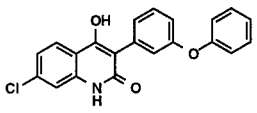
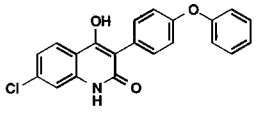
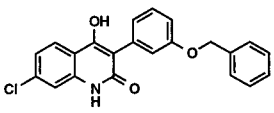
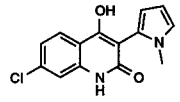
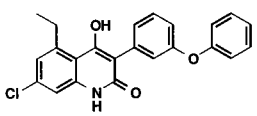
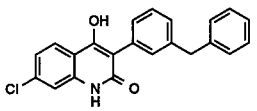
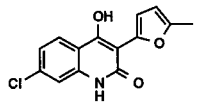
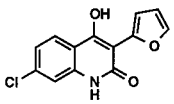
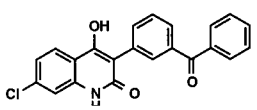
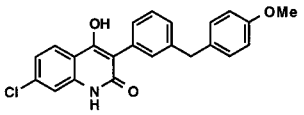
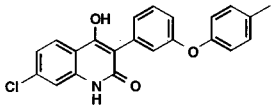
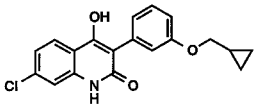
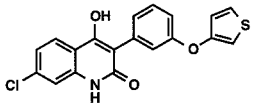
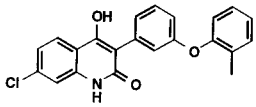
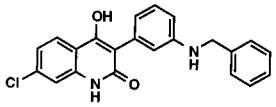
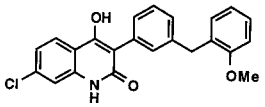
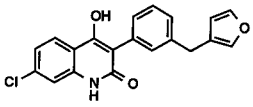
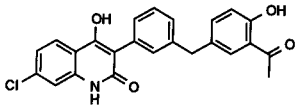
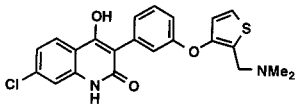
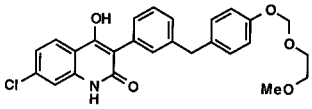
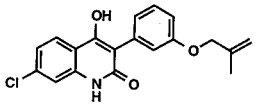
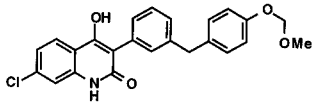
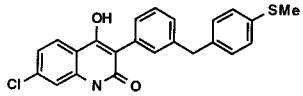
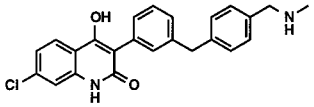
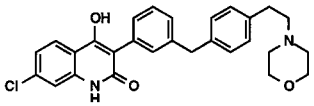
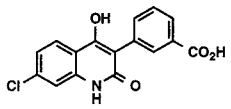
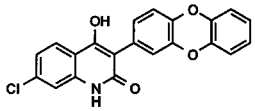
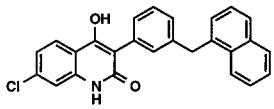
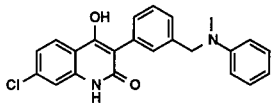
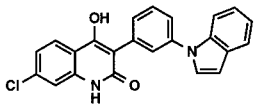
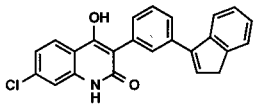
No.	Structure	HSAI[a]	PBI[b]	ED_{50}[c] mg/kg *μmol/kg*	IC_{50}[d] nM	logP[e]
1				41 *153*	16700	**0.70**
2		3	5.4	12.5 *52.2*	6450	0.24
3		1		47 *178*	25400	-0.25
4		44		25.2[f] *75.1*	3000	2.31
5		45		31.5[f] *93.9*	3000	2.51
6		6		40.1 *151*	6420	1.06
7		7		33.1 *95.9*	30000	1.11
8		213		0/8 at 20	175	**1.75**
9		3		13.1 *50.9*	2390	0.19
10		9		11.6 *42.4*	1090	0.95
11		28		31.6 *101*	932	1.66
12		59		36.8 *113*	2230	2.44
13		26		21.9 *71.1*	1520	1.66
14		40		24.8 *70.4*	954	1.81
15		6		4.1 *15.6*	419	0.35
16		10	37	4.5 *16.6*	172	**1.6**
17		11		6.9 *24.9*	352	1.45
18		8		2.7 *8.96*	421	1.58
19		14		2.1 *7.36*	84	2.16
20		13		4.3 *15.1*	391	2.16
21		8		7.7 *26.6*	658	1.74
22		10		7.7 *25.5*	204	1.58
23		2	3.1	10.9 *46.0*	7500	0.89
24		577		6.9 *18.5*	34.3	3.96
25		103		4.5 *15.0*	6.9	2.62
26		100	94	0.88 *2.42*	2	**2.9**
27		159		8.3 *22.8*	12.1	2.9
28		118		2.1 *5.56*	12.2	2.95
29		3		16.7 *60.8*	818	1.15
30		837		1.7 *4.34*	1.95	3.92
31		126	758	1.3[f] *3.60*	4	2.83
32		8	7.8	1.7[f] *6.17*	481	**1.05**
33		6		3.2 *11.8*	747	0.49
34		58		2/8 at 5	3.6	2.65

Table 1 (Continued)

No.	Structure	HSAI[a]	PBI[b]	ED_{50}[c] mg/kg *μmol/kg*	IC_{50}[d] nM	logP[e]
35		97	193	0.7 *1.79*	4.5	**2.81**
36		117		5/8 at 5	22.8	2.78
37		310		3.6 *9.54*	7.8	3.46
38		21		3.6 *10.5*	37.8	2.72
39		82	127	0.75 *2.03*	1.4	2.75
40		163		1.1 *2.91*	1.3	3.46
41		76		2.1 *5.58*	9.6	**2.57**
42		118		6/8 at 5	8	2.81
43		50		3/8 at 1	8.9	1.94
44		75		3/8 at 5	42.7	**3.31**
45		13		2/8 at 5	32.6	**1.73**
46		56		8/8 at 5 1/8 at 1	3.6	2.81
47		31		0.9 *2.64*	10.9	2.60
48		78		0.5 *1.19*	2.2	**3.34**
49		267		7/8 at 5	2.4	3.44
50		13		0/8 at 5	124	**1.25**
51		49		1/8 at 5	9	**3.01**
52		9		2/8 at 20	75.6	1.28
53		260		NT[g]	3000	**4.5**
54		629		NT[g]	27.9	4.06
55		140		NT[g]	11.7	2.96
56		322		NT[g]	10.2	**3.63**
57		571		NT[g]	6	3.75
58		68		NT[g]	3.3	1.96

[a] Human serum albumin index, as measured by retention time on an HPLC column containing human albumin. [b] Protein binding index, the ratio of binding affinities for the displacement of [^{3}H]glycine from rat cortical membranes in the presence and absence of 0.2% human serum albumin. [c] ED_{50} for protection against audiogenic seizure in DBA/2 mouse or (number protected)/(number tested at dose). ED_{50}'s are quoted in mg/kg (plain text) and in μmol/kg (italics). [d] IC_{50} for displacement of [^{3}H]L-689,560 (**65**) from rat cortical membranes. [e] log *P* either measured (in bold) or calculated[47] from tables of π values. [f] Tested 10 min postdosing. [g] Not tested.

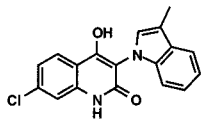
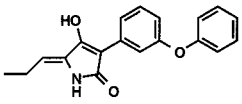
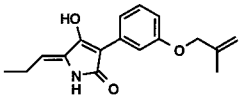
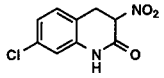
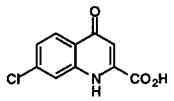
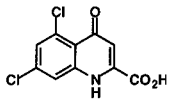
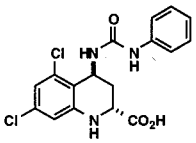
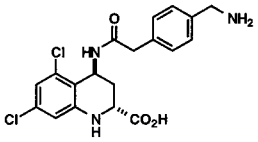
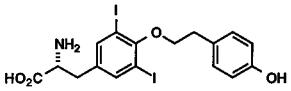
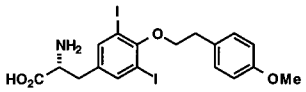
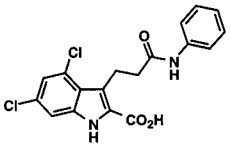
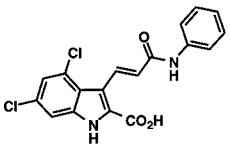
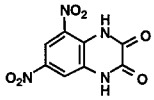
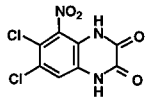
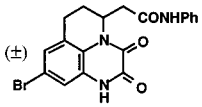
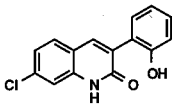
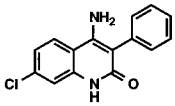
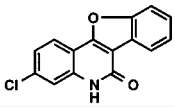
curve is shifted to the right in the presence of HSA, with the affinity of **26** reduced from 2 nM in the absence of HSA to 194 nM in the presence of HSA. This allows the calculation of a protein binding index (PBI) (eq 1), defined as the ratio of the IC_{50} values of the test compound for displacement of [^{3}H]glycine in the presence and absence of 0.2% (w/v) human serum albumin.

$$\text{PBI} = IC_{50}(\text{with albumin})/IC_{50}(\text{without albumin}) \quad (1)$$

The PBI for **26** is therefore 97. PBI values were measured for 18 compounds and are shown in Tables 1 and 2. While the binding assay to determine PBI values was more efficient than measurement of an affinity constant by equilibrium dialysis and quicker than measuring functional potency both in the presence and in the absence of albumin, it was still somewhat cumbersome because it required the use of [^{3}H]glycine to measure affinities and therefore requires centrifugation rather than filtration.

The preferred assay that we developed used HPLC to measure the relative binding of compounds to serum albumin. This was performed by comparison of HPLC

Table 2. Other Series of Glycine/NMDA Antagonists

No.	Structure	HSAI[a]	PBI[b]	ED_{50}[c] mg/kg *μmol/kg*	IC_{50}[d] nM	logP[e]
59		57		45 *138*	157	**1.46**
60		42	32	2/8 at 50	684	**2.21**
61		13	12.1	NT[f]	3030	1.91
62		7		13.5[g] *59.6*	414	**0.62**
63		2		NT[f]	320	**-1.15**
64		5	26.5	0/8 at 250	64	-0.44
65		18[h]	8.4	0/8 at 100	4	**1.08**
66		2	1.1	30 *73.5*	38.5	-0.49
67		16	41	0/8 at 100	426	**2.00**
68		32	11.7	0/8 at 100	545	2.65
69		142		NT[f]	36.4	**2.11**
70		197		0/8 at 10	1	**2.24**
71		4		0/8 at 10	170	NT[i]
72		28		3.3 *11.9*	2.8	**0.21**[k]
73		24		NT[j]	24	**2.78**
74		15	8.2	0/8 at 20	563	**3.20**
75		7	3.7	16.2 *59.8*	6750	**2.95**
76		70	28.5	0/8 at 20	8340	**4.34**

[a–e] See corresponding footnotes in Table 1. [f] Not tested. [g] This compound has activity at AMPA receptors which may contribute to its anticonvulsant effect. [h] One enantiomer. The other enantiomer has an HSAI of 23. [i] Not tested due to insolubility. [j] Not tested in this work, but workers at Sumitomo[26] report no activity at 100 mg/kg against NMDA-induced seizures. [k] Reference 48.

retention volumes using a column with a stationary phase of HSA immobilized on silica gel.[40] Since the major interaction governing retention on these phases is with the protein and it is known that the immobilized protein binds drugs in a fashion similar to that found in free solution, the retention volume should reflect the extent of binding to the protein.[41] This method allows a much higher throughput of compounds and was therefore used more extensively than either the functional or the binding assay. To allow a direct comparison between each compound, an HPLC human serum albumin binding index (HSAI) was determined. This represents the degree of drug−protein binding measured relative to an arbitrarily chosen standard compound, **26**. This figure is calculated as the ratio of the retention volume of the compound to the retention volume of **26**, normalized to 100. The retention volume is defined as

$$\text{retention volume} = (\text{retention time} - \text{system dead time}) \times \text{flow rate}$$

and the HSAI for compound **X** is

$$\text{HSAI}(\mathbf{X}) = 100 \times \text{retention volume}(\mathbf{X}) / \text{retention volume}(\mathbf{26})$$

Hence **26** has a standardized HSAI of 100, and it follows that compounds with HSAI > 100 are more highly protein bound to albumin than **26** and conversely those which are less bound will have HSAI < 100.

Correlation of Protein Binding Assays. Tables 1 and 2 show the compounds under discussion along with their results in the binding and HPLC assays. The compounds in Table 1 are 4-hydroxy-2-quinolones with a carbon substituent at the 3-position. These compounds are all acidic, with pK_a values of around 5 or below. Measured pK_a values for **1**, **26**, and **32** are 4.4, 5.2, and 4.6, respectively. Table 2 shows a number of other structural types. These include the N-linked indole (**59**), tetramic acids (**60**, **61**), 3-nitrodihydroquinolone (**62**), kynurenic acid derivatives (**63**, **64**), tetrahydroquinolines (**65**, **66**), a new series of tyrosine-based ligands (**67**, **68**), indoles (**69**, **70**), and quinoxalinediones (**71**, **72**). The last four compounds in Table 2 have considerably reduced acidity compared to the other examples. These are a derivative (**73**) of the quinoxalinediones from Sumitomo (pK_a 8.6), phenol (**74**) in which the acidic functionality of the 4-hydroxy-2-

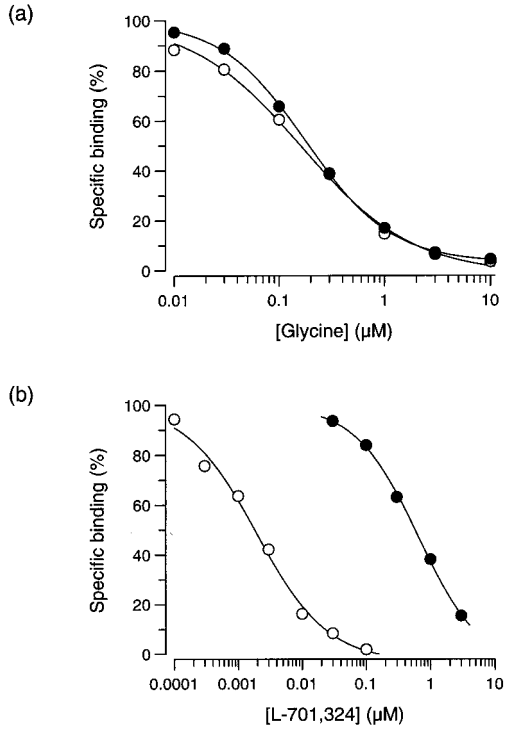


Figure 1. Inhibition of [^{3}H]glycine binding to rat cortex/hippocampus membranes by (a) glycine and (b) L-701,324, in the absence (○) and presence (●) of 0.2% human serum albumin. Inhibition curves are from the same experiment which was repeated several times with similar results.

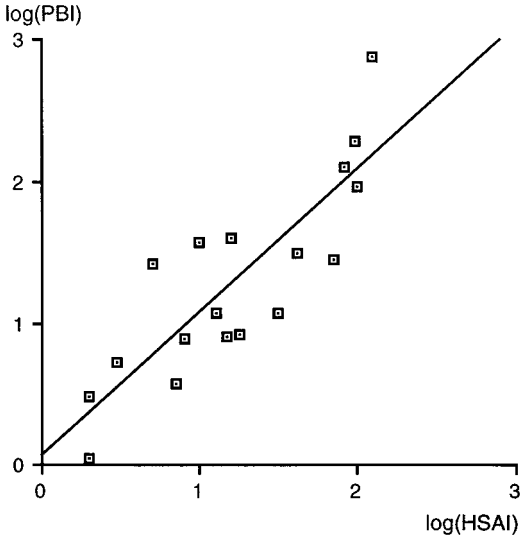


Figure 2. Correlation of the two measures of plasma protein binding: protein binding index (PBI) and HPLC human serum albumin index (HSAI).

quinolone has been moved to the pendent phenyl ring, 4-amino-2-quinolone (**75**) (pK_a > 11) in which the hydroxyl has been replaced with the less acidic amino group, and finally the compound (**76**) in which the acidic hydroxyl group of the 4-hydroxy-2-quinolone has been blocked by cyclization, to produce a nonacidic compound.

Since the HPLC assay is performed under reverse-phase conditions, where, for a nonpolar stationary phase more lipophilic compounds have longer retention times, it is important to establish that the HSAI is a measure of a compound's ability to bind to albumin, rather than just a direct measure of its lipophilicity. Figure 2 shows the correlation between the two measures of a compound's plasma protein binding, its PBI and HSAI. Data are taken from both tables and include all compounds for which there are measured values for PBI. Linear regression gives the fit:

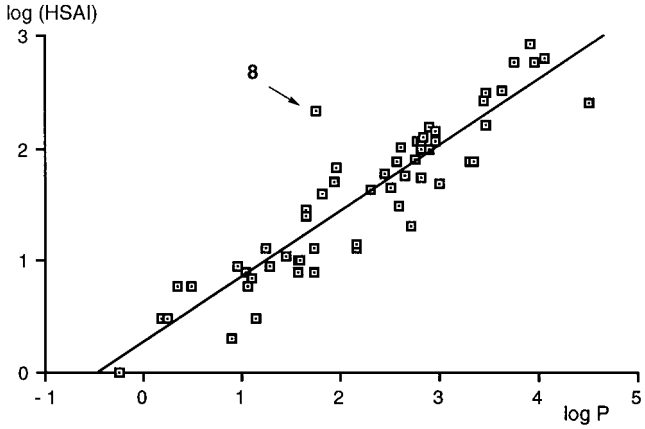


Figure 3. Correlation of plasma protein binding with log *P* for the 4-hydroxyquinolones in Table 1.

$$\log(\mathrm{PBI}) = 1.01(\pm 0.16)\log(\mathrm{HSAI}) + 0.06(\pm 0.22) \quad (2)$$

$$n = 18,\ r = 0.84,\ F = 40,\ s = 0.39$$

Errors in parentheses are the standard deviations in the coefficients.

These compounds cover the range of structural types, including acidic and nonacidic molecules. It can be seen that there is a highly significant correlation between the prediction of binding to plasma protein from these two measurements. Factors such as the different temperature and slight solvent differences in the two assays may explain some of the residual variance. A further factor in the measurement of the PBI is solubility. For compounds that have tight binding to plasma protein, it is necessary to attain high concentrations to measure their affinity in the presence of protein: achieving full solubility may be a problem (the solubility of **26** in water is less than 100 ng/mL), and therefore the PBI will, if anything, be overestimated for these compounds. For reasons of experimental ease and to overcome the solubility problems, we have therefore used the HPLC binding assay as a measure of a compound's ability to bind to plasma protein, in the knowledge that both the measures are equivalent.

4-Hydroxyquinolones. It has been known for some time[7] that there is a correlation between affinity for plasma protein and lipophilicity within a series of compounds. This is also the case for 4-hydroxyquinolones: Figure 3 shows this correlation, as a plot of log(HSAI) versus log *P* (octanol/pH 7.4 buffer) for the 4-hydroxy-2-quinolones with carbon substituents in the 3-position (compounds **2**−**58,** Table 1). There is a highly significant correlation between log(HSAI) and log *P*, with the line fitted by linear regression being

$$\log(\mathrm{HSAI}) = 0.59(\pm 0.03)\log P + 0.27(\pm 0.08) \quad (3)$$

$$n = 57,\ r = 0.92,\ F = 283,\ s = 0.28$$

The obvious outlier in this correlation is **8** with higher binding to plasma protein than would be predicted just on the basis of log *P*. This compound has a phenolic hydroxyl group in a region of the molecule where most of the others have just lipophilic groups—a possible interpretation is that this hydroxyl group picks up a specific interaction with the protein.

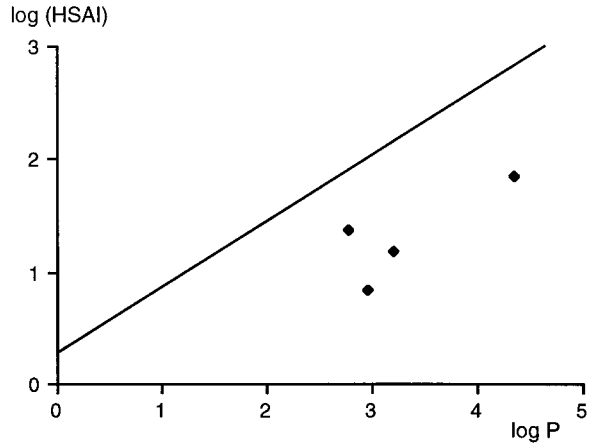


Figure 4. Nonacidic compounds **73**−**77** compared with Figure 3.

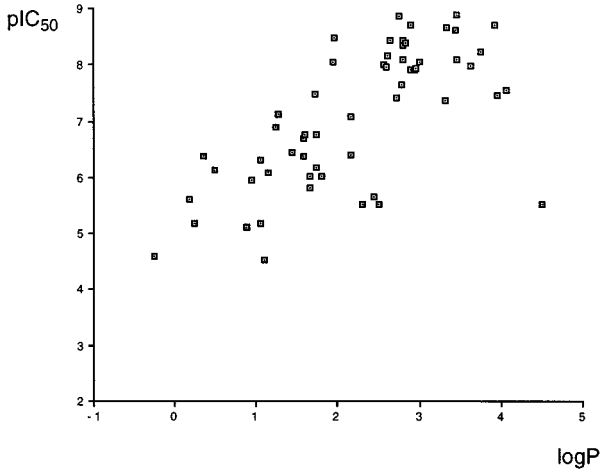


Figure 5. Effect of log *P* on glycine site binding affinity.

This correlation between log(HSAI) and log *P* does not hold for all compounds. Figure 4 shows the four less acidic compounds (**73**−**76**, Table 2) in comparison to eq 3. As can be seen, these compounds all show considerably less protein binding in relation to their log *P* values than the hydroxyquinolones, confirming that measurement of protein binding by HPLC is not in fact just a measurement of log *P*. Multiple regression analysis (vide infra) shows that the correlation between log(HSAI) and log *P* for nonacidic compounds is significantly different from that of the 4-hydroxyquinolones. Since we are using pH 7.4 buffer as the aqueous phase for the log *P* determinations, the acidic compounds are very largely ionized during the experiment. It is possible to calculate the log *P* values for the neutral species if the pK_a is known, but since ultimately we are interested in the fate of compounds in vivo where they will be present in the ionized form, this does not seem appropriate.

It was found in the medicinal chemistry program that increasing the lipophilicity in the region of the 3-substituent of the 4-hydroxyquinolones (**2**−**58**) increased binding affinity. As a general trend it was found that affinity increases up until a log *P* value around 3 and then levels off and decreases with large lipophilic substituents, indicating some steric inhibition of binding. This is illustrated graphically in Figure 5, which plots the binding affinity at the glycine site of the NMDA receptor against log *P*. The maximum affinity found by variation of the 3-position substituent was about 1 nM.[22] If all other factors remained equal, then

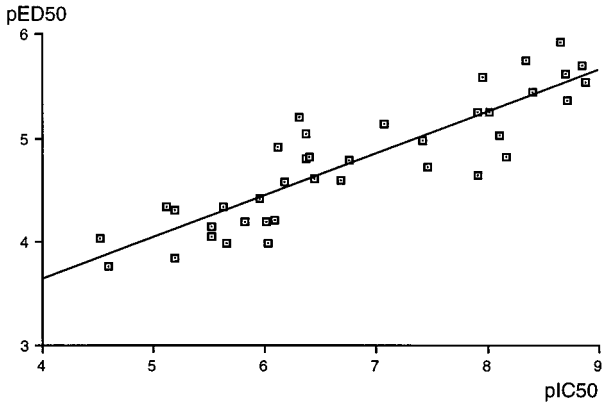


Figure 6. Correlation of in vivo activity with binding affinity.

it would be expected that an increase in binding affinity at the glycine site would translate directly to an increase in in vivo activity in the anticonvulsant assay, i.e.,

$$\mathrm{pED_{50}} = \mathrm{pIC_{50}} + k \qquad (4)$$

where *k* is a constant. Examination of the data for all the hydroxyquinolones that have measured ED_{50} values (Figure 6) shows that although the correlation between in vivo and in vitro activity is very good, there is clearly not a one-to-one improvement in pED_{50} with pIC_{50}.

$$\mathrm{pED_{50}} = 0.40(\pm 0.03)\mathrm{pIC_{50}} + 2.02(\pm 0.24) \qquad (5)$$

$$n = 39,\ r = 0.88,\ F = 130,\ s = 0.28$$

(For the correlation the ED_{50} values have been converted to mol/kg, which affects the intercept of this line but not the gradient.) It can be seen that the trend referred to for **2** and **26** holds out across the range of compounds in Table 1: there is an increase in anticonvulsant activity as binding affinity increases, but the slope of the graph is well below 1. Thus, there is some factor that is stopping the compounds from realizing the full effect of the increase in binding affinity at the glycine site.

It has already been shown that, along with glycine site binding affinity, binding to plasma protein increases with log *P*. This binding to albumin has the consequence of reducing the glycine site binding affinity in the experiments performed in the presence of albumin, as seen in the PBI. In these experiments the reduction in affinity is a direct result of there being less drug available in the aqueous component for binding to receptors (or a competition between albumin and the glycine site for drug). Substituting eq 2 [using log(PBI) = log(HSAI)] into eq 1, one obtains

$$\mathrm{pIC_{50}(with\ albumin)} = \mathrm{pIC_{50}(without\ albumin)} - \log(\mathrm{HSAI}) \qquad (6)$$

In the in vivo experiments, if the drug is getting into the brain by passive diffusion, then it is driven in by a concentration gradient across the blood−brain barrier. The reduction in the concentration in the aqueous portion of blood (free for diffusion) caused by binding to plasma protein will be proportional to the reduction in the concentration in the aqueous phase of the binding experiment caused by binding to plasma protein (assuming no other factors keep the drug from the aqueous phase). It is therefore reasonable to use the term for pIC_{50}(with albumin) from eq 6 in eq 4 giving

$$pED_{50} = [pIC_{50} - \log(HSAI)] + k \quad (7)$$

as an expectation for the relationship of in vivo and in vitro properties, if the binding to plasma protein is an important factor in reducing brain penetration.

The data are plotted in this way in Figure 7 and fitted by

$$pED_{50} = 0.60(\pm 0.05)[pIC_{50} - \log(HSAI)] + 1.50(\pm 0.25) \quad (8)$$

$$n = 39,\ r = 0.89,\ F = 143,\ s = 0.27$$

It can be seen that the fit of the data is improved when protein binding is taken into account, and the slope of the graph is closer to unity. It is the slope of the graph that is important, since it is a correlation between in vitro and in vivo measures of activity that is being sought in order to explain the weaker than expected anticonvulsant activity for these compounds.

The gradient is still not equal to unity, which is what might be expected if all the lack of in vivo activity were to be explained by protein binding. It must be remembered however that, while the values obtained for the HSAI and those for the PBI are essentially the same (eq 2), the glycine binding experiments are performed in the presence of only 0.2% (w/v) plasma protein. Higher amounts cannot be used in the assay because the binding affinity of [^{3}H]glycine is then reduced, but physiological concentrations are 20-fold higher than this. If it were possible to estimate the effect on binding in the presence of more physiologically appropriate amounts of protein, then the gradient of such a graph would be even higher. Of course there are probably other factors affecting brain penetration and in vivo activity in these compounds, including partitioning into lipid both systemically and in the brain and possibly drug metabolism, but eq 8 provides good evidence that plasma protein binding plays a major role in restricting in vivo activity in 4-hydroxyquinolones.

Further evidence for the role of plasma protein binding in determining brain penetration and in vivo activity has been obtained from a number of other experiments. The brain/blood concentration ratios have been measured directly by HPLC for three compounds (**2**, **16**, **26**); 12 rats were dosed iv with the compound under investigation and then sacrificed in groups of 3 at 5, 15, 30, and 60 min. The brains were removed, and the amount of drug in plasma and brain was measured by HPLC. Allowance was made for residual blood in the brain (using a figure of 4.8 μL/g), and figures were corrected for this. Calculations of areas under the curve for both brain and plasma were made, and the brain/blood ratio was estimated from these. Results are in Table 3 and are expressed as percentages. It can be seen that qualitatively the results are the same as those found for the anticonvulsant assay, with the brain penetration decreasing with increasing lipophilicity of the compound, so that for **2** the figure is 8%, but this falls to 4% for **16** and to around 1−2% for the highly lipophilic **26**. Since it is known that protein binding increases with increasing lipophilicity, one suggestion from this experiment is that increasing binding to plasma protein reduces brain penetration.

It is possible to competitively displace the binding of a drug to plasma protein with another compound that binds to the same site. This has been investigated for

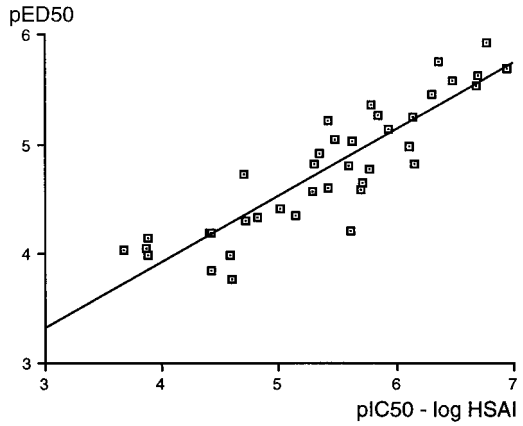


Figure 7. Correlation of in vivo activity with binding affinity, taking plasma protein binding into account.

Table 3. Brain/Plasma Ratios Measured Directly by HPLC

no.	HSAI	dose, mg/kg iv	brain/plasma ratio, %
2	3	20	8
16	10	10	4
26	100	3	1−2

Table 4. Anticonvulsant Effect[a] of **26** in the Presence and Absence of Warfarin

dose of warfarin,[b] mg/kg iv	ED_{50} of **26**,[c] mg/kg iv	limits,[d] mg/kg
0	3.6	2.1−5.4
60	1.9	1.3−2.5
100	0.64	0.48−0.77

[a] To prevent seizure induced by NMDLA (500 mg/kg sc) in Swiss Webster mice. [b] Given 15 min prior to **26**. [c] Given 15 min prior to NMDLA. [d] 95% confidence limits.

26 by testing its anticonvulsant profile in the presence of warfarin, which is known to bind to albumin.[23] The ability of **26** to prevent seizures in Swiss Webster mice induced by *N*-methyl-D,L-aspartate[42] was tested on its own and when warfarin was given 15 min before **26**. Warfarin (100 mg/kg iv) on its own does not protect against seizure (0/8 animals protected). Results of these experiments are presented in Table 4. It can be seen that warfarin dose-dependently reduces the amount of **26** needed for protection against seizure in the mice, with 100 mg/kg warfarin giving some 6-fold reduction in the ED_{50}. This indicates that when **26** is displaced from plasma protein by a competitive binding drug, more **26** is then free in plasma to penetrate into the brain.

Takasato et al. have developed[43] a method for directly measuring cerebrovascular penetration in the rat, and we have used this method, with modifications described by Gratton,[44] to investigate the brain penetration of compounds **2**, **26**, and **32**. In this experiment drug is introduced directly into the carotid artery of rats in the absence of blood flow from the heart and fluid flow maintained at a constant rate and pressure using a syringe pump. The concentration of drug is kept constant at 100 μg/mL, and the experimental time is kept short, so the flow across the blood−brain barrier is essentially unidirectional. Drug in saline was perfused for 10 s and in plasma for 60 s, so brain concentrations are low but measurable. After this period of time the amount of drug in the brain can be analyzed and a clearance from the perfusate calculated. The advantage of this method is that either rat plasma or phosphate-buffered saline can be used to dissolve the

Table 5. Clearances into Cortex from Plasma and Saline Using in Situ Brain Perfusion

compd	saline,[a] mL s^{-1} g^{-1} × 10^4	SEM[c]	plasma,[b] mL s^{-1} g^{-1} × 10^4	SEM[c]
2	410	8	2.8	0.7
26	340	38	0	0
32	450	43	4.4	0.5
diazepam	590	32	300	23
thiourea	2			

[a] Clearance from phosphate-buffered saline into cortex. Perfusion was over 10 s. [b] Clearance from rat plasma into cortex. Perfusion was over 60 s. [c] Standard error of the mean ($n = 4$ for each experiment).

drug, giving values for clearance into the brain in the presence and absence of plasma protein. The results of these experiments are shown in Table 5 along with control values for diazepam, which is highly brain penetrant, and thiourea, a compound that is considered poorly penetrant. Values found for clearance into frontal cortex for diazepam and thiourea are similar to those found by Gratton.[44]

It can be seen that clearance for **2**, **26**, **32**, and diazepam from saline into cortex is high and similar for all compounds. This shows that in the absence of plasma protein the glycine antagonists cross the blood−brain barrier as effectively as diazepam. The picture is very different when rat plasma is used as the vehicle. Clearance of **2** and **32** falls by 2 orders of magnitude, and clearance of **26** is too low to be measured in this experiment. The value for diazepam is reduced by only a factor of 2. In an equilibrium dialysis experiment diazepam was found[45] to have an association constant of about 5×10^4 L/mol, some 10-fold lower than that of **26**. The results for **2**, **32**, and **26** are in qualitative agreement with their binding to plasma protein as measured by the HSAI, where the value for **26** is 50-fold higher than that for **2** and 10-fold higher than that of **32**. This experiment provides direct evidence that binding to plasma protein modulates the brain penetration of **2**, **26**, and **32**.

It has therefore been shown that binding to plasma protein is implicated in reducing in vivo activity for **26** and reducing penetration of the blood−brain barrier for **2**, **26**, and **32**. Also decreasing brain concentrations as measured directly by HPLC for **2**, **16**, and **26** correlate with increasing lipophilicity, which has been shown to improve binding to serum albumin. When combined with these results, the correlations of anticonvulsant activity with in vitro binding experiments provide overwhelming evidence that plasma protein plays a major role in limiting the availability of the 4-hydroxyquinolone series of glycine site antagonists for activity in the central nervous system.

These correlations have been used in guiding the medicinal chemistry effort on this project. The compounds of most interest are the 3-aryl-4-hydroxyquinolones (**16**−**58**), as these consistently have the best affinities and anticonvulsant activities. It has been shown in eq 8 that there is a linear relationship between pIC_{50} − log(HSAI) and ED_{50}. Because pIC_{50} − log(HSAI) can be calculated for all compounds from in vitro measurements whereas the ED_{50} is often not measurable, we have explored the dependence of this in vitro measure on physicochemical properties. The relationship between pIC_{50} − log(HSAI) and log P is shown in Figure 8 and can be fitted by a quadratic equation:

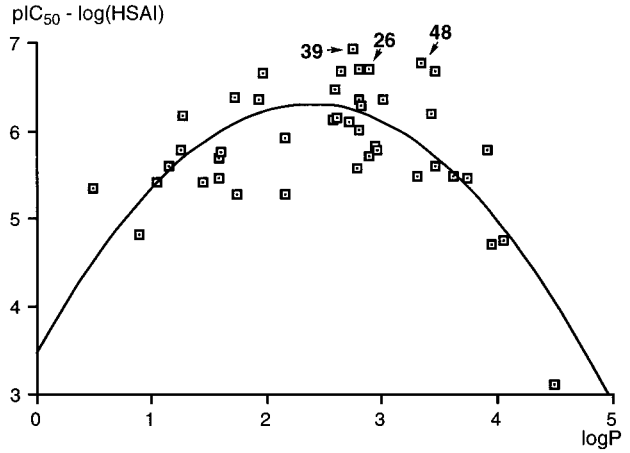


Figure 8. In vitro measure of expected in vivo activity plotted against log P for 3-aryl-4-hydroxyquinolones **16**−**58**, fitted with a quadratic equation.

$$[pIC_{50} - \log(HSAI)] = 2.39 \log P - 0.50(\log P)^2 + 3.4$$

$$n = 43,\ r = 0.73$$

which has a maximum at log $P = 2.39$. Using statistical methods to fit pIC_{50} − log(HSAI) to nth order polynomials in log P, it was found that a significant improvement was made by introducing a term in $(\log P)^2$ to the equation, but higher order terms did not explain any more of the variance in the data. The fit to a second-order polynomial in log P is therefore statistically significant. We have made a very large number of compounds with log P values in the region of 2−4 to explore structure−activity relationships, and the best three compounds in the anticonvulsant assay, **48** (ED_{50} 0.5 mg/kg), **39** (ED_{50} 0.75 mg/kg), and **26** (ED_{50} 0.88 mg/kg), are noted on Figure 8. These lie at the top of the curve, and we have reached the conclusion that these represent the optimal in vivo activity that can be achieved in this series by manipulation of a lipophilic 3-substituent. Compound **26** went into preclinical development as a result of the structure−activity relationship studies on 4-hydroxyquinolones.[22]

The value of log P that gives a maximum predicted in vivo activity (log $P = 2.39$) is remarkably similar to the log P that Hansch found[1] for the best anaesthetic activity in volatile ethers (log $P_0 = 2.35$). He has also summarized a number of findings for CNS-acting depressants[2] (including nonspecific and specific compounds such as benzodiazepines) and found that optimal log P's normally lie in a range around 2 ± 0.5. Hansch comments that for nonspecific hypnotics, optimum hydrophobicity is related primarily with movement through the body so that log P_0 says little about intrinsic potency. In the case of our glycine site antagonists two factors contribute to the existence of an optimal log P for in vivo activity: First, binding affinity increases with log P up to a certain point but then does not continue to rise (Figure 5). Second, there is a concomitant increase in factors that decrease the availability of drug to receptors in the CNS, in which binding to plasma protein plays a major role. The requirements of a receptor for lipophilic binding will be different for different receptors, and the relationship of log P to protein binding will vary from series to series, so the optimal log P found for 4-hydroxyquinolones might not be applicable to other series or other receptors.

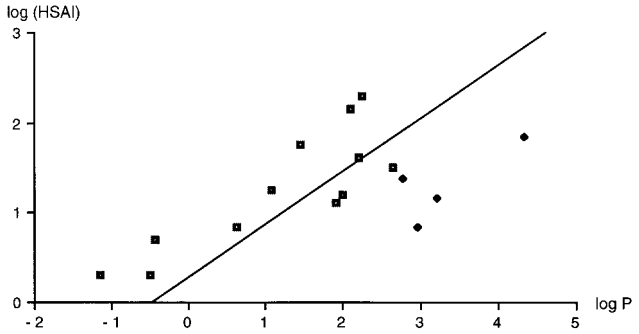


Figure 9. Plasma protein binding and log *P* values for compounds in Table 2. Acidic compounds (■) and neutral compounds (◆) are separated.

Other Series of Glycine/NMDA Antagonists. To examine the interaction of other types of glycine site antagonists with plasma protein, representative examples of different structural series are shown in Table 2. The results of comparing plasma protein binding with log P are shown in Figure 9, with the points divided into two sets: those for acidic compounds and those for nonacidic compounds (which have already been seen in Figure 4). The line shown on the graph is that fitted for the 4-hydroxyquinolones. Multiple linear regression with dummy variables was used to see if the relationship between log(HSAI) and log *P* differed for 4-hydroxyquinolones (**2**−**58**), acids (**59**−**72**), and neutral compounds (**73**−**76**). As a first step the equation was calculated using all three compound types. The resulting equation was highly significant with 71% of the variation in log(HSAI) predicted from log *P* ($r = 0.85$, $P < 0.0001$). The contrast between 4-hydroxyquinolones and acids was then entered into this equation but was not significant ($p = 0.48$). However, entry of the 4-hydroxyquinolone/neutral contrast at this stage would have produced a highly significant ($P < 0.001$) improvement in the equation with a 10% increase in variance predicted. These results show that the relationship between log(HSAI) and log *P* does not differ for 4-hydroxyquinolones and acids (**59**−**72**) but does differ significantly between these and the neutral compounds.

This analysis has important consequences for in vivo activity. The compounds in Table 2 with the best activity in the DBA/2 mouse model are the quinoxalinedione **72**, the 3-nitrodihydroquinolone **62** (ED_{50} 13.5 mg/kg, IC_{50} 414 nM, HSAI 7), and the aminoquinolone **75** (ED_{50} 16.2 mg/kg, IC_{50} 6750 nM, HSAI 7). Compounds **62** and **75** maintain reasonable anticonvulsant activity despite their relative poor binding affinity, presumably due to the low plasma protein binding. Compound **62** keeps plasma protein binding low with the absence of lipophilic groups reducing log *P*, and **75** has low protein binding despite a relatively high log *P* because of its high pK_a. A further compound of interest is **66** (ED_{50} 30 mg/kg, IC_{50} 13.5 nM, HSAI 2). This is one of the few tetrahydroquinolines that had measurable activity when dosed systemically in the anticonvulsant assay.[28] In comparison to 4-hydroxyquinolones the in vivo activity is poor despite reasonable affinity, but it is the only compound containing a carboxylic acid that has measurable activity in this assay. For **66** the HSAI is kept very low by reducing log *P* with the presence of a polar group (CH_2NH_2) in an area of the molecule tolerated by the glycine site. The low HSAI presumably removes the obstacle provided by protein binding that is present in other compounds. In contrast to these, the indole **70** suffers from the presence of a carboxylic acid and a high HSAI. This means that despite nanomolar affinity it does not have activity in the DBA/2 anticonvulsant assay.[46]

Quinoxalinediones **72** (ED_{50} 3.3 mg/kg, IC_{50} 2.8 nM, HSAI 28) and **73** (IC_{50} 24 nM, HSAI 24) illustrate the point that other factors need to be taken into account as well as protein binding. The first of these has affinity similar to that of **26** with lower protein binding. Despite this, the anticonvulsant potency is lower than that of **26**. Compound **73** was not active at 100 mg/kg against NMDA-induced seizures despite good affinity and a low HSAI. Compound **72** has two amide units and compound **73** has three, with each having high hydrogen-bonding ability, and they might be expected to have poor brain penetration based on that fact.[10]

Conclusion

It has been shown by direct methods that selected 4-hydroxyquinolone glycine antagonists readily cross the blood−brain barrier in the absence of plasma protein but that binding to serum albumin reduces brain penetration and in vivo activity for these compounds. This conclusion is supported by correlations of in vitro measurements of protein and glycine site binding with in vivo activity with a larger group of 42 4-hydroxyquinolones. The binding to plasma protein increases directly with log *P*. Because of steric limitations on the size of the 3-substituents in this series, there is an upper limit on binding affinity. The anticonvulsant activity therefore has a parabolic relationship with log P, and a maximum is reached with a log *P* value around 2.4.

The relationship between binding to plasma protein and log *P* is not significantly different in every other series of acidic glycine antagonists that have been investigated, and in these compounds good in vivo/in vitro ratios are only achieved when plasma protein binding is kept low. This can be achieved in two ways. The first, and less appealing, is to introduce polar substituents into the molecules in areas tolerated by the receptor. This has the disadvantage that polar, or hydrogen-bonding, groups are intrinsically poor for brain penetration. The second is to reduce the acidity of the compounds. In the absence of acidic functionality, binding to the major plasma protein, albumin, is much reduced. Until recently there were no examples of nonacidic glycine site ligands, but compounds **75** and **73** show that this can be achieved. Although neither is yet optimal for in vivo activity, the target of a high-affinity nonacidic glycine site antagonist is a highly desirable objective.

The compounds analyzed in this paper have all been glycine/NMDA site antagonists. However, the problem of brain penetration is one that affects all central nervous system-based medicinal chemistry programs, and the relevance of plasma protein binding to brain penetration should be considered on a wider scale. None of the physicochemical models for brain penetration that have been proposed specifically take protein binding into account, but it should be a factor when trying to design drugs that are to act within the blood−brain barrier.

Experimental Section

Melting points were taken on a Reichert Thermovar apparatus and are uncorrected. Proton NMR were measured on Bruker AM 360 or AC 250 spectrometers, and chemical shifts are reported in parts per million (δ) downfield from tetra-

methylsilane as internal standard; coupling constants are in hertz. Mass spectra were recorded on a VG 70/250 spectrometer. Merck Kieselgel (230−400 mesh) was used for column chromatography. For reactions, dry solvents were used as bought from Aldrich. Organic solutions were dried with anhydrous magnesium sulfate. Elemental analyses were done by Elemental Analysis Ltd., Okehampton, Devon, U.K., and Butterworth Laboratories Ltd, Teddington, Middlesex, U.K.

The general methods for the syntheses of 4-hydroxy-2(1*H*)-quinolone esters and ketones are described in ref 21.

2-(3-Thienyl)ethyl 7-chloro-4-hydroxy-2(1*H*)-oxo-quinoline-3-carboxylate (5): white needles (from DMF/H_2O); mp 293−295 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.04 (2 H, t, J = 6.7, CH_2Ar), 4.46 (2 H, t, J = 6.7, CO_2CH_2), 7.19 (1 H, dd, J = 1.5, 4.8, 4-thienyl), 7.25 (1 H, dd, J = 1.9, 8.7, H-6), 7.30 (1 H, d, J = 1.9, H-8), 7.41 (1 H, dd, J = 1.5, 3, 2-thienyl), 7.45 (1 H, dd, J = 3, 4.8, 5-thienyl), 7.94 (1 H, d, J = 8.7, H-5), 11.59 (1 H, s), 13.32 (1 H, br s); MS (CI^+, NH_3) m/z = 350 (M^+ + H). Anal. ($C_{16}H_{12}ClNO_4S$) C, H, N.

Ethyl 7-iodo-4-hydroxy-2(1*H*)-oxo-quinoline-3-carboxylate (7): white needles (from DMF); mp >300 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.30 (3 H, t, J = 7.1, CH_3), 4.33 (2 H, q, J = 7.1, CH_2), 7.53 (1 H, dd, J = 1.5, 8.5, H-6), 7.65 (1 H, d, J = 1.5, H-8), 7.67 (1 H, d, J = 8.5, H-5), 11.45 (1 H, br s); MS (EI) m/z = 359 (M^+). Anal. ($C_{12}H_{10}INO_4$) C, H, N.

3-(2-Thienylcarbonyl)-7-chloro-4-hydroxy-2(1*H*)-quinolone (10): yellow needles (from DMF); mp 314−315 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.18 (1 H, dd, J = 4, 5, H-4′), 7.31 (1 H, dd, J = 2, 8.5, H-6), 7.34 (1 H, d, J = 2, H-8), 7.71 (1 H, 1 H, dd, J = 1, 4, H-3′), 7.52 (1 H, d, J = 8.5, H-5), 8.02 (1 H, dd, J = 1, 5, H-5′), 11.6 (1 H, br s); MS (EI) m/z = 305 (M^+). Anal. ($C_{14}H_8ClNO_3S$) C, H, N.

3-(Benzo[*b*]thien-3-ylcarbonyl)-7-chloro-4-hydroxy-2(1*H*)-quinolone (12): white needles (from DMF); mp 306−307 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.21 (1 H, dd, J = 1, 11, H-6), 7.36 (1 H, d, J = 1, H-8), 7.45−7.5 (2 H, m, H-5′, H-6′), 7.98 (1 H, d, J = 11, H-5), 8.03 (1 H, d, J = 9, H-4′), 8.45 (1 H, s, H-2′), 8.47 (1 H, d, J = 9, H-7′), 10.8 (1 H, br s); MS (EI) m/z = 355 (M^+). Anal. ($C_{18}H_{10}ClNO_3S$) C, H, N.

3-[(5-Chlorothien-2-yl)carbonyl]-7-chloro-4-hydroxy-2(1*H*)-quinolone (13): white needles (from DMF); mp 304−305 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.21 (1 H, d, J = 1, H-4′), 7.24 (1 H, dd, J = 1, 10, H-6), 7.32 (1 H, d, J = 1, H-8), 7.65 (1 H, d, J = 1, H-3′), 7.94 (1 H, d, J = 10, H-5); MS (EI) m/z = 340 (M^+). Anal. ($C_{14}H_7Cl_2NO_3S$) C, H, N.

3-[(5-Bromothien-2-yl)carbonyl]-7-chloro-4-hydroxy-2(1*H*)-quinolone (14): white needles (from DMF); mp 309−310 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.25 (1 H, dd, J = 1, 10, H-6), 7.32 (1 H, d, J = 2, H-4′), 7.33 (1 H, d, J = 1, H-8), 7.60 (1 H, d, J = 2, H-3′), 7.93 (1 H, d, J = 10, H-5); MS (EI) m/z = 384 (M^+). Anal. ($C_{14}H_7BrClNO_3S$) C, H, N.

7-Chloro-4-hydroxy-3-(5-methyl-2-furyl)-2(1*H*)-quinolone (32). Bis(2-oxo-3-oxazolidinyl)phosphinic chloride (21.68 g, 113.6 mmol) was added to a stirred solution of methyl 4-chloroanthranilate (10.5 g, 56.8 mmol), 5-methyl furan-2-acetic acid (7.95 g, 56.8 mmol), and triethylamine (15.8 mL 113.6 mmol) in dry 1,2-dichloroethane (100 mL) at room temperature. The resulting mixture was stirred at room temperature for 18 h. The reaction mixture was then washed with aqueous 1 N HCl (2 × 50 mL), the organic layers were separated and dried, and the solvent was removed under reduced pressure to afford a red oil. The oil was dissolved in dry THF (100 mL) and treated with a solution of potassium hexamethyldisilazide (57.3 mL of 0.5 M solution in toluene, 28.4 mmol) at room temperature for 1 h. The reaction was quenched by the addition of methanol (50 mL) followed by aqueous 1 N HCl which resulted in the formation of a white precipitate. The solid was filtered, dried, and recrystallized from DMF to give **32** as white needles: mp 280−282 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.39 (3 H, s, Me), 6.19 (1 H, d, J = 1, furan H-3), 6.66 (1 H, d, J = 1, furan H-4), 7.21 (1 H, dd, J = 10, 1, H-6), 7.31 (1 H, d, J = 1, H-8), 7.91 (1 H, d, J = 10, H-5), 10.3 (1 H, br s, NH), 11.61 (1 H, s, OH); MS (EI) m/z = 275 (M^+). Anal. ($C_{14}H_{10}ClNO_3$) C, H, N.

The following compounds were prepared in a similar fashion.

7-Chloro-3-(3-thienyl)-4-hydroxy-2(1*H*)-quinolone (17): white needles; mp 341 °C dec (from DMF); ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.22 (1 H, dd, J = 8.5, 2, H-6), 7.31 (1 H, s, H-8), 7.43 (1 H, d, J = 4.9, thiophene-H-5), 7.49−7.51 (1 H, m, thiophene-H-4), 7.76 (1 H, s, thiophene-H-2), 7.98 (1 H, d, J = 8.5, H-5), 10.80 (1 H, br s, NH), 11.55 (1 H, s, OH); MS (EI) m/z = 277 (M^+). Anal. ($C_{13}H_8ClNO_2S$) C, H, N.

7-Chloro-4-hydroxy-3-(4-methoxyphenyl)-2(1*H*)-quinolone (18): mp >350 °C (from DMF/H_2O); ^{1}H NMR (250 MHz, DMSO-d_6) δ 3.81 (3 H, s, Me), 6.96 (2 H, d, J = 7.2, H-2′), 7.21 (1 H, dd, J = 8.7, 2.1, H-6), 7.29 (2 H, d, J = 7.2, H-3′), 7.33 (1 H, d, J = 2.1, H-8), 7.96 (1 H, d, J = 8.7, H-5), 11.49 (1 H, s, NH); MS (EI) m/z = 301 (M^+) (found, m/z = 301.0478; $C_{16}H_{12}ClNO_3$ requires m/z = 301.0507).

7-Chloro-4-hydroxy-3-(3-methylphenyl)-2(1*H*)-quinolone (19): mp >350 °C (from DMF/H_2O); ^{1}H NMR (250 MHz, DMSO-d_6) δ 2.35 (3 H, s, Me), 7.11−7.17 (4 H, m, ArH), 7.26−7.31 (2 H, m, ArH), 7.91 (1 H, d, J = 8.6, H-5), 11.49 (1 H, s, NH); MS (EI) m/z = 285 (M^+). Anal. ($C_{16}H_{12}ClNO_2$) C, H, N.

7-Chloro-4-hydroxy-3-(4-methylphenyl)-2(1*H*)-quinolone (20): mp >350 °C (from DMF); ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.34 (3 H, s, Me), 7.2−7.25 (5 H, m, ArH), 7.30 (1 H, d, J = 2.0, H-8), 7.91 (1 H, d, J = 8.6, H-5), 11.49 (1 H, s, NH); MS (EI) m/z = 285 (M^+). Anal. ($C_{16}H_{12}ClNO_2 \cdot 0.25H_2O$) C, H, N.

7-Chloro-3-(4-fluorophenyl)-4-hydroxy-2(1*H*)-quinolone (21): mp 335−337 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.13−7.18 (3 H, m, ArH), 7.32 (1 H, s, H-8), 7.38−7.42 (2 H, m, ArH), 7.92−7.94 (1 H, d, J = 8.6, H-5), 10.19 (1 H, br s, OH), 11.54 (1 H, s, NH); MS (EI) m/z = 289 (M^+). Anal. ($C_{15}H_9ClFNO_2$) C, H, N.

7-Chloro-4-hydroxy-3-(3-methoxyphenyl)-2(1*H*)-quinolone (22): mp >320 °C dec; ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.76 (3 H, s, Me), 6.88−6.94 (3 H, m, ArH), 7.20 (1 H, dd, J = 8.7, 2.1, H-6), 7.28−7.32 (2 H, m, ArH), 7.91 (1 H, d, J = 8.7, H-5), 10.24 (1 H, br s, OH), 11.48 (1 H, s, NH); MS (EI) m/z = 301 (M^+). Anal. ($C_{16}H_{12}ClNO_3$) C, H, N.

4-Hydroxy-3-phenyl-2(1*H*)-quinolone (23): mp 206 °C (from DMF); ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.17 (1 H, t, J = 7.1, H-7), 7.2−7.4 (6 H, m, ArH), 7.50 (1 H, td, J = 7.1, 1, H-6), 7.94 (1 dd, J = 7.1, 1, H-5), 10.09 (1 H, br s, OH), 11.43 (1 H, s, NH); MS (EI) m/z = 237 (M^+). Anal. ($C_{15}H_{11}NO_2$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(phenylethynyl)phenyl]-2(1*H*)-quinolone (24): mp 297−300 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.23 (1 H, dd, J = 8.6, 2.0, H-6), 7.33 (1 H, d, J = 2.0, H-8), 7.41−7.51 (9 H, m, ArH), 7.95 (1 H, d, J = 8.6, H-5), 10.50 (1 H, br s, OH), 11.58 (1 H, s, NH); MS (EI) m/z = 371 (M^+). Anal. ($C_{23}H_{14}ClNO_2$) C, H, N.

7-Chloro-5-ethyl-4-hydroxy-3-phenyl-2(1*H*)-quinolone (25): mp 284−288 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.20 (3 H, t, J = 7.3, Me), 3.12 (2 H, q, J = 7.3, CH_2), 6.99 (1 H, d, J = 2.1, H-6 or H-8), 7.20 (1 H, d, J = 2.1, H-6 or H-8), 7.30−7.43 (5 H, m, ArH), 9.83 (1 H, br s, OH), 11.49 (1 H, s, NH); MS (EI) m/z = 299 (M^+). Anal. ($C_{17}H_{14}ClNO_2$) C, H, N.

7-Chloro-4-hydroxy-3-(3-phenoxyphenyl)-2(1*H*)-quinolone (26): mp 303−306 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 6.96 (1 H, dd, J = 7.4, 1.8, H-4′), 7.01 (1 H, s, H-2′), 7.08−7.14 (4 H, m, ArH), 7.20 (1 H, dd, J = 8.8, 2.0, H-6), 7.31 (1 H, d, J = 2.0, H-8), 7.36−7.43 (3 H, m, ArH), 7.92 (1 H, d, J = 8.8, H-5), 10.40 (1 H, br s, OH), 11.53 (1 H, s, NH); MS (EI) m/z = 363 (M^+). Anal. ($C_{21}H_{14}ClNO_3$) C, H, N.

7-Chloro-4-hydroxy-3-(4-phenoxyphenyl)-2(1*H*)-quinolone (27): mp 274−276 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.01−7.08 (4 H, m, ArH), 7.15 (1 H, t, J = 7.5, H-4″), 7.21 (1 H, dd, J = 8.7, 2.1, H-6), 7.32 (1 H, d, J = 2.1, H-8), 7.37−7.44 (4 H, m, ArH), 7.93 (1 H, d, J = 8.7, H-5), 10.29 (1 H, br s, OH), 11.55 (1 H, s, NH); MS (EI) m/z = 363 (M^+). Anal. ($C_{21}H_{14}ClNO_3 \cdot 0.125H_2O$) C, H, N.

3-[3-(Benzyloxy)phenyl]-7-chloro-4-hydroxy-2(1*H*)-quinolone (28): mp 293−295 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 5.10 (2 H, s, CH_2), 6.93−7.08 (3 H, m, ArH), 7.21 (1 H, dd, J = 8.6, 2.0, H-6), 7.19−7.47 (7 H, m, ArH),

7.92 (1 H, d, J = 8.6, H-5), 10.26 (1 H, br s, OH), 11.52 (1 H, s, NH); MS (EI) m/z = 377 (M^+). Anal. ($C_{22}H_{16}ClNO_3 \cdot 0.25H_2O$) C, H, N.

7-Chloro-4-hydroxy-3-(1-methyl-2-pyrrolyl)-2(1*H*)-quinolone (29): white needles; mp >340 °C (from DMF); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.41 (3 H, s, Me), 5.90 (1 H, dd, J = 3, 1.5, pyrrole-H), 6.05 (1 H, dd, J = 3, 1.5, pyrrole-H), 6.84 (1 H, d, J = 1.5, pyrrole-H-5), 7.19 (1 H, dd, J = 8, 1.5, H-6), 7.31 (1 H, d, J = 1, H-8), 7.89 (1 H, d, J = 8, H-5), 10.18 (1 H, br s, NH), 11.50 (1 H, s, OH); MS (EI) m/z = 274 (M^+). Anal. ($C_{14}H_{11}ClN_2O_2 \cdot 0.4H_2O$) C, H, N.

7-Chloro-5-ethyl-4-hydroxy-3-(3-phenoxyphenyl)-2(1*H*)-quinolone (30): mp 222−224 °C (from ethyl acetate/hexane); ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.21 (3 H, t, J = 7.4, Me), 3.14 (2 H, q, J = 7.4, CH_2), 6.97−6.99 (3 H, m, Ar-H), 7.08−7.13 (4H, m, Ar-H), 7.22 (1 H, d, J = 2.2, Ar-H), 7.36−7.44 (3 H, m, Ar-H), 9.8 (1 H, br s, OH), 11.35 (1 H, br s, NH); MS (EI) m/z = 391 (M^+). Anal. ($C_{23}H_{18}ClNO_3$) C, H, N.

7-Chloro-4-hydroxy-3-(3-benzylphenyl)-2(1*H*)-quinolone (31): mp 304−306 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.96 (2 H, s, CH_2), 7.1−7.4 (11 H, m, ArH), 7.92 (1 H, d, J = 8.6, H-5), 10.2 (1 H, s, OH), 11.50 (1 H, s, NH); MS (CI^+, NH_3) m/z = 362 (M^+ + H). Anal. ($C_{22}H_{16}ClNO_2 \bullet 0.5H_2O$) C, H, N.

7-Chloro-3-(2-furyl)-4-hydroxy-2(1*H*)-quinolone (33): white needles; mp 246−250 °C (from DMF); ^{1}H NMR (360 MHz, DMSO-d_6) δ 6.62 (1 H, dd, J = 2, 1, furan H-4), 6.98 (1 H, d, J = 2, furan H-3), 7.21 (1 H, dd, J = 10, 1.5, H-6), 7.30 (1 H, d, J = 1.5, H-8), 7.79 (1 H, d, J = 1, furan H-5), 8.01 (1 H, d, J = 10, H-5), 11.0 (1 H, br s, NH), 11.63 (1 H, s, OH); MS (EI) m/z = 261 (M^+). Anal. ($C_{13}H_8ClNO_3 \cdot 0.4H_2O$) C, H, N.

7-Chloro-4-hydroxy-3-(3-benzoylphenyl)-2(1*H*)-quinolone (34): mp 294−295 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.31 (1 H, dd, J = 8.7, 2.0, H-6), 7.32 (1 H, d, J = 2.0, H-8), 7.5−7.9 (9 H, m, ArH), 7.96 (1 H, d, J = 8.7, H-5), 10.6 (1 H, s, OH), 11.6 (1 H, s, NH); MS (EI) m/z = 375 (M^+). Anal. ($C_{22}H_{14}ClNO_3 \cdot 0.4H_2O$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(4-methoxybenzyl)phenyl]-2(1*H*)-quinolone (35): mp 282−284 °C (from DMF); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.71 (3 H, s, OMe), 3.90 (2 H, s, CH_2), 6.83 (2 H, d, J = 7, H-3″), 7.1−7.3 (8 H, m, ArH), 7.91 (1 H, d, J = 8.6, H-5), 10.23 (1 H, br s, OH), 11.50 (1 H, s, NH); MS (EI) m/z = 391 (M^+). Anal. ($C_{23}H_{18}ClNO_3$) C, H, N.

7-Chloro-4-hydroxy-3-(3′-biphenylyl)-2(1*H*)-quinolone (36): mp >345 °C dec (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.22 (1 H, dd, J = 8.6, 1.9, H-6), 7.34−7.38 (3 H, m, ArH), 7.45−7.52 (3 H, m, ArH), 7.60−7.68 (4 H, m, ArH), 7.96 (1 H, d, J = 8.6, H-5), 10.39 (1 H, br s, OH), 11.57 (1 H, s, NH); MS (EI) m/z = 347 (M^+). Anal. ($C_{21}H_{14}ClNO_2 \cdot 0.25H_2O$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(4-methylphenoxy)phenyl]-2(1*H*)-quinolone (37): mp 296−298 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.28 (3 H, s, Me), 6.90−6.99 (4 H, m, ArH), 7.11 (1 H, d, J = 6.6, H-6), 7.17−7.22 (3 H, m, ArH), 7.31 (1 H, s, H-8), 7.38 (1 H, t, J = 7.9, ArH), 7.92 (1 H, d, J = 8.6, H-5), 11.53 (1 H, br s, NH); MS (EI) m/z = 376 (M^+). Anal. ($C_{22}H_{16}ClNO_3 \bullet 0.15H_2O$) C, H, N.

3-[3-(Cyclopropylmethoxy)phenyl]-7-chloro-4-hydroxy-2(1*H*)-quinolone (38): mp 320−322 °C (from DMF); ^{1}H NMR (360 MHz, DMSO-d_6) δ 0.30−0.35 (2 H, m, cyclopropyl CH_2), 0.55−0.60 (2 H, m, cyclopropyl CH_2), 1.25−1.30 (1 H, m, cyclopropyl CH), 3.81 (2 H, d, J = 7, OCH_2), 6.86−6.91 (3 H, m, ArH), 7.21 (1 H, dd, J = 2.0, 8.7, H-6), 7.26−7.31 (2 H, m, ArH), 7.91 (1 H, d, J = 8.7 H-5), 10.2 (1 H, br s, OH), 11.5 (1 H, s, NH); MS (EI) m/z = 397 (M^+). Anal. ($C_{19}H_{16}ClNO_3$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(3-thienyloxy)phenyl]-2(1*H*)-quinolone (39): mp >315 °C dec (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 6.93 (3 H, m, ArH), 7.05 (1 H, br s, ArH), 7.14 (1 H, d, J = 8, ArH), 7.21 (1 H, dd, J = 8.6, 2, H-6), 7.31 (1 H, d, J = 2, H-8), 7.37 (1 H, t, J = 8, H-5′), 7.56 (1 H, dd, J = 5, 3, ArH), 7.93 (1 H, d, J = 8.6, H-5), 10.37 (1 H, br s, OH), 11.52 (1 H, s, NH); MS (EI) m/z = 369 (M^+). Anal. ($C_{19}H_{12}ClNO_3S$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(2-methylphenoxy)phenyl]-2(1*H*)-quinolone (40): mp 288−290 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.22 (3 H, s, Me), 6.84 (1 H, dd, J = 8.0, 1.7, ArH), 6.89 (1 H, s, ArH), 6.96 (1 H, d, J = 8.0, Ar-H), 7.05−7.10 (2 H, m, ArH), 7.18−7.22 (2 H, m, ArH), 7.30−7.36 (3 H, m, ArH), 7.92 (1 H, d, J = 8.6, H-5), 11.49 (1 H, s, NH); MS (EI) m/z = 377 (M^+). Anal. ($C_{22}H_{16}ClNO_3 \cdot 0.15H_2O$) C, H, N.

3-[3-(*N*-Benzylamino)phenyl]-7-chloro-4-hydroxy-2(1*H*)-quinolone (41): mp 259−260 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 4.27 (2 H, d, J = 4.5, CH_2), 6.16 (1 H, t, J = 5.9, N*H*CH_2), 6.50 (2 H, dd, J = 7.7, 1.7, ArH), 6.62 (1 H, s, H-2′), 7.06 (1 H, t, J = 7.7, ArH), 7.17−7.24 (2 H, m, ArH), 7.28−7.39 (5 H, m, ArH), 7.88 (1 H, d, J = 8.7, ArH), 10.00 (1 H, br s, OH), 11.44 (1 H, br s, NH); MS (EI) m/z = 377 (M^+). Anal. ($C_{22}H_{17}ClN_2O_2$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(2-methoxybenzyl)phenyl]-2(1*H*)-quinolone (42): mp 265−270 °C (from DMF/acetone); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.79 (3 H, s, OMe), 3.92 (2 H, s, CH_2), 6.86 (1 H, dt, J = 7.5, 1, H-5″), 6.97 (1 H, d, J = 7.9, H-3″), 7.1−7.3 (8 H, m, ArH), 7.92 (1 H, d, J = 8.7, H-5), 10.2 (1 H, br s, OH), 11.50 (1 H, s, NH); MS (EI) m/z = 391 (M^+). Anal. ($C_{23}H_{18}ClNO_3$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(3-furanylmethyl)phenyl]-2(1*H*)-quinolone (43): mp >300 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.76 (2 H, s, CH_2), 6.38 (1 H, s, H-4″), 7.15−7.25 (4 H, m, ArH), 7.30−7.35 (2 H, m, ArH), 7.50 (1 H, s, H-2″ or H-5″), 7.56 (1 H, s, H-5″ or H-2″), 7.92 (1 H, d, J = 8.7, H-5), 10.2 (1 H, br s, OH), 11.50 (1 H, s, NH); MS (EI) m/z = 351 (M^+). Anal. ($C_{20}H_{14}ClNO_3$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(3-acetyl-4-hydroxybenzyl)-phenyl]-2(1*H*)-quinolone (44): mp 268−270 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.63 (3 H, s, Me), 3.95 (2 H, s, CH_2), 6.89 (1 H, d, J = 8.4, H-5″), 7.18−7.22 (3 H, m, ArH), 7.26 (1 H, s, H-2′), 7.30−7.34 (2 H, m, ArH), 7.42 (1 H, dd, J = 2.1, 8.4, H-6″), 7.82 (1 H, d, J = 2.1, H-2″), 7.92 (1 H, d, J = 8.6, H-5), 10.2 (1 H, br s, OH), 11.52 (1 H, s, NH), 11.8 (1 H, br s, OH); MS (CI^-, NH_3) m/z = 419 (M^-) (found, m/z = 419.0897; $C_{24}H_{18}ClNO_4$ requires m/z = 419.0924).

3-[3-[[2-[(Dimethylamino)methyl]-3-thienyl]oxy]phenyl]-7-chloro-4-hydroxy-2(1*H*)-quinolone (45): hydrochloride salt, mp 216−219 °C (from ^{1}PrOH); ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.75 (6 H, s, Me), 4.41 (2 H, s, CH_2), 6.91 (1 H, d, J = 5.5, thiophene H-4), 7.0−7.05 (2 H, m, ArH), 7.18 (1 H, d, J = 8, ArH), 7.22 (1 H, dd, J = 2.0, 8.7, H-6), 7.34 (1 H, d, J = 2.0, H-8), 7.42 (1 H, t, J = 8, phenyl H-5), 7.75 (1 H, d, J = 5.5, thiophene H-5), 7.98 (1 H, d, J = 8.7, H-5), 10.4 (1 H, br s, OH), 11.6 (1 H, s, NH); MS (EI) m/z = 426 (M^+). Anal. ($C_{22}H_{20}Cl_2N_2O_3S$) C, H, N.

7-Chloro-4-hydroxy-3-[3-[4-[(2-methoxyethoxy)methoxy]-benzyl]phenyl]-2(1*H*)-quinolone (46): mp 239−241 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.20 (2 H, s, Me), 3.42−3.45 (2 H, m, OCH_2), 3.68−3.70 (2 H, m, OCH_2), 3.90 (2 H, s, $ArCH_2$), 5.19 (2 H, s, OCH_2O), 6.94 (2 H, d, J = 8.5, H-3″), 7.1−7.2 (6 H, m, ArH), 7.28−7.32 (2 H, m, ArH), 7.91 (1 H, d, J = 8.6, H-5), 10.2 (1 H, br s, OH), 11.51 (1 H, s, NH); MS (EI) m/z = 465 (M^+). Anal. ($C_{26}H_{24}ClNO_5$) C, H, N.

7-Chloro-4-hydroxy-3-[3-[(2-methylprop-2-enyl)oxy]-phenyl]-2(1*H*)- quinolone (47): mp 297−299 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.23 (3 H, s, Me), 4.40 (2 H, s, OCH_2), 4.96 (1 H, s, CCH_2), 5.08 (1 H, s, CCH_2), 6.70−6.94 (3 H, m, ArH), 7.20 (1 H, dd, J = 8.6, 1.9, H-6), 7.27−7.31 (2 H, m, ArH, H-8), 7.92 (1 H, d, J = 8.6, H-5), 11.50 (1 H, s, NH); MS m/z = 342 (M^+). Anal. ($C_{19}H_{16}ClNO_3$) C, H, N.

7-Chloro-4-hydroxy-3-[3-[4-(methoxymethoxy)benzyl]-phenyl]-2(1*H*)-quinolone (48): mp 261−264 °C (from DMF/acetone/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.35 (3 H, s, Me), 3.90 (2 H, s, $ArCH_2Ar$), 5.13 (2 H, s, O CH_2O), 6.93 (2 H, d, J = 8.6, H-3″), 7.13−7.22 (6 H, m, ArH), 7.28−7.32 (2 H, m, ArH), 7.92 (1 H, d, J = 8.6, H-5), 10.2 (1 H, br s, OH), 11.51 (1 H, s, NH); MS (EI) m/z = 421 (M^+). Anal. ($C_{24}H_{20}ClNO_4$) C, H, N.

7-Chloro-4-hydroxy-3-[3-[4-(methylthio)benzyl]phenyl]-2(1*H*)-quinolone (49): mp 296−297 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.43 (3 H, s, Me), 3.92 (2 H, s, CH_2), 7.1−7.3 (10 H, m, ArH), 7.92 (1 H, d, J = 8.7, H-5), 10.1 (1 H, br s, OH), 11.51 (1 H, s, NH); MS (EI) m/z = (EI) 407 (M^+).

7-Chloro-4-hydroxy-3-[3-[4-[(methylamino)methyl]benzyl]phenyl]-2(1*H*)-quinolone (50): hydrochloride; mp 254−256 °C (from MeOH/H_2O); ^{1}H NMR (360 MHz, CD_3OD) δ 2.68 (3 H, s, Me), 4.06 (2 H, s, CH_2), 4.12 (2 H, s, CH_2), 7.21−7.24 (4 H, m, ArH), 7.35−7.40 (6 H, m, ArH), 7.95 (1 H, d, J = 8.5, H-5); MS (EI) m/z = 404 (M^+). Anal. ($C_{24}H_{21}ClN_2O_2 \cdot 1.7HCl$) C, H, N.

7-Chloro-4-hydroxy-3-[3-[4-(*N*-morpholinoethyl)benzyl]-phenyl]-2(1*H*)-quinolone (51): hydrochloride salt; mp 273−276 °C (from MeOH); ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.94−3.10 (6 H, m, NCH_2CH_2O, NCH_2CH_2Ar), 3.10−3.30 (2 H, m, NCH_2CH_2Ar), 3.84−3.94 (4 H, m, NCH_2CH_2O), 3.97 (2 H, s, $ArCH_2Ar$), 7.14−7.33 (9 H, m, $ArCH_2Ar$, H-6), 7.38 (1 H, d, J = 2.0, H-8), 7.95 (1 H, d, J = 8.6, H-5), 11.52 (1 H, s, NH); MS (CI^+) m/z = 475 ($M^+ + H$). Anal. ($C_{28}H_{27}ClN_2O_3 \cdot HCl$) C, H, N.

3-(3-Carboxyphenyl)-7-chloro-4-hydroxy-2(1*H*)-quinolone (52): mp >340 °C (from DMF); ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.23 (1 H, dd, J = 8.7, 2.2, H-6), 7.34 (1 H, d, J = 2, H-8), 7.52 (1 H, app t, J = 7.9, H-5′), 7.63 (1 H, dt, J = 7.8, 1.5, H-6′), 7.89 (1 H, dt, J = 7.8, 1.5, H-4′), 7.95−7.97 (2.25 H, m, H-5, H-2′, DMF), 10.48 (1 H, br s, OH), 11.61 (1 H, s, NH), 12.88 (1 H, br s, COOH); MS (CI^+, NH_3) m/z = 333 ($M + NH_4^+$). Anal. ($C_{16}H_{10}ClNO_4 \cdot 0.25DMF$) C, H, N.

3-(Dibenzo[*b*,*e*][1,4]dioxin-3-yl)-7-chloro-4-hydroxy-2(1*H*)-quinolone (53): mp >390 °C dec; ^{1}H NMR (360 MHz, DMSO-d_6) δ 6.9−7.0 (7 H, m, ArH), 7.21 (1 H, dd, J = 2.0, 8.7 H-6), 7.31 (1 H, d, J = 2.0, H-8), 7.93 (1 H, d, J = 8.7, H-5), 10.4 (1 H, br s, OH), 11.55 (1 H, s, NH); MS (CI^+, NH_3) m/z = 397 ($M + NH_4^+$). Anal. ($C_{21}H_{12}ClNO_4$) C, H, N.

7-Chloro-4-hydroxy-3-[3-(1-naphthylmethyl)phenyl]-2(1*H*)-quinolone (54): sodium salt; mp >310 °C (from ^{i}PrOH); ^{1}H NMR (360 MHz, DMSO-d_6) δ 4.35 (2 H, s, CH_2), 6.60 (1 H, d, J = 7.6, ArH), 6.82 (1 H, dd, J = 8.4, 2, ArH), 6.99 (1 H, t, J = 7.6, ArH), 7.02 (1 H, d, J = 2, ArH), 7.4−7.6 (5 H, m, ArH), 7.8−7.9 (3 H, m, ArH), 7.90−7.95 (1 H, m, ArH), 8.10−8.15 (1 H, m, ArH), 9.75 (1 H, s, NH); MS (CI^+, NH_3) m/z = 429 ($M + NH_4^+$). Anal. ($C_{26}H_{17}ClNNaO_2$) C, H, N.

7-Chloro-4-hydroxy-3-[3-[(*N*-methyl-*N*-phenylamino)-methyl]phenyl]-2(1*H*)-quinolone (55): sodium salt; mp >300 °C (from ^{i}PrOH); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.00 (3 H, s, Me), 4.47 (2 H, s, CH_2), 6.57 (1 H, t, J = 7.2, H-4″), 6.74 (2 H, t, J = 8, H-2″), 6.83 (1 H, dd, J = 2, 8.4, H-6), 7.0−7.2 (3 H, m, ArH), 7.61 (1 H, d, J = 7.8, ArH), 7.71 (1 H, d, J = 2, ARH), 7.86 (1 H, d, J = 8.4, H-5), 9.76 (1 H, s, NH); MS (CI^+, NH_3) m/z = 391 ($M^+ + H$). Anal. ($C_{24}H_{16}ClNO_2 \cdot 0.5H_2O$) C, H, N.

7-Chloro-4-hydroxy-3-(3-indol-1-ylphenyl)-2(1*H*)-quinolone (56): mp 290 °C dec (from AcOH); ^{1}H NMR (360 MHz, DMSO-d_6) δ 6.71 (1H, d, J = 3.1, indolyl H-2), 7.13 (1 H, t, J = 7.3, ArH), 7.18−7.25 (2 H, m, ArH), 7.42 (1 H, s, H-2′), 7.43 (1 H, d, J = 7.5, ArH), 7.52 (1 H, d, J = 7.0, ArH), 7.58−7.67 (4 H, m, ArH), 7.72 (1 H, d, J = 8.1, ArH), 7.98 (1 H, d, J = 8.7, H-5), 10.58 (1 H, br s, OH), 11.60, (1 H, s, NH); MS (EI$^-$) m/z = 386 ($M^{\bullet -}$). Anal. ($C_{23}H_{15}ClN_2O_2 \cdot 0.25H_2O$) C, H, N.

7-Chloro-4-hydroxy-3-(3-indenylphenyl)-2(1*H*)-quinolone (57): red solid; mp 258−260 °C (from EtOH); ^{1}H NMR (360 MHz, DMSO-d_6) δ 3.56 (2 H, s, CH_2), 6.73 (1 H, s, $CHCH_2$), 7.1−7.7 (10 H, m, ArH), 7.97 (1 H, d, J = 8.7, H-5), 10.5 (1 H, br s, OH), 11.6 (1 H, s, NH); MS (CI^+, NH_3) m/z = 388 ($M^+ + H$). Anal. ($C_{24}H_{16}ClNO_2 \bullet 0.7H_2O$) C, H, N.

7-Chloro-4-hydroxy-3-[3-[(3-thienylmethyl)amino]phenyl]-2(1*H*)-quinolone (58): mp 254−256 °C (from DMF/H_2O); ^{1}H NMR (360 MHz, DMSO-d_6) δ 4.26 (2 H, s, CH_2), 6.04 (1 H, br s, N*H*CHAr), 6.51 (1 H, d, J = 7.5, H-6′), 6.58 (1 H, d, J = 8.1, H-4′), 6.64 (1 H, s, H-2′), 7.10 (1 H, t, J = 7.8, H-5′), 7.12 (1 H, dd, J = 6, 0.8, thienyl H-5), 7.20 (1 H, dd, J = 8.6, 1.9, H-6), 7.30 (1 H, d, J = 1.9, H-8), 7.37 (1 H, s, thienyl H-2), 7.47 (1 H, dd, J = 4.9, 2.8, thienyl H-4), 7.89 (1 H, d, J = 8.6, H-5), 10.00 (1 H, br s, OH), 1.48 (1 H, s, NH); MS (CI^+, NH_3) m/z = 383 ($M^+ + H$). Anal. ($C_{20}H_{15}ClN_2O_2S$) C, H, N.

3-(3-Methylindol-1-yl)-7-chloro-4-hydroxy-2(1*H*)-quinolone (59): mp 290 °C dec; ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.31 (3 H, s, Me), 6.92−6.96 (1 H, m, indole H-7), 7.03 (1 H, d, J = 0.9, indole H-2), 7.04−7.09 (2 H, m, indole H-5,6), 7.27 (1 H, dd, J = 1.9, 8.6 H-6), 7.36 (1 H, d, J = 1.9, H-8), 7.51−7.55 (1 H, m, indole H-4), 7.95 (1 H, d, J = 8.6, H-5), 11.2 (1 H, br s, OH), 11.3 (1 H, s, NH); MS (EI) m/z = 324 (M^+). Anal. ($C_{18}H_{13}ClN_2O_2 \cdot 0.1H_2O$) C, H, N.

3-(2-Hydroxyphenyl)-7-chloroquinolin-2(1*H*)-one (74). Oxalyl chloride (2.95 mL, 4.31 g, 34 mmol) was added to 2-methoxyphenylacetic acid (4.7 g, 28.3 mmol) in CH_2Cl_2 (80 mL) containing DMF (3 drops) at room temperature. After 90 min the mixture was evaporated, the residue was dissolved in dichloroethane (80 mL), and 2-amino-4-chlorobenzyl alcohol (**77**; 2 g, 12.9 mmol) was added. The mixture was refluxed for 90 min, cooled, filtered, washed with $NaHCO_3$, evaporated, and purified by flash chromatography, eluting with 1:1 EtOAc−hexane to give 2-(3-methoxyphenylacetamido)-4-chlorobenzyl 2-methoxyphenylacetate (2.9 g, 50%) as a white solid. This was dissolved in MeOH (200 mL) and stirred with K_2CO_3 (5 g) at room temperature for 16 h. The mixture was partitioned between EtOAc and water; then the organic phase was washed with brine, dried, evaporated, and purified by flash chromatography, eluting with 1:1 EtOAc−hexane to give 2-(3-methoxyphenylacetamido)-4-chlorobenzyl alcohol (**78**; 1.47 g, 75%) as a white solid; 850 mg (2.8 mmol) of this solid was dissolved in CH_2Cl_2 (80 mL) with PCC (1.38 g, 6.4 mmol) and stirred for 3 h, before being diluted with EtOAc (70 mL) and filtered through silica gel. The solution was evaporated to give 870 mg of 2-(3-methoxyphenylacetamido)-4-chlorobenzaldehyde as a brown waxy solid which was used without further purification. The aldehyde was dissolved in MeOH (30 mL) with NaOMe (230 mg, 4.3 mmol) and stirred for 18 h. The mixture was poured into a citric acid solution and extracted three times with EtOAc, giving a white suspension of the product in the organic solvent. This suspension was washed with water and evaporated to give a yellow solid which was recrystallized from MeOH to give 3-(2-methoxyphenyl)-7-chloroquinolin-2(1*H*)-one (**79**; 560 mg, 68%) as fine white needles, mp 235−236 °C; 300 mg (1.05 mmol) of this solid was suspended in CH_2Cl_2 (30 mL), and BBr_3 (1 M in CH_2Cl_2, 3.2 mL) was added at 0 °C. After 30 min the reaction mixture was poured into water and extracted with EtOAc (2 × 70 mL). The combined organic layers were washed with water and brine, dried, and evaporated, and the residue was recrystallized from MeOH to give **74** (238 mg, 83%) as yellow crystals: mp 246−248 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 6.87 (1 H, t, J = 8, H-5′), 6.90 (1 H, d, J = 8, H-3′), 7.22 (1 H, dt, J = 1.7, 8, H-4′), 7.26 (1 H, dd, J = 2.1, 8.4, H-6), 7.31 (1 H, dd, J = 1.7, 8, H-6′), 7.39 (1 H, d, J = 2.1, H-8), 7.77 (1 H, d, J = 8.4, H-5), 8.03 (1 H, s, H-4), 9.56 (1 H, br s, OH), 12.1 (1 H, br s, NH); MS (EI) m/z = 271 (M^+). Anal. ($C_{15}H_{10}ClNO_2$) C, H, N.

4-Amino-7-chloro-3-phenylquinolin-2(1*H*)-one (75). Methyl 2-amino-4-chlorobenzoate (**80**; 20 g, 107 mmol) was heated at 150 °C in MeOH (300 mL) saturated with NH_3 in an autoclave for 3 days, then cooled, and evaporated to a brown solid. This was washed with ether, suspended in 1 M NaOH, and filtered to give 2-amino-4-chlorobenzamide (11.2 g, 61%). Triethylamine (34 mL, 245 mmol) was added to a solution of the amide (9.5 g, 56 mmol) at 0 °C in THF (200 mL) followed by trifluoroacetic anhydride (20.5 mL, 145 mmol) in THF (50 mL). After 30 min water (200 mL) was added, and the mixture was extracted with Et_2O (2 × 200 mL). The Et_2O layer was dried and evaporated, and the residue was dissolved in MeOH−water (1:1, 200 mL) containing K_2CO_3 (15 g). The mixture was heated at 70 °C for 24 h, cooled, and extracted with EtOAc (2 × 200 mL), and the extracts were washed with water and brine, dried, and evaporated to give 2-amino-4-chlorobenzonitrile (**81**; 8.5 g, 100%): ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.47 (2 H, br s, NH_2), 6.72 (1 H, dd, J = 2.6, 11.9, H-5), 6.75 (1 H, d, J = 2.6, H-3), 7.31 (1 H, d, J = 11.9, H-6); MS (CI^+, NH_3) m/z = 152 ($M^+ + H$). The nitrile (0.9 g, 5.9 mmol) and phenylacetyl chloride (0.78 mL, 5.9 mmol) were refluxed in dichloroethane (40 mL) for 16 h, cooled, and evaporated, and the residue was recrystallized from MeOH to give the amide **82** (0.7 g, 44%). This was heated in DMF (20 mL) with NaH (0.23 g, 80% in oil, 8 mmol) at 100 °C for 3 h, cooled, and poured into water; the solid was collected and recrystallized from DMF/water to give **75** (0.23 g, 33%) as white crystals: mp 337−338 °C; ^{1}H NMR (360 MHz, DMSO-

d_6) δ 5.96 (2 H, br s, NH_2), 7.14−8.04 (8 H, m, ArH), 11.1 (1 H, br s, NH); MS (EI) $m/z = 270$ (M^+). Anal. ($C_{15}H_{11}ClN_2O$) C, H, N.

[^{3}H]Glycine Binding. Assays were performed in polypropylene tubes containing 150 μg of P_2 membrane protein (rat cortex/hippocampus[36]), 50 nM [^{3}H]glycine (35.1−51.3 Ci/mmol; DuPont/NEN), and 50 mM Tris-acetate buffer (pH 7.0). Drugs were dissolved in dimethyl sulfoxide (final assay concentration of 1%) and were added to the membranes on ice before initiation of the incubation by addition of the radioligand. Nonspecific binding was determined in the presence of 1 mM glycine. Inhibition curves to glycine site ligands were constructed in the presence or absence of 0.2% human serum albumin (HSA). Tubes were incubated at 4 °C for 30 min before centrifugation at 50000*g* for 5 min, to separate free radioactivity from bound. The supernatant was discarded, and the pellet was superficially washed with 2 × 2 mL of assay buffer. Pellets containing bound radioactivity were solubilized overnight in 0.5 mL of 2% sodium dodecyl sulfate, and 0.4 mL of the resulting solution was added to 10 mL of scintillation fluid, for quantification of radioactivity using a β-counter. Inhibition curves were analyzed for a one-site model by the equation $\% I = \% I_{max}/1 + (IC_{50}/[L])^{nH}$, using Research System 1 software (Bolt, Beranek and Newman, Inc., Cambridge, MA). The degree of binding is expressed as the protein binding index (PBI) which is the (mean IC_{50} in the presence of HSA)/(mean IC_{50} in the absence of HSA). Binding experiments were repeated three times, with the maximum standard error calculated from the pIC_{50} values always less than 5% of the mean. PBI values were then calculated from the mean binding figures.

HPLC HSA Binding Index. The relative binding of compounds to albumin was performed by comparison of HPLC retention volumes using an HPLC column with a stationary phase of HSA immobilized on silica gel.[40] Since the major interaction governing retention on these phases is with the protein and it is known that the immobilized protein binds drugs in a fashion similar to that found in free solution, the retention volume should reflect the extent of binding to the protein.[32] Briefly, samples were dissolved at a concentration of 1 mg mL^{-1} in DMSO, and 5 μL injections were made on to an HSA column (30 mm × 4.6 mm i.d., 5 μm) (Shandon HPLC, Cheshire, U.K.) with a mobile phase of 50 mM K_2HPO_4, 50 mM KH_2PO_4 adjusted to pH 7.4 (1 M NaOH) with 10% propan-1-ol. All analyses were performed in duplicate at a flow rate of 2 mL min^{-1} and at a column temperature of 37 °C. Reproducibility was excellent, with retention times of repeat injections typically being within 0.1 min of the original, irrespective of elution time.

Effect of Warfarin on the Anticonvulsant Activity of 26. Experiments were performed on male Swiss Webster mice (20−30 g; Bantin and Kingman, Hull, U.K). Animals were housed in groups of 5 under a 12-h light/dark cycle (lights on at 7 a.m.). Groups of 8 mice were injected iv with warfarin (dissolved in 1 M NaOH, adjusted to pH 7 with HCl) or vehicle. After 15 min animals were injected iv with **26** (dissolved in 25%, w/v, β-cyclodextrin in water at pH 7) and then 15 min later with 500 mg/kg *N*-methyl-D,L-aspartic acid (NMDLA) subcutaneously. The latency to onset of tonic seizures was noted over the next 30 min, and animals not convulsing within this period were considered protected. ED_{50} values were determined by Probit analysis.

In Situ Brain Perfusion Studies in the Anesthetized Rat. These experiments were performed using the method of Takasato[43] with modifications described by Gratton.[44] Rats were anesthetized, and a cannula was placed in the right common carotid artery, with the external carotid artery ligated just below the occipital branch. A programmable syringe pump was used to control the flow of perfusate from the syringe and maintained a pressure of 120 mmHg (100 mmHg rat blood pressure and 20 mmHg tip pressure) during the perfusion. This perfusion pressure is typical of cerebral perfusion pressure and produces perfusion flow rates typical of cerebral blood flow in intact anesthetized rats.

At the beginning of the experiment the ventricles of the heart were removed, thus allowing the free flow of perfusate, and the pump was started. The drug concentration in the perfusate was 100 μg/mL. Drug was perfused in saline for 10 s or in plasma for 60 s during the experiment. The experiment was ended by decapitation and the brain removed for analysis. The procedure was repeated for groups of 4 female rats with drugs dissolved in phosphate-buffered saline or rat plasma.

Statistics. Statistical analyses were performed using BMDP New System for Windows, version 1.1, on a Compaq Deskpro 386/20e computer.

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Supporting Information Available: Microanalyses on novel compounds and details of the π values used to calculate log *P*'s in Tables 1 and 2 (15 pages). Ordering information is given on any current masthead page.

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