Pergamon

Biochemical Pharmacology, Vol. 49, No. 6, pp. 791–797, 1995.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(94)00541-9

VESAMICOL ANALOGUES AS SIGMA LIGANDS

MOLECULAR DETERMINANTS OF SELECTIVITY AT THE VESAMICOL RECEPTOR

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(*Received* 20 *June* 1994; *accepted* 10 *October* 1994)

Abstract—The present study compares the affinities of 2-(4-phenylpiperidino)cyclohexanol (vesamicol, **1**) and selected analogues of the latter at the vesamicol receptor (VR) with the corresponding affinities at $\sigma 1$ and $\sigma 2$ binding sites. For this study, the parent structure **1** was divided into three fragments: A (cyclohexyl), B (piperidyl) and C (phenyl). Vesamicol analogues were then selected to reflect structural modifications in these fragments. Consistent with earlier reports, vesamicol was found to exhibit nanomolar affinities at the VR and $\sigma 1$ and $\sigma 2$ sites, resulting in poor selectivity for the VR over the sigma sites. Vesamicol analogues characterized by an acyclic A-fragment showed moderate to low affinities at the VR and moderate to high affinities at $\sigma 1$ and $\sigma 2$ sites. As a result, many of these analogues showed poor selectivity for the VR. Replacement of the C4 carbon of **1** with a halobenzyl amine resulted in higher affinities at the VR coupled with moderate to low affinities at $\sigma 1$ and $\sigma 2$ sites. The introduction of a benzofused substituent at the C4 and C5 positions of **1** (compound **2**) resulted in a 200-fold increase in affinity at the VR accompanied by a 5- to 6-fold decrease in affinity at $\sigma 1$ and $\sigma 2$ sites relative to the parent structure. Consequently, compound **2** showed 12,000-fold higher affinity at the VR than at sigma sites. Restricting the rotation of fragment C relative to B (by means of alkyl and alkenyl bridges) generally yielded analogues with subnanomolar affinities at the VR. The corresponding affinities of these spirofused conformationally restricted analogues were moderate to poor at $\sigma 1$ and $\sigma 2$ sites when fragment A was preserved. In contrast, the affinities at $\sigma 1$ and $\sigma 2$ sites were decreased 3- to 11-fold when fragment A was modified at position C4 and decreased up to 100-fold with benzofusion at the C4 and C5 positions of fragment A. Consequently, the spirofused analogues **15–19** were among the most selective VR ligands examined. Thus, the effect of conformational restriction in fragments A and B–C is to increase affinity at the VR while decreasing affinity at $\sigma 1$ and $\sigma 2$ sites, and thereby increasing selectivity for the VR over the sigma sites.

Key words: acetylcholine transport; vesamicol receptor; vesamicol analogs; sigma receptor; selectivity

ChAT** catalyzes the synthesis of ACh within the cytosol of cholinergic neurons. Newly synthesized ACh is transported into synaptic vesicles for storage and subsequent impulse-driven quantal release. Since cholinergic neurotransmission is driven largely by quantal release, the storage of ACh and its subsequent impulse-mediated release constitute an important component of presynaptic cholinergic mechanisms. The VR [1], a unique site on the cholinergic synaptic vesicle, is linked functionally to the vesicular ACh transporter [2, 3]. Accordingly, the blockade of this receptor by compounds such as the prototypical VR ligand 2-(4-phenylpiperidino)cyclohexanol (**1**, vesamicol, AH5183) results in the inhibition of vesicular ACh transport and the subsequent quantal release of ACh from presynaptic cholinergic terminals (reviewed in Refs 4–6). In animals, this blockade results in respiratory paralysis and death [7, 8]. Given the effectiveness of vesamicol-induced cholinergic blockade, and the importance of storage/release mechanisms in neurotransmission, further investigation of the pharmacology of vesamicol and its analogues may provide new insights into the mechanism underlying the regulation of chemical neurotransmission at the cholinergic synapse.

In spite of its nanomolar affinity for the VR, at higher concentrations vesamicol also binds to α-adrenoceptors [9]. In addition, **1** is a high-affinity ligand for σ binding sites (σR) [10]. The marginal selectivity of this ligand has prompted the search for more selective VR ligands. In previous reports [11–16], we and others detailed the synthesis and vesicular ACh-transport-inhibiting properties of several vesamicol analogues. Although many of these exhibit nanomolar and picomolar affinities for the VR, subsequent investigations [10, 17, 18] suggested that, like vesamicol, several of these

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** Abbreviations: ChAT, choline acetyltransferase; ACh, acetylcholine; DTG, 1,3-di(2-tolyl)guanidine; VR, vesamicol receptor; and σR, sigma receptor.

potent analogues also bind to σ sites with nanomolar affinity. In an effort to develop vesamicol analogues characterized by high VR/σR selectivity, we undertook the present investigation in order to gain further insight into the structural elements that determine selectivity for the VR relative to the σR. For the purpose of this discussion, VR/σR selectivity is defined as $K_i(\sigma R)/K_i(VR)$ or $K_i(\sigma R)/IC_{50}$ (VR).

MATERIALS AND METHODS

The synthesis of vesamicol and all analogues described in this study has been reported [11, 13, 14, 16]. All compounds were tested in the form of the corresponding hydrochlorides.

Sigma receptor binding

Sigma-1 binding sites were labeled with the σ1-selective radioligand [^{3}H]-(+)-SKF 10,047 (Dupont-NEN) in guinea pig brain membranes (Rockland Biological) according to published procedures [19, 20]. Sigma-2 sites were assayed in rat liver membranes [20], a rich source of these sites, with [^{3}H]DTG (Dupont-NEN) in the presence of (+)-pentazocine (100 nM).

Membrane preparation. The crude P2 membrane fraction was prepared from frozen guinea pig brains minus cerebellum. Brains were allowed to thaw slowly on ice before homogenization. The crude P2 membrane fraction was also prepared from the livers of male Sprague–Dawley rats (175–225 g). Animals were killed by decapitation, and the livers were removed and minced before homogenization.

Tissue homogenization was carried out at 4° in 10 mL/g tissue weight of 10 mM Tris–HCl/0.32 M sucrose, pH 7.4, using a Polytron Teflon–glass homogenizer. The crude homogenate was centrifuged for 10 min at 1000 *g*, and the supernatant was saved on ice. The pellet was resuspended in 2 mL/g tissue weight of ice-cold 10 mM Tris–HCl/0.32 M sucrose, pH 7.4, by vortexing. After centrifuging at 1000 *g* for 10 min, the pellet was discarded, and the supernatants were combined and centrifuged at 31,000 *g* for 15 min. The pellet was resuspended in 3 mL/g of 10 mM Tris–HCl, pH 7.4, by vortexing, and the suspension was allowed to incubate at 25° for 15 min. Following centrifugation at 31,000 *g* for 15 min, the pellet was resuspended by gentle homogenization to 1.53 mL/g in 10 mM Tris–HCl, pH 7.4, and aliquots were stored at −80° until used. The protein concentration of the suspension was determined by the method of Bradford [21] and generally ranged from 20 to 25 mg protein/mL.

Sigma-1 (σ1) binding assay. Guinea pig membranes (325–500 μg protein) were incubated with 3 nM [^{3}H]-(+)-SKF 10,047 (54.0 Ci/mmol) in 0.5 mL of 50 mM Tris–HCl for 120 min at 25°. Test compounds were dissolved in ethanol and added to the membrane suspension at the beginning of the incubation to yield concentrations ranging from 0.05 to 2000 nM in a total incubation volume of 0.5 mL. Assays were terminated by the addition of 5 mL of ice-cold 10 mM Tris–HCl, pH 8.0, followed by rapid filtration through Whatman GF/B glass fiber filters (presoaked in 0.5% polyethylenimine) using a Brandel cell harvester (Gaithersburg, MD). Filters were washed twice with 5 mL of ice-cold buffer. Nonspecific binding was determined in the presence of 10 μM (+)-pentazocine. Liquid scintillation counting was carried out in Ecoscint (ICN, Costa Mesa, CA) using a Beckman spectrometer with a counting efficiency of 50%.

Sigma-2 (σ2) binding assay. Rat liver membranes (160–200 μg protein) were incubated with 3 nM [^{3}H]-DTG (37.2 Ci/mmol) in the presence of 1 μM (+)-pentazocine to mask σ1 sites. Incubations were carried out in 50 mM Tris–HCl, pH 8.0, for 120 min at 25°. Test compounds were added in concentrations ranging from 0.05 to 2000 nM, and the total incubation volume was adjusted to 0.5 mL with additional buffer. Assays were terminated by the addition of 5 mL of ice-cold 10 mM Tris–HCl, pH 8.0, followed by rapid filtration through Whatman GF/B glass fiber filters that had been presoaked in 0.5% polyethylenimine. Filters were then washed twice with 5 mL of ice-cold buffer. Nonspecific binding was determined in the presence of 5 μM DTG. Liquid scintillation counting was carried out in Ecoscint (ICN), using a Beckman spectrometer with a counting efficiency of 50%.

Analysis. The IC_{50} values at sigma sites were determined in triplicate from log-logit transformation of binding data using 5–7 concentrations of each analogue. K_i values were calculated from the IC_{50} values using the Cheng–Prusoff equation [22] and represent mean values ± SEM of at least three separate determinations.

Vesamicol receptor binding

Reserve synaptic vesicles isolated from the electric organ of *Torpedo californica* were used to carry out competition assays with unlabeled analogues and [^{3}H]vesamicol as described [15]. Vesamicol exhibits similar affinity for rat and Torpedo VR [15, 23–25].

RESULTS

For the purpose of this discussion, the structure of **1** has been divided into three major fragments: A (cyclohexyl), B (piperidyl) and C (phenyl) (Fig. 1). In addition, the long axis of the molecule, C4″-C1″-C4′-N1′-C2-C5, is also shown.

The affinities of compounds **1–21** at the VR, σ1 and σ2 binding sites are shown in Table 1. The structure–activity relationships of these compounds at the VR were discussed earlier [11, 13, 14, 16]. Since vesamicol exhibits similar affinity for Torpedo and rat VR [15, 23–25], it assumed that VR affinities obtained in Torpedo can be compared safely with the corresponding sigma site affinities obtained in the rat.

As suggested in a previous report [10], compound **1** exhibited high affinity for σ binding sites. However, **1** displayed no subtype selectivity; the K_i values determined for σ1 and σ2 binding sites were 26 ± 8 and 34 ± 2 nM, respectively. The lack of subtype selectivity was a common feature of all analogues examined. Replacement of the cyclohexyl group with a propylene bridge (compounds **3–10**) only resulted in small changes in affinity for sigma binding sites. With the exception of **10**, affinities for the σ1 site were either unchanged (compound (−)-**3**) or

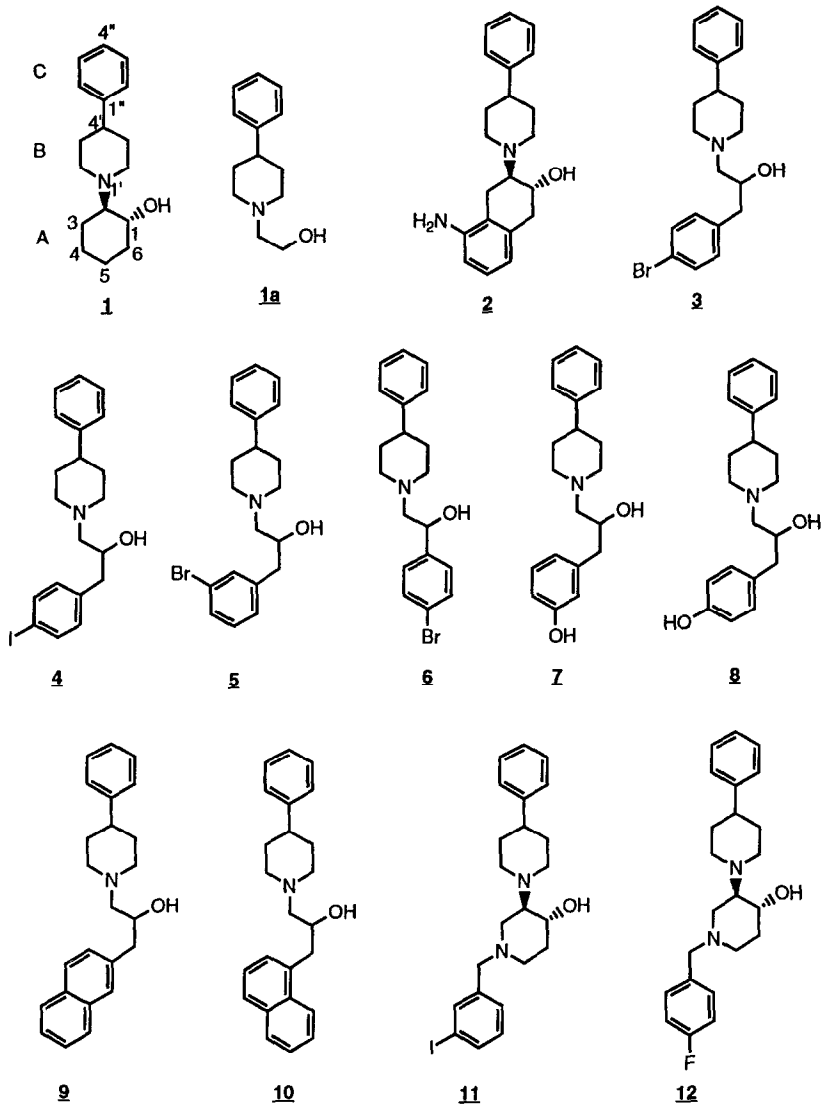

Fig. 1. Vesamicol and its structurally related analogues.

increased by no more than 2-fold. Affinity for the $\sigma 2$ site was generally unchanged except for compounds **6** and **8**. Owing to these small changes in affinity, the propylene-bridged analogues displayed 2-fold selectivity for $\sigma 1$ over $\sigma 2$. Replacement of the cyclohexyl moiety with the substituted piperidyl fragment resulted in a significant decrease in affinity at both $\sigma 1$ and $\sigma 2$ binding sites when the substituent was *m*-iodobenzyl (compare **1** vs **11**). However, when the substituent was *p*-fluorobenzyl (**12**), the affinity for $\sigma 1$ increased 2-fold relative to **1**, but the affinity for $\sigma 2$ was unchanged. Both **11** and **12** were also found to exhibit 2- to 3-fold selectivity for the $\sigma 1$ over $\sigma 2$ site.

Compounds **13** and **14** (Fig. 2) were designed as conformationally restricted analogues of vesamicol in which the plane of fragment B is fixed in an orthogonal or near-orthogonal orientation relative to the plane of fragment C. As evident in Table 1, conformational restriction achieved by the introduction of a vinyl or ethylene bridge failed to alter affinity for $\sigma 1$ and $\sigma 2$ binding sites (**1** vs **13** and **14**). However, conformational restriction of the B–C fragment combined with the fusion of a benzene ring onto fragment A resulted in 16- and 20-fold reductions in affinity at $\sigma 1$ and $\sigma 2$ sites, respectively (**1** vs **15**). The reduction in affinity was exacerbated by the introduction of a bromine atom into fragment B–C of the rigid system (compare **15** vs **16**). As a result, compound **16** displayed the lowest affinity for sigma binding sites of all compounds examined. Significant changes in affinity were also observed when conformational restriction of the B–C fragment was combined with the introduction of a nitrogen atom into fragment A. In this connection, compound **17** displayed 8-fold and 2-fold lower affinity for $\sigma 1$ and $\sigma 2$ sites, respectively, relative to **12**. In contrast, compound **20** was half as potent as **12** at $\sigma 1$ and $\sigma 2$

Table 1. Affinities (nM) of vesamicol and analogues at the vesamicol receptor (VR) and σ binding sites.*

Compounds	IC_{50} (VR)[2]†	K_i (VR)	K_i (σ1)	K_i (σ2)
1	40	2.0 ± 1.0	26 ± 8	34 ± 2
2		0.013 ± 0.001	157 ± 22	207 ± 30
(+)-**3**	328 ± 108		8 ± 2	25 ± 8
(−)-**3**	36 ± 5		19 ± 6	20 ± 5
(−)-**4**	26 ± 11		13 ± 4	24 ± 4
5	73 ± 17		11 ± 2	23 ± 19
6	30 ± 5		13 ± 0	7 ± 2
7	520 ± 30		10 ± 4	39 ± 1
8	220 ± 54		10 ± 1	11 ± 1
9	145 ± 15		14 ± 6	27 ± 2
10	1400 ± 300		63 ± 20	43 ± 10
11		0.13 ± 0.03	92 ± 27	190 ± 8
12		0.44 ± 0.11	10 ± 3	36 ± 6
13		6.93 ± 2.05	31 ± 5	37 ± 5
14		7.60 ± 0.99	21 ± 4	30 ± 8
15		0.12 ± 0.03	338 ± 6	610 ± 41
16		0.21 ± 0.06	>2000	>2000
17		0.25 ± 0.03	80 ± 2	80 ± 5
18		0.26 ± 0.08	148 ± 6	278 ± 10
19		0.67 ± 0.19	283 ± 20	224 ± 49
20		0.36 ± 0.10	25 ± 1	67 ± 7
21		2.60 ± 0.70	55 ± 8	99 ± 7

* Values are means ± range, N = 2.

† Data from Efange *et al.* [13] and Rogers *et al.* [11, 15]. The IC_{50} values were obtained in earlier work utilizing a rapid screen of binding that involved relatively high receptor concentrations. Values of IC_{50} greater than about 50 nM are approximately equal to K_I values, but values below 50 nM are too high.

sites. Moreover, compound **18** was essentially equipotent with **11** at both sites. However, reduction of the vinyl bridge in **18** resulted in a 3-fold reduction in affinity for σ1 with no change in affinity for the σ2 site (compare **11** vs **18** and **19**). Finally, extension of the bridge by one methylene group decreased affinity for σ1 by 2-fold without altering affinity for σ2 (compare **20** vs **21**).

DISCUSSION

Following the identification and subsequent characterization of the VR [1], it appeared early on that this site would be potentially useful for studying presynaptic cholinergic mechanisms. However, subsequent studies of [^{3}H]vesamicol binding in rodent models of cholinergic hypofunction and in Alzheimer's disease yielded puzzling results. In rat forebrain sections [26], the binding of [^{3}H]vesamicol showed a regional heterogeneity that was correlated with ChAT activity and [^{3}H]hemicholinium-3 binding (an indicator of sodium-dependent high-affinity transport). Specifically, high levels of [^{3}H]-vesamicol binding were reported in the diagonal band of Broca and the olfactory tubercle, while moderate to low levels were reported over the caudate-putamen, nucleus accumbens and cortex [26, 27]. However, following the transection of the fimbria, which was accompanied by a 61% reduction in hippocampal levels of ChAT, [^{3}H]vesamicol binding was only reduced by 33% [26, 27]. This discrepancy was later confirmed by other researchers. Ruberg *et al.* [28] and Holley *et al.* [29] reported finding no change in rat cortical [^{3}H]vesamicol binding following a chemical lesion of the nucleus basalis. In addition, cortical [^{3}H]vesamicol binding as determined by postmortem of an Alzheimer's diseased brain, was essentially unchanged in spite of significant reductions in ChAT activity [28, 30]. Although these results appeared to suggest that the VR was not a suitable presynaptic cholinergic marker, we later demonstrated that vesamicol is a high-affinity ligand for sigma sites [10]. On the basis of these and other observations, we concluded that problems encountered in the earlier studies could be attributed in large part to the marginal selectivity of this ligand. The identification of sigma sites as a confounding factor in studies involving VR ligands suggested that a better definition of those structural elements that determine selectivity for VR over σ binding sites would further the development of selective ligands for the VR and the investigation of mechanisms of presynaptic cholinergic function.

An inspection of the analogues examined in the present study (Fig. 1) reveals the following structural modifications relative to **1**: conformational restriction of fragment A (compound **2**); replacement of the cyclohexyl moiety (fragment A) with a substituted ethyl or propyl group (**3–10**); single-point substitution in fragment A (**11** and **12**); conformational restriction of the B–C fragment (**13** and **14**); simultaneous conformational restriction of the B–C and A fragments (**15** and **16**); and conformational restriction of the B–C fragment combined with single-point

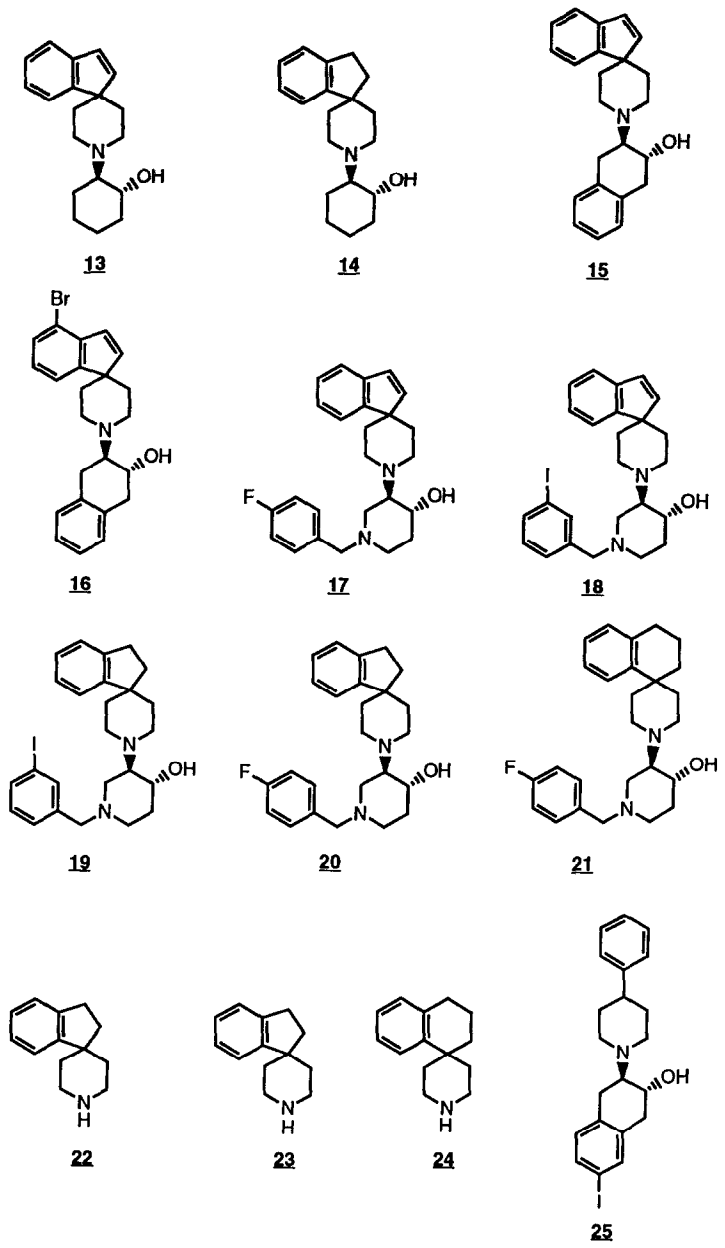

Fig. 2. Conformationally restricted vesamicol analogues.

substitution in fragment A (**17–21**). As revealed by the results of the present study, these modifications result in compounds that display a wide range of affinities of σ binding sites. In general, replacement of the cyclohexyl moiety in fragment A with bulky flexible groups results in compounds that display comparable or slightly higher affinities for σ binding sites relative to **1**. The foregoing suggests that binding at σ sites does not require a cyclic moiety in fragment A. The latter view is consistent with previous reports [31] regarding the structural requirements for binding at σ sites (*vide infra*). Single-point substitution at the C-4 position of **1** (compounds **11** and **12**) failed to display any obvious trends in affinity for σ sites. However, increasing the rigidity of the core structure did result in significant changes in affinity for σ binding sites. In view of the comparable affinities of compounds **1**, **13** and **14**, we conclude that restricting the orientation of fragment B relative to fragment C is not necessarily incompatible with high-affinity binding at $\sigma 1$ and $\sigma 2$ sites. This conclusion is consistent with an earlier study [31] describing a series of *N*-arylalkyl analogues of compounds **22–24** as potent ligands for σ binding sites. However, the noticeable decline in affinity associated with the tetrahydronaphthyl analogues **2**, **15** and **16** suggests that the size and shape of the N-substituent may play an important role in binding.

In this connection, molecular modelling studies* and X-ray crystallographic studies [11] of **1** reveal a structure in which the cyclohexyl group exists in a chair conformation while the 4-phenylpiperidyl (B–C) and hydroxyl groups are disposed *trans*-diequatorially about C1 and C2. However, as revealed by the superposition of **1** and **2** (or **13** and **15**) (data not shown), replacement of the cyclohexyl moiety with the tetrahydronaphthyl group causes a flattening of the cyclohexyl portion of fragment A. In addition, the benzo moiety is found to project further out into space, reflecting the increased steric bulk of the new structure. Given the large spatial separation between fragments A and B–C, it is reasonable to assume that there is no through-space interaction between these groups. Since the benzo moiety is distal to C1 and C2, we conclude that the reduction in affinities of **2**, **15** and **16** (relative to **1** or **13**) probably derives from the increased steric bulk and rigidity at the distal end of fragment A. However, the situation may be rather more complex, as suggested by other studies (*vide infra*).

Previous studies by Rogers *et al.* [11] have revealed that **1a** is responsible for molecular recognition at the VR (Fig. 1). Furthermore, the presence of bulky lipophilic substituents at the C-1 position of this fragment was found to increase affinity at the VR [11]. Ironically, in an independent study of σR ligands [31], the 4-phenylpiperidyl moiety was also identified as the primary pharmacophore at this receptor. In addition, these workers observed that N-substitution of the primary pharmacophore with bulky lipophilic groups markedly increases affinity for the σR. The structural similarity between the two pharmacophores is indicative of a high degree of similarity between the VR and σ binding sites. Since vesamicol and its analogues are N-substituted 4-phenylpiperidines, it is clear that all VR ligands of this structural class are potentially high-affinity ligands for σ binding sites. The latter notwithstanding, we found that the analogues examined in the present study exhibited a wide range of selectivities for the VR and σ binding sites, thereby suggesting the involvement of higher-order effects.

As shown in a previous report [16], when rotation of fragment C relative to fragment B is restricted by means of a vinyl bridge, affinity for the VR decreased 3-fold (compare **1** vs **13**). However, as the present study shows, affinity for σ sites remains unchanged. This disparity results in a 3-fold reduction in VR/σR selectivity relative to **1**. Although the latter observation would appear to suggest that restriction of the B–C fragment does not enhance VR/σR selectivity, further examination of the data suggests otherwise. With the exception of **13**, **14** and **21**, all those analogues that are characterized by conformational restriction in the B–C fragment exhibited higher affinity for the VR relative to **1**. However, their corresponding affinities for σ sites were generally lower relative to **1**. The result is that these conformationally restricted analogues generally displayed a higher selectivity for the VR over the σ sites. In contrast, all the ring A-deficient compounds exhibited comparable affinities for the VR and σ sites, resulting in poor selectivity for the VR. In fact, four of these compounds ((+)-**3**, **7**, **8** and **10**) displayed greater than 20-fold selectivity for the σ sites over the VR. Although the use of IC_{50} values may lead to underestimation of VR/σR selectivity for (−)-**3**, (−)-**4** and **6** (see footnote in Table 1), other data [10] suggest that these compounds do indeed exhibit poor selectivity for the VR. Since the replacement of the cyclohexyl group (fragment A) with an arylalkyl moiety increases the flexibility of these molecules, allowing them to adopt those conformations that are optimal for binding at either the VR or sigma binding sites, these results suggest that conformational mobility is an important determinant of VR/σR selectivity. In view of the high degree of selectivity associated with the relatively rigid analogues **2**, **15** and **16**, it would be reasonable to suggest that the size and shape of fragment A are key determinants of selectivity. However, differences between the VR and σ sites in the region of fragment A appear to be very subtle. The subtlety is illustrated clearly by a recent study [32], which reports that (−)-**25** is a potent VR ligand, whereas its antipode (+)-**25** binds selectively to σ sites.

In contrast to the VR for which only vesamicol and its analogues are high-affinity ligands, potent σ ligands have been identified from several structural classes. While the latter observation suggests that the requirements for binding to σ sites are less stringent than those for binding at the VR, the apparent permissiveness of the σ site may also be related to the existence of multiple subtypes. Given the similarity between the VR and σ pharmacophores and the apparent permissiveness of the σ site, all vesamicol analogues would be expected to display high affinity at σ sites. Therefore, the identification of highly selective VR ligands such as **2**, **15** and **16** suggests the existence of mutually exclusive regions (located within the vicinity of fragment A) in the binding domains of these two sites. The precise description of these regions must await further study.

Acknowledgements—Financial support for this work was provided by the National Institute of Neurological Disorders & Stroke under Grant NS28711 (S.M.N.E.) and NS31907 (R.H.M.).

* Efange SMN, unpublished results.

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