J. Pharm. Pharmacol. 1987, 39: 877–882
Received March 9, 1987

The activity of 25 paroxetine/femoxetine structure variants in various reactions, assumed to be important for the effect of antidepressants

PER PLENGE*, ERLING T. MELLERUP, TAGE HONORÉ†, AND PIERRE LE FÈVRE HONORÉ†

Psychochemistry Institute, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen Ø, and †Research Division, The Danish Ferrosan Group, DK-2860, Soeborg, Denmark

Structure-activity relationships for 25 structural variants around the 5-hydroxytryptamine (5-HT) uptake inhibitors paroxetine and femoxetine have been investigated. Three parameters related to the 5-HT system were investigated: (i) The inhibition of [^{3}H]5-HT uptake into rat brain synaptosomes, (ii) the inhibition of [^{3}H]paroxetine binding to rat neuronal membranes and (iii) the effect of the compounds on the affinity of [^{3}H]imipramine for the human platelet membrane binding site, measured as the dissociation rate of the [^{3}H]imipramine human platelet membrane binding site complex. A highly significant correlation was found for 5-HT uptake inhibition and inhibition of [^{3}H]paroxetine binding for the different substances, indicating that the two parameters are closely connected. However the slope of the regression line was only 0·6 and not 1·0; this may indicate that [^{3}H]paroxetine binding is necessary, but not sufficient for 5-HT uptake inhibition. No correlation was found between the inhibition of [^{3}H]paroxetine binding and the affinity of the compounds for the [^{3}H]imipramine binding site complex. The two binding sites are therefore probably situated on different parts of the 5-HT transport system, the [^{3}H]paroxetine binding site being part of the 5-HT transport mechanism whereas the [^{3}H]imipramine binding site may represent a site modulating the activity of, and affinity for, 5-HT in the 5-HT transport mechanism. Structure-activity relationships among the substances showed that stereochemical changes from (−)- to (+)-*trans* changed the activity towards both 5-HT uptake inhibition and [^{3}H]paroxetine displacement for most of the (−)-/(+)-pairs. The substitution of −H with −F or $-CH_3$ also affected the activity. The results showed that in this series of compounds, (−)-*trans*-4-(*p*-fluorophenyl)-3-(3,4-methylenedioxyphenoxymethyl)piperidine (paroxetine) is an optimal structure for influencing the chosen parameters.

While the causes of depression as well as the psychotropic mechanism of antidepressant drugs are poorly understood, several biochemical effects of antidepressant drugs are well-known, particularly the inhibition of presynaptic reuptake of 5-hydroxytryptamine (5-HT) and noradrenaline. That knowledge has formed the basis of hypotheses about depression and of research strategies and guidelines in the development of new antidepressant drugs. Various biochemical mechanisms are related to the reuptake process. The finding of a specific, high-affinity binding site for imipramine on the 5-HT transport mechanism in neuronal and human platelet membranes (Raisman et al 1980; Paul et al 1980) is pertinent in this respect and has led to studies of the relations between the effect of various antidepressants on 5-HT uptake and imipramine binding (Langer et al 1980).

* Correspondence.

Recently a third effect of antidepressants on the 5-HT transport system has been described (Wennogle & Meyerson 1985; Plenge & Mellerup 1985). This consists of a change in the affinity between imipramine or paroxetine (a selective and very potent 5-HT uptake inhibitor, Buus Lassen 1978) and their respective binding sites on the 5-HT transport mechanism. This may be caused by conformational changes in or around the binding sites, thereby indicating that antidepressants, besides being competitive inhibitors of 5-HT uptake, may also cause allosteric changes in the structure of the 5-HT uptake mechanism.

In the present study the three variables related to 5-HT transport—(i) direct inhibition, (ii) binding of imipramine and paroxetine, and (iii) affinity change—have been studied for 25 derivatives of 3-hydroxymethyl-4-phenylpiperidine, which are close analogues of femoxetine and paroxetine, both currently under development as antidepressants.

METHODS

Human platelet membranes

Human platelets were obtained from 500 mL blood to which sodium citrate was added as an anticoagulant. Erythrocytes and leukocytes were removed by low speed centrifugation (200*g*, 10 min, 20 °C). All the following steps were performed at 0–4 °C. Platelets were precipitated from the platelet-rich plasma by centrifugation at 3000*g*, 10 min. The platelets were washed twice with 8 mL buffer (mM; 50 Tris/HCl, 150 NaCl, 20 EDTA, pH 7·5), and frozen as a dry pellet at −80 °C. Lysis and homogenization of the platelets took place in 8 mL buffer (mM; 5 Tris/HCl, 5 EDTA, pH 7·5) using an Ultra-Thurrax homogenizer (5 s high speed). Platelet membranes were washed twice with 8 mL buffer (mM; 70 Tris/HCl, pH 7·5) (centrifugation 30 000*g*, 10 min) and finally resuspended in buffer (mM; 50 Tris/HCl, 120 NaCl, 5 KCl, pH 7·5) to a concentration between 0·5 and 1·0 mg protein mL^{-1}. The samples were kept at −80 °C until analysis.

Rat brain membranes

Rats were decapitated, the brains removed and placed on ice, and whole brain, except brainstem and cerebellum, was homogenized in 10 mL ice-cold buffer (mM; 50 Tris/HCl, 120 NaCl, 5 KCl, pH 7·5) using an Ultra-Thurrax homogenizer. The homogenate was washed twice in the same buffer by resuspension and centrifugation at 10 000*g*, 10 min. The final pellet was resuspended in the buffer to give a protein concentration of about 4 mg membrane protein mL^{-1} (corresponding to about 100 mg brain tissue mL^{-1}). The membranes were stored at −80 °C for the determination of K_i against [^{3}H]paroxetine for the 25 structural compounds studied (Table 1).

Rat brain synaptosomal membranes

Whole rat forebrain was homogenized in 20 volumes of ice-cold 0·23 M sucrose by 4 strokes at 500 rev min^{-1} in a Potter-Elvedhjem homogenizer fitted with a Teflon piston. The homogenate was centrifuged for 10 min at 1000*g*. The supernatant was used for 5-HT uptake assays.

Protein analysis

The protein concentrations in the membrane preparations were determined by the method of Lowry et al (1951) modified for membrane proteins according to Peterson (1977).

Drugs

[^{3}H]Imipramine (24 Ci $mmol^{-1}$ Amersham, UK), [^{3}H]paroxetine (21 Ci $mmol^{-1}$ NEN, USA) and [^{3}H]5-HT (1·04 Ci $mmol^{-1}$ NEN, USA) were purchased; the structural analogues of paroxetine and femoxetine were synthesized and provided by Ferrosan, Denmark.

Dissociation rate determinations

Human platelet membrane suspension (40 mL) was adjusted to about 0·5 mg protein mL^{-1} and incubated at 0 °C for 2 h with 4 nM [^{3}H]imipramine. At that time aliquots (200 µL) were diluted (50×) to 10 mL 0 °C buffer (mM; 50 Tris/HCl, 120 NaCl, 5 KCl, pH 7·5) containing 200 µM of the various compounds. The diluted samples were filtered 20 to 150 min after the dilution step through Whatman GF/F glass-fibre filters, and washed with 3 × 5 mL 0 °C buffer. Determinations were in triplicate, and the median values used to determine the dissociation rates. In each assay the dissociation rate in the presence of 200 µM imipramine was used as a control, giving half lives of 66, 65, 65 and 66 min in the four experimental series used in the present investigation. The effects of the drugs on the dissociation rates were determined in single experiments using 10 different time intervals between dilution and filtration. To determine the non-specific binding, tubes from each dissociation rate determination were heated to 25 °C for 3 h, ensuring that all specifically bound ligand had dissociated from the binding site. The slopes of the curves in the semilogarithmic plots were calculated using a least square fit to the experimental points.

The filters were dried overnight and counted in Pico-Fluor 15 using conventional scintillation techniques.

Determination of K_i values

Rat brain membranes (final concentration about 0·4 mg mL^{-1}) were incubated in 900 µL buffer (mM; 50 Tris/HCl, 120 NaCl, 5 KCl, pH 7·5) containing 0·4 nM [^{3}H]paroxetine and the different drugs in concentrations, increasing stepwise by a factor 3, around the IC50 value for the different compounds. Non-specific binding was determined using 1 µM paroxetine as displacer. All determinations were done in triplicate, and the median values were used. The IC50 was defined as the drug concentration displacing 50% of the specifically bound [^{3}H]paroxetine. From the IC50 values K_i was calculated: K_i = IC50/(1 + L/KD), where KD was 0·08 nM and L the concentration of free [^{3}H]paroxetine at 50% displacement. The experiments were repeated three times. The samples were incubated for 3 h at 20 °C. After incubation, 5 mL ice-cold buffer was added,

and the samples were rapidly filtered through Whatman GF/F glass-fibre filters and washed 4 times with 5 mL ice-cold buffer. The filters were dried overnight and counted as mentioned before.

5-HT uptake inhibition

Aliquots (200 μL) of synaptosomal membrane preparation were incubated at 37 °C for 10 min with 100 μL [^{3}H]5-HT (final concentration at 47 nM), 100 μL test solution and 5 mL buffer (mM; 122 NaCl, 4·8 KCl, 1·3 $CaCl_2$, 1·2 $MgSO_4$, 16 sodium phosphate, 1 ascorbic acid, 11 glucose, 0·2 EDTA, pH 7·4). The buffer was oxygenated before addition of [^{3}H]5-HT, ascorbic acid and glucose).

The uptake was terminated by cooling the tubes on ice followed by filtration of the contents through Whatman GF/F glass-fibre filters. These were washed with 2 × 10 mL ice-cold saline. Bound radioactivity on filters was determined by liquid scintillation counting. Correction for blanks was made using mixtures incubated at 0 °C.

Chemical structures

The compounds had a basic structure with substituents R1, R2 and R3 as shown in Table 1. There are two asymmetrical carbon atoms at the (★) which give the possibility of (−)/(+)*cis* and (−)/(+)*trans* structures.

The two compounds under development as antidepressants are no. 5, paroxetine and no. 15, femoxetine.

RESULTS

The results for the 25 compounds are presented in Table 1. Compounds nos 1 to 11 are analogues of paroxetine, and nos 12 to 22 of femoxetine. The main differences between femoxetine and paroxetine are the substituents in the R3 position, being 4-methoxyphenyl and 3,4-methylene dioxyphenyl, respectively.

Fig. 1 shows dissociation rate curves for two (−)-/(+)-*trans* pairs of compounds (nos 5, 6, 14, 15) to illustrate the methodological aspects of this determination.

Fig. 2 shows the correlation for the individual compounds of inhibition of [^{3}H]5-HT uptake into rat brain synaptosomes and inhibition of [^{3}H]paroxetine binding to rat brain membranes. IC50 and K_i for the individual drugs are plotted as ordinate and abscissa, respectively, on double-log paper. A good correlation is seen for the two parameters with an r value of 0·93 ($P < 0·001$), but the slope of the line is 0·6 and not 1.

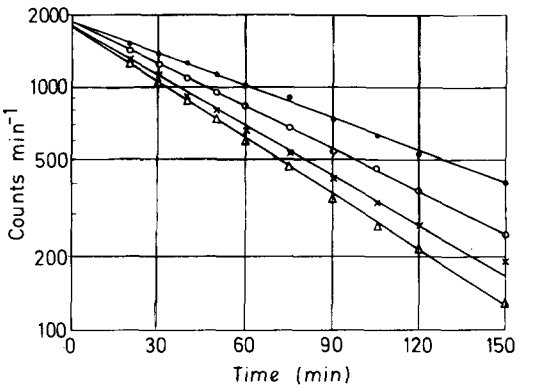


FIG. 1. Dissociation rates of [^{3}H]imipramine human platelet membrane binding site complexes influenced by two (−)-/(+)-*trans* pairs of drugs from Table 1. △——△ no. 5 (paroxetine), ×——× no. 6, ○——○ no. 14 and ●——● no. 15 (femoxetine). Ordinate: counts min^{-1} in sample minus non-specific binding on a logarithmic scale. Abscissa: time (min) after the preincubated membranes were diluted 50× with buffer containing the various compounds under investigation at a concentration of 200 μM.

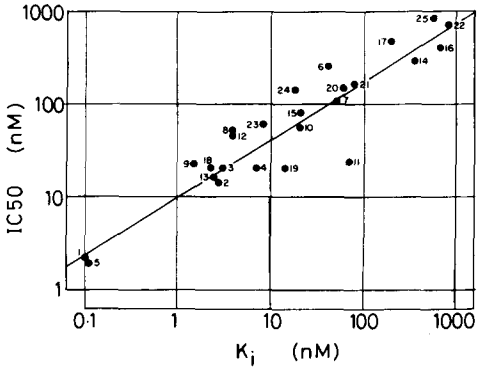


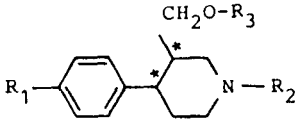
FIG. 2. Comparison of the inhibition of [^{3}H]5-HT uptake (IC50) into rat brain synaptosomes (ordinate) with the inhibition of [^{3}H]paroxetine binding (K_i) to rat neuronal membranes (abscissa) by various compounds from Table 1. Each point represents a different compound, identified by number in Table 1. The regression line is fitted by the method of least squares; log IC50 = 1·025 + 0·613 logK_i, r = 0·93, $P < 0·001$.

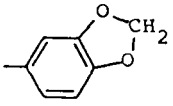
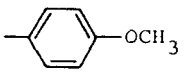
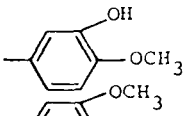
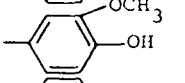
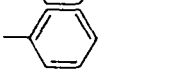
From Fig. 3 it can be seen that there is no correlation of the effect of the compounds on the half-life of the [^{3}H]imipramine-platelet membrane binding ($t_{\frac{1}{2}}$) site and their inhibition of [^{3}H]paroxetine binding to rat brain membranes (log K_i).

DISCUSSION

Most 5-HT uptake inhibitors have been found independently by different pharmaceutial companies and they are structurally very different. The 5-HT

Table 1. A series of 25 compounds based on the paroxetine (no. 5) and femoxetine (no. 15) structures. R_1, R_2 and R_3 are substituents. IC50 refers to inhibition of 5-HT uptake into rat brain synaptosomes. K_i is the inhibition of [^{3}H]paroxetine binding to rat neuronal membranes. $t_{\frac{1}{2}}$ is the half life of human platelet membrane-bound [^{3}H]imipramine under the influence of 200 μM of the different compounds.



Compounds	R_1	R_2	R_3	Optical activity	Configuration	IC50 nM	K_i nM	Dissociation rate $t_{\frac{1}{2}}$ min
1	H	H		−	*trans*	2·2	0·10	40
2	H	H		+	*trans*	14	2·7	40
3	H	CH_3		−	*trans*	20	3	65
4	H	CH_3		+	*trans*	20	7	44
5 (paroxetine)	F	H		−	*trans*	1·9	0·11	39
6	F	H		+	*trans*	250	40	43
7	F	H		−	*cis*	105	50	51
8	F	H		+	*cis*	50	6	68
9	F	CH_3		−	*trans*	22	1·5	40
10	F	CH_3		+	*trans*	55	20	42
11	F	CH_3		+	*cis*	24	70	44
12	H	H		−	*trans*	50	3·6	48
13	H	H		+	*trans*	16	2·4	44
14	H	CH_3		−	*trans*	285	350	50
15 (femoxetine)	H	CH_3		+	*trans*	80	20	66
16	H	CH_3		−	*cis*	415	670	47
17	H	CH_3		+	*cis*	470	195	58
18	F	H		−	*trans*	20	2·2	41
19	F	H		+	*trans*	20	14	61
20	F	CH_3		−	*trans*	150	60	55
21	F	CH_3		+	*trans*	130	75	55
22	F	CH_3		−	*cis*	700	850	47
23	F	H		−	*trans*	60	8	95
24	F	H		−	*trans*	140	18	75
25	H	H		−	*trans*	850	600	47

transport complex is a multimolecular structure (Ross 1982) so although the 5-HT uptake inhibitors obviously have that inhibition in common, the point of interaction within the transport mechanism probably varies. This has been demonstrated for imipramine and paroxetine, as both the temperature dependence of their affinities and the molecular weights of the two complexes differ (Mellerup et al 1983, 1985; Plenge & Mellerup 1984). It has also been found (Wennogle & Meyerson 1985; Plenge & Mellerup 1985) that the drugs paroxetine and citalopram have opposite effects on the dissociation rate of [^{3}H]imipramine bound to human platelet membrane binding sites. This again emphasizes that two selective 5-HT uptake inhibitors act differently. Other drugs like fluoxetine, femoxetine, zimelidine and the tricyclic antidepressants have no modifying effect on this platelet model.

In the present work we have looked at structure-activity relations among related compounds including the two drugs paroxetine and femoxetine.

One of the first compounds examined was no. 25

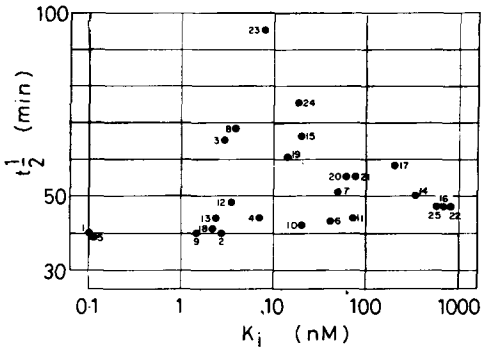


FIG. 3. Comparison of the effect on $t_{\frac{1}{2}}$ (dissociation rate of human platelet membrane-bound [^{3}H]imipramine) (ordinate) with the inhibition of [^{3}H]paroxetine binding (K_i) to rat neuronal membranes (abscissa) by various compounds, identified by number in Table 1.

(Table 1) which was able to inhibit 5-HT uptake to some extent. It has two asymmetrical carbon atoms and four isomers are therefore possible ((−)-/(+)-*trans* and (−)-/(+)-*cis*).

The absolute configuration has been established for no. 15 femoxetine ((+)-*trans*) to 3*R*,4*S* (Jones & Kennard 1979) and for no. 5 paroxetine ((−)-*trans*) to 3*S*,4*R* (unpublished). Thus the (−)-*trans* femoxetine analogues have the same absolute configuration as the (−)-*trans* analogues of paroxetine.

In the *trans* compounds the two large substituents, the phenyl-carrying R1 and the phenoxymethyl $-CH_2OR3$, can be situated either diaxially or diequatorially. The diequatorial position of the groups is energetically the most favourable, and high resolution NMR spectroscopy has confirmed that both paroxetine and femoxetine (nos 5 and 15, Table 1) are diequatorial (unpublished results). The *cis* compounds will exist as an equilibrium of the two conformers where one of the substituents in the 3- and 4-position of the piperidine ring will be axial and the other equatorial. Presumably the conformers having the phenyl equatorial will dominate. Accordingly the *cis* compounds differ from the corresponding *trans* compounds in physical characteristics like melting point.

Table 1 shows how variations in molecular structure can affect the three 5-HT-related parameters measured. To simplify the discussion, the following abbreviations will be used: IC50 refers to inhibition of [^{3}H]5-HT uptake into rat cortical synaptosomes, K_i refers to competitive inhibition of [^{3}H]paroxetine binding to rat brain membranes and $t_{\frac{1}{2}}$ is the half-life of the [^{3}H]imipramine human platelet membrane binding site complex in the presence of 200 μM of any of the different compounds.

Fig. 2 shows the correlation of 5-HT uptake and inhibition of [^{3}H]paroxetine binding (K_i) for the 25 compounds. A fairly good correlation is seen, making it probable that for both parameters the same sites are affected by the different compounds. However, the slope of the regression line in Fig. 2 is 0·6 and not 1 as found by Langer et al (1980) for a group of tricyclic and non-tricyclic 5-HT upake inhibitors. Those authors correlated the inhibition of [^{3}H]imipramine binding to rat brain membranes with the IC50 for the inhibition of [^{3}H]5-HT uptake. Our slope of 0·6 may therefore indicate that the proteins associated with [^{3}H]paroxetine binding and [^{3}H]5-HT uptake are closely connected, but not identical. Thus paroxetine receptor binding may be necessary, but not sufficient for uptake inhibition.

The effects of the compounds on $t_{\frac{1}{2}}$ on the other hand do not correlate well with K_i (Fig. 3) and there are several examples, like the pairs 3–4 and 18–19, where $t_{\frac{1}{2}}$ is very different but the differences in K_i are small. However, with the exception of nos 3 and 18, the *trans* 'paroxetines' induce a faster dissociation (shorter $t_{\frac{1}{2}}$) than do the *trans* 'femoxetines'. It should be noted that the effects on $t_{\frac{1}{2}}$ occurs in the micromolar range whereas the effects on K_i and IC50 are in the nanomolar range. The difference in concentration range shows that the same phenomenon is not being measured, but evidence exists which supports the '$t_{\frac{1}{2}}$' effect being connected with the 5-HT system, for example the difference in effect on $t_{\frac{1}{2}}$ among the individual compounds in Table 1, and the fact (Plenge & Mellerup 1985) that only drugs affecting the 5-HT system affect $t_{\frac{1}{2}}$ whereas other psychotropic drugs like MAO inhibitors and neuroleptics do not. It may be that $t_{\frac{1}{2}}$ measures a regulator site for 5-HT uptake which is stimulated (i.e. increases the affinity for 5-HT at the transport site) when the 5-HT concentration increases in the synaptic cleft following 5-HT release. It has been shown (Plenge & Mellerup 1985) that the 5-HT uptake inhibitor, citalopram, and 5-HT itself, increase, whereas paroxetine decreases, $t_{\frac{1}{2}}$ relative to the platelet imipramine effect. Therefore, citalopram and 5-HT could be considered as agonists for this regulator site, whereas paroxetine, having the effect of increasing the dissociation rate might be called an inverse agonist, by analogy with the terminology proposed for compounds binding to the benzodiazepine receptors (Braestrup et al 1983). Antagonists to the citalopram effect are not known at present; if citalopram and paroxetine are mixed,

only the citalopram effect is seen (Plenge & Mellerup 1985).

From the results in Table 1 some structural and stereochemical elements necessary for potency in displacing [^{3}H]paroxetine binding and inhibition of 5-HT uptake can be identified. (1) The change of substituent at R3 of the (−)-*trans* analogues from 4-methoxyphenyl to 3,4-methylenedioxyphenyl increases the affinity for the [^{3}H]paroxetine binding site and the inhibition of 5-HT uptake. (2) Introduction of $-CH_3$ at R_2 leads to a fall in potency for all (−)-*trans* analogues and all (+)-*trans* analogues except the pair 6/10. (3) Introduction of fluorine at R_1 leads to an increase in potency of (−)-*trans* analogues and to a fall for (+)-*trans* analogues. (4) (−)-*trans* Analogues of paroxetine are more potent than the corresponding (+)-*trans* analogues. But analogues of femoxetine in the (+)-*trans* form, i.e. 13 and 15, seem to be more potent than their corresponding (−)-*trans* forms.

Among the compounds tested, nos 1 and 5 (paroxetine) are by far the most potent compounds, as judged by K_i and IC50 values. These two compounds thus seem to be the optimal structures for a selective interaction with the 5-HT binding site.

According to Pfeiffer (1956), the more potent a drug within a series of compounds, the bigger is the difference between enantiomorphous structures if the asymmetry is situated in a central part of the molecule. This is seen with the pairs of compounds 1–2 and 5–6 for IC50 and K_i values. But these pairs have the same effect on $t_{\frac{1}{2}}$. Two other pairs of compounds, 7–8 and 14–15, show large differences in their effect on $t_{\frac{1}{2}}$ with only small differences in their effect on IC50 and K_i. This divergence may be taken to support the assumption that the $t_{\frac{1}{2}}$ effect is dependent on interaction between other parts of the paroxetine–femoxetine molecules with the 5-HT transport complex than are the IC50–K_i effects.

As seen with paroxetine (no. 5) and femoxetine (no. 15) it is also known for other psychotropic drugs that the pharmacological activity is predominantly associated with one of the enantiomers (Waldmeier 1983). However many of the newer 'non-tricyclic' antidepressant drugs contain one or more asymmetrical carbon atoms, but are marketed as the racemic mixtures. It is to be expected that higher affinity i.e. lower dose levels, and possibly less side effects, could be obtained if only the most active of the chiral structures were used in the therapy.

Acknowledgements

This study was supported by the Danish Medical Research Council. The authors would like to thank Dr M. Engelstoft for his valuable comments and supply of compounds.

REFERENCES

Braestrup, C., Nielsen, M., Honoré, T., Jensen, L. H., Petersen, E. N. (1983) Neuropharmacology 22: 1451–1457

Buss Lassen, J. (1978) Eur. J. Pharmacol. 47: 351–356

Jones, P. G., Kennard, O. (1979) Acta Cryst. B35: 1732–1735

Langer, S. Z., Moret, C., Raisman, R., Dubocovich, M. L., Briley, M. (1980) Science 210: 1133–1135

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193: 265–275

Mellerup, E. T., Plenge, P., Engelstoft, M. (1983) Eur. J. Pharmacol. 96: 303–309

Mellerup, E. T., Plenge, P., Nielsen, M. (1985) Ibid. 106: 411–413

Paul, S. M., Rehavi, M., Skolnick, P., Goodwin, F. K. (1980) Life Sci. 26: 953–959

Peterson, G. L. (1977) Anal. Biochem. 83: 346–356

Pfeiffer, C. C. (1956) Science 124: 29–31

Plenge, P., Mellerup, E. T. (1984) Biochim. Biophys. Acta 770: 22–28

Plenge, P., Mellerup, E. T. (1985) Eur. J. Pharmacol. 119: 1–8

Raisman, R., Briley, M. S., Langer, S. Z. (1980) Ibid. 61: 373–380

Ross, S. B. (1982) in: Osborne, N. N. (ed.) Biology of serotonergic transmission, John Wiley and Sons Ltd, Chichester, UK. pp 159–194

Waldmeier, P. C. (1983) Trends Pharmacol. Sci. 4: 448–449

Wennogle, L. P., Meyerson, L. R. (1985) Life Sci. 36: 1541–1550