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Structure–property relationships on histamine H_3-antagonists: binding of phenyl-substituted alkylthioimidazole derivatives to rat plasma proteins

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Abstract

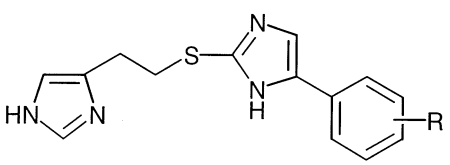
The binding of a series of H_3-antagonists to rat plasma proteins was investigated by dialysis experiments, with RP-HPLC measurement of the free ligand. The series was composed of 4(5)-phenyl-2-[[2-[4(5)-imidazolyl]ethyl]thio]imidazoles having, on the phenyl ring, *meta*- and *para*-substituents, with different physico-chemical characteristics. As high protein binding had been proposed as being one of the features limiting brain access for the reference H_3-antagonist thioperamide, the title series was employed to test the possibility of achieving lower protein binding by modulation of lipophilicity, while maintaining good receptor affinity. The compounds tested showed quotas of bound drug ranging from 60 to 97.5%, while for thioperamide a 78% bound drug quota was observed at high total concentrations, with a steep increase in bound percentage at lower concentrations. Two of the tested compounds, having a carboxamide substituent, showed lower protein binding compared to thioperamide over a wide range of total concentration, without a significant loss in affinity with respect to the parent compound. A strict dependence of protein binding on lipophilicity was observed, and a QSPR model was derived which could also account for the protein binding observed for thioperamide, while receptor affinity had been reported to be quite insensitive to phenyl ring substitution. It is therefore possible to modulate protein binding of these H_3-antagonists, through lipophilicity adjustment, without losing receptor affinity; this finding could help in the design of new compounds with improved brain access.

Keywords: H_3-Antagonists; QSPR; Lipophilicity; Protein binding

1. Introduction

A series of antagonists at the histamine H_3-receptor, characterized by a central 2-alkylthio-imidazole portion, has recently been described [1,2]. The introduction, on the 4(5)-imidazole position, of a variously substituted phenyl ring led to compounds (**1**–**11** in Fig. 1) with good receptor affinity on rat brain membranes and good antagonist potency on guinea-pig ileum [3]. The aim of the present work was to study the protein binding of these compounds, in order to devise structure–property relationships and to investigate an aspect of drug distribution which could influence their pharmacokinetic behavior.

Histamine H_3-receptor was first characterized as an autoreceptor, able to regulate the release and synthesis of histamine from histaminergic neurons [4]. In the



Compounds 1-11:

Fig. 1. General formula of compounds **1**–**11**.

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central nervous system (CNS), these neurons are located mainly in the tuberomammillary nucleus of the posterior hypothalamus, although they project to most cerebral areas [5,6]. H_3-receptors are now known to act as hetero-receptors as well, modulating the release of other neurotransmitters [7,8] (dopamine, acetylcholine, GABA, glutamate); they also seem to play an important modulating role in peripheral neuronal transmission [9,10] (airways, vessels, gastrointestinal tract).

Although no H_3-receptor-acting drug has been introduced into therapy so far, there are significant indications in the literature for the therapeutic potential of both H_3-agonists and -antagonists [11]. In particular, H_3-antagonists have been shown to be particularly promising in animal models on obesity [12] and cognitive deficits [13,14], and their possible use as antiepilepsy drugs is now under evaluation [11]. Considering the high distribution of H_3-receptors in the CNS [15], treatment with an H_3-receptor antagonist should achieve central effects with limited peripheral side-effects [16,17], but, given the possible adverse effects of a drastic histamine release into certain organs, high peripheral concentrations of H_3-antagonists may be dangerous in some conditions.

Many potent H_3-antagonists have been reported. Their general structure can be described, at least in classical compounds, as being composed of an imidazole ring connected via a spacer to a polar group, which in turn is linked to a lipophilic ending group [2].

The attention of medicinal chemists has focused on the nature and the relevance of the central polar group, since its physico-chemical properties could be critical for distribution behavior. Examples of polar groups are substituted thioureas, as in thioperamide, isothioureas, as in clobenpropit and amidines, esters, or guanidines, as in other derivatives [18]. Recently, potent H_3-antagonists lacking a polar group have been presented [19]; an unsaturated chain in the appropriate position could guarantee H_3-receptor antagonism as well as good blood-brain barrier (BBB) penetration.

Indeed, in addition to finding new, potent and selective H_3-antagonists, one of the present major concerns is to optimize brain access, in order to obtain significant action on central H_3-receptors with limited drug concentrations in the blood.

Brain access can be hampered by various phenomena. In the case of clobenpropit and other similar compounds, which have a strongly basic group, the prevalence of the charged species in solution is a clear limiting factor for crossing the BBB. Despite a very high receptor affinity ($pK_i = 9.16$) [20], in fact, clobenpropit had shown limited potency when administered peripherally ($ED_{50} = 26$ mg kg^{-1} on in vivo screening on central H_3-receptor activity after p.o. administration in mouse), while for thioperamide, which has the same or lower receptor affinity, higher in vivo potency had been observed ($ED_{50} = 1.0$ mg kg^{-1} [21]).

On the other hand, the distribution of thioperamide to rat brain proved to be lower than expected, compared to its concentration in peripheral tissues after systemic administration [22], although this compound has a neutral polar group and possesses the lipophilic requirements which should allow easy access to the CNS. In fact, it has good affinity for brain membrane phospholipids [23], and a log $P_{o/w}$ value around 2.13, which is believed to be an optimal one for brain access.

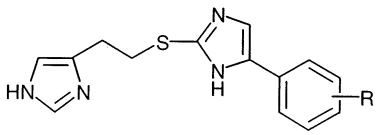
Good potency has been observed in ex vivo experiments after oral administration of thioperamide [24], but this may be due to very high receptor affinity ($pK_i = 8.5$ on rat cortex) [25], while its brain access is not as prompt as can be expected from lipophilicity. In fact, it was observed that, after i.p. administration of a dose of 10 mg/kg to rats, brain levels of thioperamide are lower than those observed in peripheral tissues [22], and that brain access suddenly increases at higher doses [26]. These observations can be related to thioperamide binding to rat plasma proteins, which is described by a complex function, accounting for a specific, saturable binding site, and a non-specific one [23]; the percentage of bound drug is therefore rather constant at relatively high total concentrations, but rapidly increases at lower concentrations, probably limiting the crossing of the BBB. Protein binding could therefore be regarded as one of the properties determining brain access for H_3-antagonists with a neutral polar group. Indeed, taking protein binding into account led to an improvement in the vitro/vivo correlation for a series of 4-hydroxy-2-quinolones with anticonvulsant activity [27]. The effect of protein binding on brain penetration has recently been reported for other classes of compounds.

Lipophilicity has been known for many years to be one of the most important factors involved in the extent of protein binding [28,29], and quantitative relationships (QSPR) between this property and the octanol-water partition coefficient (further indicated as Log P) have recently been applied to many classes of therapeutic substances [30–32]. Aromatic substitution with groups having different π values can therefore be employed to investigate the possibility of modulating protein binding in a series of compounds endowed with good receptor affinity.

In a previous paper, we presented a QSAR study relating H_3-receptor affinity on rat brain membranes and H_3-antagonist potency on guinea-pig ileum to the physico-chemical properties of a series of *para*- and *meta*-substituted 4(5)-phenyl-2-[[2-[4(5)-imidazolyl]-ethyl]thio] imidazoles [3].

By introducing substituents on the phenyl ring, we obtained a significant variation in lipophilicity, starting from a Log P around the value of 2 for the parent compound (see Table 1). While potency on guinea-pig ileum showed a complex dependence on lipophilicity and other properties, rat brain H_3-receptor affinity was

Table 1
Biological and physico-chemical data for compounds **1**–**11**



Compd	R	pK_i [a]	Log B/F	Log P [b]	π	σ	MR
1	H	7.85	0.72	2.62	0	0	1.03
2	*p*-NO_2	7.28	0.84	2.75	−0.28	0.78	7.36
3	*p*-Cl	7.45	1.08	3.66	0.71	0.23	6.03
4	*p*-*n*-C_3H_7	7.40	1.34	4.09	1.55	−0.13	14.96
5	*p*-*O*-*n*-C_4H_9	7.71	1.39	4.11	1.55	−0.32	21.66
6	*p*-$OSO_2C_6H_5$	[c]	1.46	3.72	0.93	0.33	36.70
7	*p*-$CONH_2$	7.55	0.26	1.40	−1.49	0.36	9.81
8	*m*-Br	7.85	1.24	3.76	0.86	0.39	8.88
9	*m*-NO_2	7.51	0.95	2.43	−0.28	0.71	7.36
10	*m*-*O*-*n*-C_3H_7	8.03	1.22	3.70	1.05	0.10	17.06
11	*m*-$CONH_2$	7.79	0.30	1.61	−1.49	0.28	9.81
Thioperamide		8.49	0.55	2.13			

[a] Inhibition of [^{3}H]NAMHA binding to rat brain membranes; see Ref. [3].
[b] Determined by the pH-metric method using a Sirius-PCA 101 instrument; see Ref. [3].
[c] Two-site model gave a better fit; see Ref. [3].

quite insensitive to aromatic substitution, with minor variations around the pK_i value of 7.85, observed for the parent compound (see Table 1). These results could reasonably be exploited in an optimization design regarding pharmacokinetic properties such as protein binding. Although receptor affinity of these compounds was lower than for thioperamide, its tolerance to physico-chemical properties prompted us to evaluate the possibility of obtaining a significant decrease in protein binding and, at the same time, to investigate the relationship between lipophilicity and protein binding for this series.

Therefore, we measured the binding of these compounds to rat plasma proteins by means of equilibrium dialysis, employing pooled blood samples, collected from several animals, to reduce individual variability, and different starting concentrations, in order to test the concentration-binding profile. Owing to compound solubilities, and because dialysis experiments were carried out, total (bound + free) concentrations could not be accurately adjusted; however, three different starting concentrations with doubling values, in the whole range of 15–200 μM, were prepared.

2. Experimental

2.1. Measurement of protein binding

The protein binding was evaluated by means of dialysis experiments in the following way. Pooled batches of plasma were collected from 5 to 10 Wistar rats of 80 g body weight and directly employed for the dialysis experiments. A starting solution of the tested compound in aqueous buffer (phosphate buffer 20 mM pH 7.4, EDTA 1 mM, NaCl 150 mM) was prepared, with concentrations in the 10–200 μM range; quotas of this solution were diluted to obtain two new solutions, with concentrations of 1/2 and 1/4 with respect to the initial one.

A total of 10 ml of these solutions were poured into a vessel and employed for the dialysis experiments.

In a preliminary series of experiments (controls), a sealed dialysis tube, containing 1 ml of buffer solution containg the test compound, was placed into the solution vessel and the compound was allowed to diffuse into the dialysis tube. After 24 h, when a state of equilibrium had presumably been reached, compound concentrations were measured in the external compartment (C_1).

The values obtained were significantly lower than those expected from the dilution in the whole volume (11 ml); this was attributed to the binding to the experimental apparatus, usually encountered in dialysis experiments, which was presumed to be irreversible for the tested compounds, as was found to be the case for thioperamide [23].

In a second series of experiments, 1 ml plasma filled the inner sealed dialysis tube. The concentrations in the external compartment were measured after 24 h (C_2). C_2 thus represents the free concentration of the compound at a state of equilibrium ($C_2 = F$).

For each of the three concentrations, experiments were performed in triplicate. The concentration of drug

bound to the proteins in the inner solution was calculated as:

$$B = (C_1 - C_2)(V_{ext} + V_{int})/V_{int}$$

The total concentration in the inner compartment was calculated as $B + F$, and is reported in Table 3.

2.2. Apparatus and chromatographic conditions

During the experiments, cellulose dialysis tubes (MW cut-off 5000, Visking) were employed. To remove possible interfering substances, they were cleaned three times with distilled water. To avoid complete dehydration, which could alter the diameter of the pores, they were stored at high humidity. Evaporation of the fluid was prevented by the use of a plastic cover.

Compound concentrations were measured by HPLC using a Gilson chromatograph equipped with a Rheodyne 7125 injector (20 μl). A Gilson 115 UV detector, working at wavelengths ranging from 260 to 290 nm, was used. The signal was recorded on a Hewlett-Packard HP3994A recorder. The chromatographic column was a prepacked Spherisorb ODS2 (Phase Separation, 250 × 4.6 mm, 10 μm particle size). The mobile phases were mixtures of phosphate buffers at different pH values, ranging from 5.5 to 7, and acetonitrile.

The areas were measured at room temperature; the mobile phase flow rate was 1.00 ml/min.

2.3. Calibration

Quantitative analysis of external compartment concentrations was performed using the external standard method. Calibration curves were obtained with standard solutions of the eleven compounds dissolved in methanol.

2.4. Chemicals and reagents

HPLC grade acetonitrile was obtained from Sigma Aldrich S.p.A. All the compounds were synthesized in our laboratory [3].

2.5. QSPR analysis

Log $P_{o/w}$ values for the tested compounds and for thioperamide, used as the independent variable, had been measured in previous works [3,23], and these are reported in Table 1. Other structural descriptors, i.e. the substituent constants MR for steric bulk and σ for electronic effect, were included in the analysis; the correlation matrix is reported in Table 2. The average Log B/F over the three concentrations tested was employed as the dependent variable. Regression analysis was performed with the least squares method using Excel 97 (Microsoft Corp.).

Table 2
Correlation matrix for physico-chemical properties reported in Table 1

	π	σ	MR
π	1.00	−0.54	0.45
σ	−0.54	1.00	−0.25
MR	0.45	−0.25	1.00

3. Results and discussion

The results of the dialysis experiments, reported in Table 3, revealed all the phenyl-substituted derivatives of the parent compound **1** as being more than 50% bound to rat plasma proteins, with significant differences between the various compounds.

The percentage of protein binding (%B in Table 3) generally showed little variation on the concentrations tested, which were doubled and quadrupled for each compound. The final, total concentration in the inner compartment containing plasma proteins (C_{tot}), depended on the extent of protein binding and on other experimental conditions (i.e. binding to the apparatus); the values calculated from the final concentrations in the external compartment (C_2, corresponding to the free drug concentrations, F), are reported in Table 3, with the calculated bound concentrations (B), the percentage of bound compound and the ratio B/F.

The variation in the B/F ratio versus B is a crude indication of the nature of binding, being constant for aspecific, unsaturable binding, and decreasing when a saturable binding site is present [33]; for most of the tested compounds, this ratio remained quite constant, indicating a prevalence of aspecific binding, while for a few of the compounds (**3**, **8** and **9**) a slightly negative trend was observed, indicating the possibility of an additional specific binding. A full analysis of the binding curves was, however, beyond the scope of our work.

Two of the compounds (**7** and **11**), having the carboxamide substituent in the *meta*- or *para*- position of the phenyl ring, showed reduced protein binding compared to the reference compound thioperamide, with values of %B in the range 60–68%, while thioperamide was 78% bound at concentrations corresponding to the prevalence of aspecific binding [23]. This corresponds to a huge increase in the free quota, ranging from 22% for thioperamide to 32–40% for the carboxamide derivatives cited.

On the other hand, other compounds presented a higher binding than thioperamide, especially considering the high total concentrations used in the present experiments, and thus the possibility of increased protein binding at lower concentrations, as has been observed for thioperamide itself [23].

Table 3
Rat protein binding of compounds **1–11**

	R	F [a] (μM)		B [b] (μM)	C_{tot} (μM)	%B [c]	B/F [d]
1	H	60.59	(59.85–61.81)	274.21	334.80	81.9	4.53
		27.33	(26.70–27.88)	139.29	166.62	83.6	5.10
		11.27	(11.06–11.53)	71.91	83.18	86.5	6.38
2	*p*-NO_2	42.99	(41.95–44.2)	259.68	302.67	85.8	6.04
		20.47	(20.30–20.65)	139.58	160.05	87.2	6.82
		10.03	(9.46–10.80)	79.11	89.14	88.7	7.89
3	*p*-Cl	70.90	(66.27–76.88)	546.18	617.08	88.5	7.70
		28.00	(26.63–29.01)	357.32	385.32	92.7	12.76
		11.64	(10.42–12.42)	206.48	218.12	94.7	17.74
4	*p*-*n*-C_3H_7	44.06	(41.94–47.35)	927.01	971.07	95.5	21.04
		24.59	(23.67–25.39)	532.76	557.35	95.6	21.67
		11.32	(10.97–11.75)	260.59	271.91	95.8	23.02
5	*p*-*O*-*n*-C_4H_9	[e]					
		10.96	(10.32–11.83)	222.27	233.23	95.3	20.28
		4.61	(4.35–5.11)	133.01	137.62	96.7	28.85
6	*p*-$OSO_2C_6H_5$	17.17	(16.44–18.06)	274.81	291.98	94.1	16.01
		6.60	(6.43–6.75)	259.26	265.86	97.5	39.28
		3.86	(3.40–4.60)	150.53	154.39	97.5	39.00
7	*p*-$CONH_2$	126.51	(125.23–127.82)	185.54	312.05	59.5	1.47
		57.74	(57.19–58.65)	112.93	170.67	66.2	1.96
		27.44	(26.95–28.30)	56.35	83.79	67.3	2.05
8	*m*-Br	33.30	(31.24–34.91)	465.04	498.34	93.3	13.97
		14.82	(14.21–15.4)	216.68	231.50	93.6	14.62
		4.50	(4.30–4.65)	112.51	117.01	96.2	25.00
9	*m*-NO_2	79.28	(78.31–80.48)	467.34	546.62	85.5	5.89
		33.77	(32.56–35.03)	301.52	335.29	89.9	8.93
		12.44	(12.37–12.52)	166.06	178.50	93.0	13.35
10	*m*-*O*-*n*-C_3H_7	28.88	(27.39–30.12)	485.35	514.23	94.4	16.81
		16.62	(16.03–17.49)	232.30	248.92	93.3	13.98
		7.08	(6.26–8.23)	134.24	141.32	95.0	18.96
11	*m*-$CONH_2$	77.18	(75.43–79.28)	165.37	242.55	68.2	2.14
		36.27	(35.96–36.81)	69.07	105.34	65.6	1.90
		15.23	(15.04–15.62)	30.43	45.66	66.6	2.00
Thioperamide		146.49		537.41	683.90	78.6	3.67
		36.49		134.48	170.97	78.7	3.69
		6.86		54.69	61.55	88.9	5.69
		2.52		40.23	42.75	94.1	6.69

[a] F, external equilibrium concentration in the presence of plasma proteins (see Experimental). The range of three replicates is reported in brackets.
[b] B, bound concentration to rat plasma proteins (see Experimental).
[c] %B, percentage of bound compound.
[d] B/F, bound over free ratio.
[e] Solubility problems were encountered at higher starting concentrations.

It was apparent, from the octanol–water Log P data reported in Table 1, that a close relationship between protein binding and lipophilicity could be observed for this series of compounds. With the aim of devising a quantitative relationship, protein binding was expressed as the logarithm of the B/F ratio (see Table 1), which is particularly suitable when an unspecific and apparently unsaturable binding is found, following the equation $B = KF$. In this case, Log B/F is a measure of Log K, and the binding process is strictly related to a partition one, such as that employed for the Log P measurement.

A linear relationship between Log B/F and the lipophilicity of the compounds was found, showing a positive correlation between the two properties, with a rather low slope compared to other QSPRs for known classes of therapeutic substances [28].

$$\text{Log } B/F = 0.41(\pm 0.04)\ \text{Log } P - 0.2g(\pm 0.13) \quad (1)$$

$n = 11,\ r^2 = 0.92,\ s = 0.12,\ F = 104.09$

No substituent constant other than lipophilicity descriptors could be used to explain protein binding differences within this series (see Tables 1 and 2), nor was it possible to improve the fit of the data to the model by multiple regression analysis, since the standard error of Eq. (1) was just in the range of Log B/F uncertainty. Eq. (1) well describes the case of an aspecific binding of a series of compounds to rat plasma proteins, where hydrophobic interaction seems to be the driving force of the process.

Two compounds, with the p-$OSO_2C_6H_5$ and the m-NO_2 substituents (compounds **6** and **9**), lie over the regression line (see Fig. 2), with a Log B/F higher than that expected from the trend of the other compounds. This could be due to unexplained effects, not accounted for by lipophilicity, but it should be noted that compounds **3**, **6** and **9**, having the worst residuals from the model, are characterized by a higher variation in their B/F ratio over the concentration range in comparison with the others of the series. This behavior could be related to their low fitting to the curve.

The reference compound, thioperamide, lies perfectly on the straight line, probably indicating that the QSPR observed for compounds **1**–**11** could also account for the aspecific component of its plasma protein binding.

The present data indicate a strict dependence of rat plasma protein binding on lipophilicity for this series of H_3-antagonists. This dependence only refers to the aspecific component for the binding of the whole series, and also applies for thioperamide which, although different from a structural point of view, still belongs to the class of antagonists endowed with neutral polar groups. The range of total concentrations explored (C_{tot} in Table 3), and other experimental conditions, did not lead to the characterization of specific binding sites; this does not exclude the possibility, however, that at lower total concentrations, the presence of a specific component of binding could be revealed, as has been observed for thioperamide.

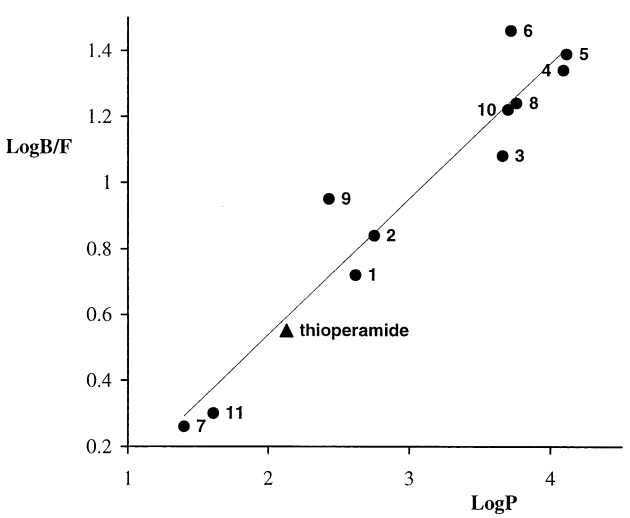


Fig. 2. Plot of protein binding vs. lipophilicity (Log $P_{o/w}$) for tested compounds (filled circles) and for thioperamide (triangle). The regression line for compounds **1**–**11** is plotted.

With these limitations, it is possible to assess that protein binding of 2-alkylthio-5-phenyl-imidazole derivatives can be markedly reduced, without dramatically affecting their affinity for the H_3-receptor, thanks to the high tolerance of the receptor to aromatic substitution (see Table 1) and to the dependence of protein binding on lipophilicity. It is therefore possible to obtain a modulation of the plasma protein binding within the series by modulating the lipophilicity of the members.

Lipophilicity has been revealed to be an important physico-chemical property, influencing not only protein binding, but also many aspects of the pharmacokinetic behavior of drugs. Processes such as absorption, elimination and distribution are strongly affected by the lipophilic/hydrophilic balance of the compound [34]. On the other hand, the capacity of a drug to have an effect on its target organ is strictly dependent on its ability to reach its site of action; for H_3-antagonists, this corresponds to the ability of accessing the CNS. The present results suggest that one of the distribution properties affecting brain access, i.e. protein binding, can be modeled by a simple physico-chemical descriptor, Log P. On the other hand, passive crossing of the BBB is a rather complex process [35], and its dependence on drug properties should be further investigated. Explicit inclusion of protein binding in brain access studies could help to unravel this complex relationship in series of well designed experiments.

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