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BINDING OF [^{3}H] SR 49059, A POTENT NONPEPTIDE VASOPRESSIN V_{1a} ANTAGONIST, TO RAT AND HUMAN LIVER MEMBRANES

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SUMMARY : The new potent and selective nonpeptide vasopressin V_{1a} antagonist, SR 49059, was tritiated and used for the characterization of rat and human liver AVP V_{1a} receptors. Binding of [^{3}H] SR 49059 was time-dependent, reversible and saturable. A single class of high affinity binding sites was identified with K_d values of 0.63 ± 0.13 and 2.95 ± 0.64 nM, in rat and human liver membranes, respectively. The maximal binding capacity (B_{max}) was about 7 times higher in rat than in human liver preparations. The relative potencies of several AVP/oxytocin agonists or antagonists to inhibit [^{3}H] SR 49059 binding confirmed that this ligand labeled a homogenous population of sites with the expected AVP V_{1a} profile. Furthermore, [^{3}H] SR 49059 or unlabeled SR 49059 displayed only slight species differences between rat and human V_{1a} receptors, whereas OPC-21268, another nonpeptide V_{1a} antagonist, exhibited a high species-related potency with more than 500 fold higher affinity for rat than for human liver V_{1a} receptors. Thus, [^{3}H] SR 49059 is the first nonpeptide AVP V_{1a} ligand reported having highly specific activity, stability, specificity and affinity. This makes it a suitable probe for labeling AVP V_{1a} receptors in rat and also in human tissues.

Vasopressin (AVP) is a nonapeptide well-known for its pressor and antidiuretic activities. However AVP has a wide spectrum of biological actions, acting on distinct tissues through three receptor subtypes : V_{1a} receptors, mainly in brain, vasculature, platelets, liver, adrenals and uterus ; V_{1b} receptors in anterior pituitary; V_2 receptors mainly in the kidney (1). AVP may be involved in several disorders such as cardiovascular diseases, diabetes insipidus, dysmenorrhea (2, 3) and the development of selective AVP antagonists is essential for further elucidating the pathophysiological role of AVP and for generating new therapeutic agents. In this field, recent years have marked a turning point with the discovery of the first orally-effective nonpeptide antagonists selective at AVP V_{1a} or V_2 receptors, OPC-21268 and OPC-31260, respectively (4, 5). More recently, we reported the pharmacological characterization of

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the most potent, selective AVP V_{1a} antagonist, SR 49059, active *in vitro* at rat and human AVP V_{1a} receptors and displaying good oral bioavailability *in vivo* (6).

The aim of this study was to characterize liver AVP V_{1a} receptors using tritiated SR 49059. Marked species differences have been reported for AVP receptors on the bases of binding and functional potencies of certain AVP analogues (7), and more recently the cloning of rat and human AVP V_{1a} receptors confirmed differences in the aminoacid sequences in these two species (8, 9). Therefore, it was of interest to characterize [^{3}H] SR 49059 binding to rat and human liver tissues. Comparison was established versus two other tritiated ligands, the native hormone [^{3}H] AVP and the selective peptide AVP V_{1a} antagonist, [^{3}H] d(CH_2)$_5$Tyr(Me)AVP.

MATERIALS AND METHODS

Chemicals : SR 49059, its enantiomer (SR 49770), OPC-21268 and OPC-31260 were synthesized at Sanofi Recherche, Montpellier. All compounds were initially dissolved in DMSO at a concentration of 10^{-2}M, then diluted in the appropriate test solvent. AVP, d(CH_2)$_5$Tyr(Me)AVP (SKF-100273), oxytocin, DDAVP and bacitracin were from Sigma Chemical Co (L'Isle d'Abeau). Bovine serum albumin (BSA) was obtained from IBF (Paris, France). All other chemicals were from Prolabo (France). [^{3}H] AVP and [^{3}H] d(CH_2)$_5$Tyr(Me)AVP were purchased from New England Nuclear (Paris, France) ; [^{3}H] SR 49059 (54 Ci/mmol) was synthesized by Amersham.

Biological material : Human liver tissues were obtained from patients undergoing lobectomy. Frozen samples were collected in conformity with national ethical rules. Male Sprague Dawley rats (Iffa-Credo, Lyon, France) were used.

Membrane preparation : Rat and human liver plasma membranes were prepared by the method of Prpic (10) and stored as aliquots in liquid nitrogen until used.

Binding assays : Liver plasma membranes (75 µg/assay for rat and 130 µg/assay for human) were incubated for 30 min at 30°C in a 50 mM Tris-HCl buffer pH 7.4 containing 10 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml bovine serum albumin, 1 mg/ml bacitracin and 2.5 nM [^{3}H] SR 49059. The incubation was stopped by adding 4 ml of ice-cold buffer. The content of the assay tubes was filtered rapidly through Whatmann GF/B filters then rinsed with 2 x 4 ml of buffer. Non-specific binding was determined by incubating with 1 µM SR 49059. Saturation experiments were performed with increasing concentrations of [^{3}H] SR 49059, [^{3}H] AVP or [^{3}H] d(CH_2)$_5$Tyr(Me)AVP from 0.1 to 25 nM. Data from association, saturation and competition experiments were analyzed using a non-linear regression program (11).

RESULTS

Kinetic experiments : As shown in figure 1, binding of [^{3}H] SR 49059 to its receptor on rat (A) or human (B) liver membranes was time-dependent and reached an apparent equilibrium in about 15 min according to a monoexponential process. Once steady-state was reached, dissociation was initiated after 30 min by the addition of an excess amount of unlabeled SR 49059 (1 µM). Full dissociation of the ligand-receptor complex occurred rapidly both in rat and human tissues. During the incubation period, the stability of [^{3}H] SR 49059 was assessed by HPLC analysis of the supernatant obtained

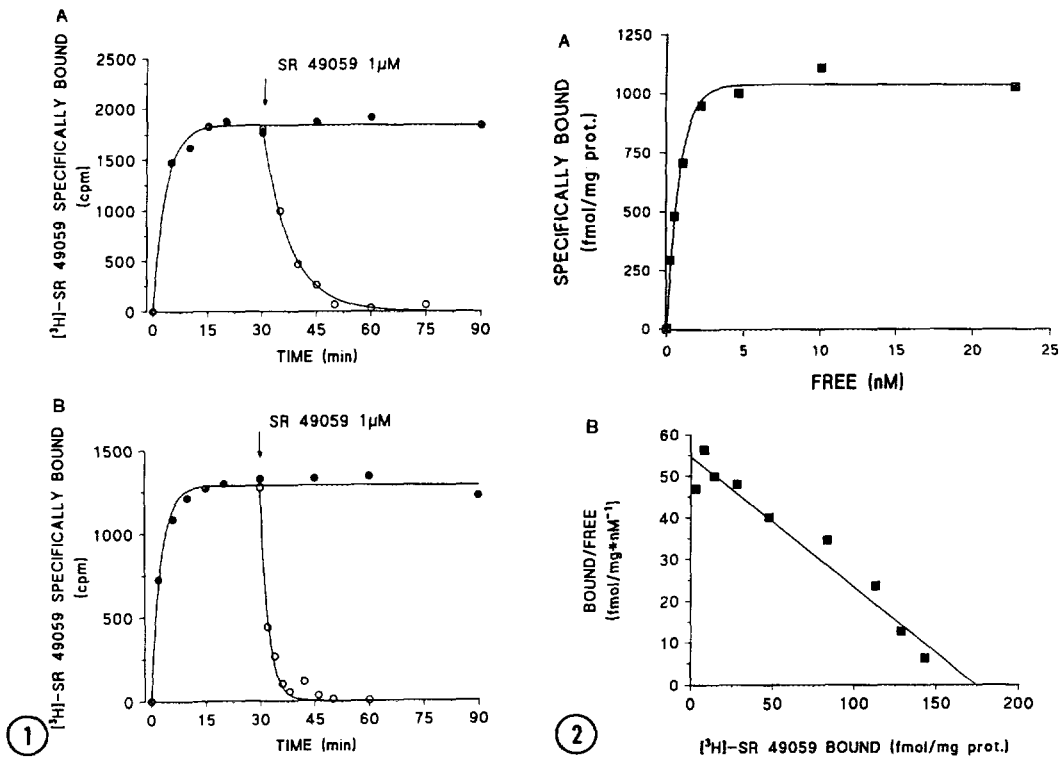

Figure 1 . Time-course of association (●) and dissociation (○) of [^{3}H] SR 49059 to rat (A) or human (B) liver membranes.
The arrow indicates the time at which unlabeled SR 49059 (1 µM) was added to initiate the dissociation process. Results represent data from a typical experiment performed in duplicate repeated 3 times without noticeable modifications.

Figure 2 . Specific binding of [^{3}H] SR 49059 to human liver membranes at varying concentrations of [^{3}H] SR 49059.
(A) saturation isotherm - (B) Scatchard plot.
Results represent data of a typical experiment performed in duplicate repeated 3 times with similar results.

after filtration of the incubation medium. Under standard conditions, [^{3}H] SR 49059 displayed high stability since no degradation of this ligand was observed after a 4 hour incubation period (data not shown). Non-specific binding, determined in the presence of 1 µM SR 49059, was markedly higher in human than in rat liver membranes and represented respectively 60 and 10 % of the bound radioactivity.

Saturation experiments : Specific binding of [^{3}H] SR 49059 in both rat and human liver membranes was dose-dependent and saturable. Scatchard analysis of data gave linear plots consistent with the presence of a single class of high-affinity binding sites (Fig. 2 and 3). For comparison, results from similar saturation experiments performed simultaneously, under the same experimental conditions, on both tissues using [^{3}H] AVP or [^{3}H] d(CH_2)$_5$Tyr(Me)AVP as ligands, are reported in Table 1. Like [^{3}H] AVP or [^{3}H] d(CH_2)$_5$Tyr(Me)AVP, [^{3}H] SR 49059 displayed nanomolar (human) and even

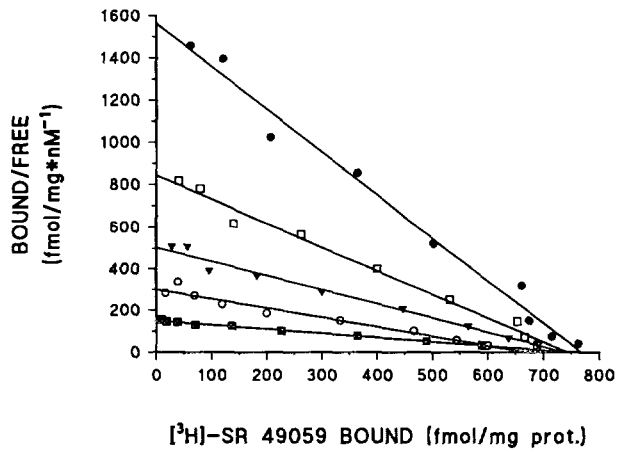

Figure 3 . Scatchard plots of [^{3}H] SR 49059 binding to rat liver membranes without (●) or with 0.5 (□), 1 (▼), 2 (○) or 4 (■) nM unlabeled SR 49059.
Results represent data from a typical experiment performed in duplicate repeated 3 times.

subnanomolar (rat) affinity for liver V_{1a} receptors. The maximal binding capacity (B_{max}) of [^{3}H] SR 49059 was considerably higher (x 7) for rat liver than for human liver ; this ratio was also observed with the other tritiated ligands used.

Furthermore, saturation binding experiments were performed on rat and human liver membranes in the absence or presence of SR 49059 (0.5, 1, 2 and 4 nM). As illustrated in Figure 3, in rat tissue, we observed a significant increase in the K_d value in function of SR 49059 concentrations whereas no change in the maximal binding capacity occurred, suggesting competitive inhibition of tritiated SR 49059 binding to its site by the unlabeled molecule, SR 49059. The same results were obtained with human liver tissue (data not shown).

Competition experiments : The binding of [^{3}H] SR 49059 to its site on rat and human liver membranes was further characterized by studying the relative potencies of several peptide and nonpeptide AVP/oxytocin agonists and antagonists (Table 2). The

Table 1 : Equilibrium binding parameters (K_d, B_{max}) of [^{3}H] SR 49059, [^{3}H] AVP and [^{3}H] d(CH_2)$_5$Tyr(Me)AVP for rat and human liver vasopressin V_{1a} receptors

	K_d (nM)		B_{max} (fmol/mg prot.)	
	Rat	Human	Rat	Human
[^{3}H] SR 49059	0.63 ± 0.13	2.95 ± 0.64	918 ± 160	130 ± 40
[^{3}H] AVP	1.57 ± 0.49	0.80 ± 0.15	908 ± 223	183 ± 45
[^{3}H] d(CH_2)$_5$Tyr(Me)AVP	0.25 ± 0.11	1.34 ± 0.40	820 ± 110	108 ± 30

Values are means ± SD obtained from 3 or 4 independent experiments performed in duplicate.

Table 2 : Inhibition of [^{3}H] SR 49059 binding to rat and human liver membranes by AVP or oxytocin agonist and antagonist compounds

Compounds	K_i (nM)	
	Rat	Human
Peptide		
AVP	0.43 ± 0.10	0.63 ± 0.12
OT	26 ± 4	9.0 ± 4.4
DDAVP	213 ± 34	224 ± 93
[^{3}H] d(CH$_2$)$_5$Tyr(Me)AVP	0.20 ± 0.01	2.58 ± 1.64
Nonpeptide		
SR 49059	0.79 ± 0.26	3.2 ± 0.8
SR 49770	16 ± 5	168 ± 35
OPC-21268	61 ± 20	26700 ± 1855
OPC 31260	290 ± 57	250 ± 48

Inhibition constants (K_i) were calculated according to the equation of Cheng and Prusoff (12). Values are means ± SD obtained from 3 or 5 independent experiments performed in duplicate.

receptor identified by [^{3}H] SR 49059 is of the AVP V_{1a} type since AVP and d(CH$_2$)$_5$Tyr(Me)AVP, the selective V_{1a} antagonist, showed nanomolar affinities, whereas the specific V_2 agonist, DDAVP, the nonpeptide AVP V_2 antagonist, OPC-31260 (5) and oxytocin were less potent. The nonpeptide V_{1a} antagonist, OPC-21268 dose-dependently inhibited specific binding of [^{3}H] SR 49059 to rat liver membranes with a *Ki* value of 60 nM. In contrast, it is of interest to note that under the same experimental conditions, OPC-21268 displayed only very weak affinity for human liver V_1 receptors (Ki value of 25700 nM) (Fig. 4A, 4B); affinity of the other compounds tested differed by no more than 10 fold (d(CH$_2$)$_5$Tyr(Me)AVP, SR 49770). Furthermore, [^{3}H] SR 49059 binding to V_{1a} receptors both in rat and human liver membranes was highly stereospecific since SR 49059's enantiomer, SR 49770, had 20 to 50 fold less affinity than SR 49059 (Table 1 ; Fig. 4A, 4B). Furthermore, GTP and the nonhydrolyzable GTP analog [Gpp(NH)p] were completely inactive up to 100 µM, consistent with an antagonist G-protein-coupled receptor binding (data not shown).

CONCLUSION

Our study demonstrated that [^{3}H] SR 49059 binds with high affinity to rat and human liver membranes and that the single population of binding sites identified has the AVP V_{1a} pattern. The binding equilibrium dissociation constant (K_d) values calculated from kinetic, saturation and competition experiments were similar and evidenced that [^{3}H] SR 49059 recognizes the AVP V_{1a} sites with nanomolar affinity, as do other tritiated peptide ligands (14, 15). Only recently-developed radioiodinated peptide ligands derived from the specific V_{1a} antagonist, d(CH$_2$)$_5$Tyr(Me)AVP, displayed better affinity

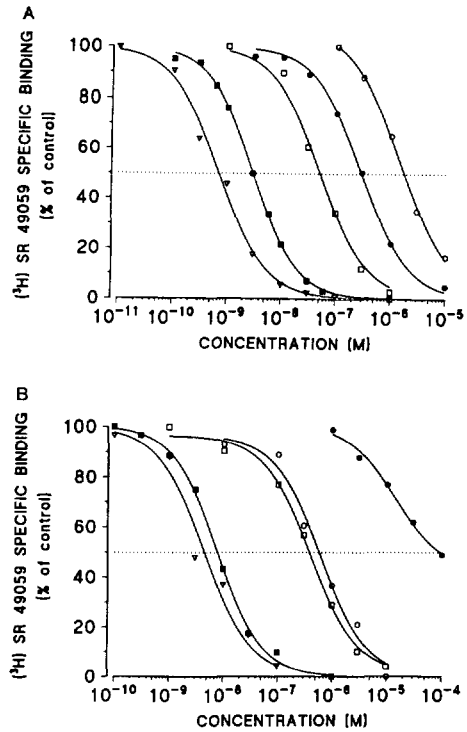

Figure 4 . Inhibition of specific [^{3}H] SR 49059 binding to rat (A) and human (B) liver membranes by d(CH_2)$_5$Tyr(Me)AVP (▽), SR 49059 (■), SR 49770 (□), OPC-21268 (●) and OPC-31260 (○).
Results represent data of a typical experiment performed in duplicate repeated 3 or 4 times without noticeable modifications.

than tritiated ligands for rat liver V1a receptors (K_d of 0.06 nM) (16). But [^{3}H] SR 49059 is the first selective nonpeptide molecule in this field showing extremely high stability due to its synthetic nature.

The comparison with other tritiated ligands ([^{3}H] AVP, [^{3}H] d(CH_2)$_5$Tyr(Me)AVP) showed similarity with the maximal binding capacity (Bmax) of [^{3}H] SR 49059 and evidenced a density of sites considerably higher (x 7) in rat than in human liver membranes. This relatively low density of sites in human liver was also reported by Petitbone et *al* (13) and could be related to the weak glycogenic effect of AVP observed in human liver tissues *in vitro* (17). However, the fact that frozen human tissues were used versus fresh rat livers for the same membrane preparations might influence the maximal binding capacity.

The relative potencies of several peptide AVP and oxytocin agonists or antagonists to inhibit [^{3}H] SR 49059 binding to rat and human liver tissues clearly indicated that [^{3}H] SR 49059 labeled a homogenous population of sites (nHill about unity) with the expected AVP V_{1a} receptor profile : d(CH_2)$_5$Tyr(Me)AVP and AVP displayed nanomolar affinities in this binding whereas oxytocin and DDAVP had weaker potencies. The potent selective V_{1a} antagonist profile of unlabeled SR 49059,

observed in several biochemical and pharmacological experiments (6), fully explained the high affinity and specificity of the tritiated molecule. Another important observation was the stereospecificity of the binding of [^{3}H] SR 49059 to liver V_{1a} receptors since SR 49059's enantiomer (SR 49770) was 20 and 50 fold less potent than SR 49059 in rat and human liver tissues, respectively.

In the rat liver, the nonpeptide V_{1a} antagonist, OPC-21268, inhibited [^{3}H] SR 49059 binding with an affinity (Ki = 60 nM) close to that already reported for rat liver V_{1a} receptors using [^{3}H] AVP as a ligand (4, 13). As expected, OPC-31260, the first synthetic V_2 antagonist described (5) displayed only weak affinity (Ki of 270 nM).

Another important observation was the species differences existing between rat and human V_{1a} receptors evidenced through the relative potency of several compounds and in particular of OPC-21268. This molecule displayed high species-related potency with more than 500 fold higher affinity for rat than for human liver V_{1a} receptors ; other molecules such as d(CH_2)$_5$Tyr(Me)AVP, SR 49770 and SR 49059 showed slight discrimination between the two tissues (x 5 to 10). This pattern of OPC-21268 in [^{3}H] SR 49059 binding to human tissue was consistent with previous results obtained by Petitbone et *al* (13) in the binding of [^{3}H] AVP to human liver and uterus, and by our team (6) in human platelets, adrenals and also in non-pregnant uterus. The discrimination made by OPC-21268, and not by SR 49059, for human V_{1a} receptors suggested that these two small synthetic compounds recognized different domains of the receptor. Furthermore, recent findings in the cloning of AVP V_{1a} receptors in human tissue confirmed species differences since rat liver and human vascular smooth muscle cell AVP V_{1a} receptors displayed 83 % nucleotide sequence homology (18).

Thus, our study clearly demonstrates the similarity between the sites identified by [^{3}H] SR 49059 and other tritiated ligands, since the affinity, binding capacity and AVP agonist/antagonist relative potencies were highly similar both in rat and human liver tissues. Consequently, [^{3}H] SR 49059 is the first nonpeptide AVP V_{1a} ligand described so far. With its highly specific activity, specificity, affinity and stability, [^{3}H] SR 49059 is an interesting probe for labeling AVP V_{1a} receptors in rat and in human tissues.

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