[^{3}H]BHDP as a novel and selective ligand for σ1 receptors in liver mitochondria and brain synaptosomes of the rat

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Abstract The binding profile of [^{3}H]BHDP ([^{3}H]*N*-benzyl-*N*′-(2-hydroxy-3,4-dimethoxybenzyl)-piperazine) was evaluated. [^{3}H]BHDP labelled a single class of binding sites with high affinity (K_d = 2–3 nM) in rat liver mitochondria and synaptic membranes. The pharmacological characterization of these sites using σ reference compounds revealed that these sites are σ receptors and, more particularly, σ1 receptors. Indeed, BHDP inhibited [^{3}H]pentazocine binding, a marker for σ1 receptors, with high affinity in a competitive manner. BHDP is selective for σ1 receptors since it did not show any relevant affinity for most of the other receptors, ion channels or transporters tested. Moreover, in an in vitro model of cellular hypoxia, BHDP prevented the fall in adenosine triphosphate (ATP) levels caused by 24 h hypoxia in cultured astrocytes. Taken together, these results demonstrate that [^{3}H]BHDP is a potent and selective ligand for σ1 receptors showing cytoprotective effects in astrocytes.

Key words: σ Receptor; Liver mitochondrion; Synaptosome; Binding; Hypoxia; Astrocyte

1. Introduction

It is now well established that σ receptors are distinct entities and differ from any other known receptor [1]. These receptors are expressed in various areas of the brain and in peripheral organs including liver, testis, kidney, spleen and intestine. σ Receptors have been classified at least into two subtypes, termed σ1 and σ2, which were distinguished by biochemical and pharmacological studies [2]. The first one shows high affinity for benzomorphans, a stereoselectivity for the (+)-isomers, and is usually labelled with the radioligand [^{3}H](+)-pentazocine [3,4] whereas the second exhibits a lower affinity for (+)-pentazocine and a reverse stereoselectivity. Usually σ2 binding sites are probed with 1,3-di(2-tolyl)guanidine (DTG) which binds to both subtypes 1 and 2 in roughly equal amounts.

The pattern of distribution of these receptors among subcellular fractions of different organs indicates that these receptors are highly concentrated and mainly located on microsomal and plasma membranes [3,5]. However, recent studies also demonstrated the presence of σ receptors in rat brain and liver mitochondrial membranes [6,7]. These receptors were located on the outer mitochondrial membrane but their pharmacological significance and their role on mitochondrial function remain unknown. In order to study the role of these receptors we search for a selective ligand devoid of mitochondrial toxicity. Indeed, usual ligands such as haloperidol or pentazocine, were shown to alter mitochondrial respiration [8] which hampered their use.

We identified among a series of piperazine derivatives synthesized by two of us (M.F. Boussard, M. Wierzbicki) a compound, *N*-benzyl-*N*′-(2-hydroxy-3,4-dimethoxybenzyl)-piperazine (BHDP) (Fig. 1), which exhibited several properties indicating that it could be a σ ligand. This drug is structurally related to *N*-substituted 4-benzylpiperidines, such as ifenprodil and eliprodil, which exhibit high affinity for σ1 receptors [9,10], and its chemical structure is similar to that of SA4503 which has recently been shown to be a potent and selective σ1 receptor agonist [11]. In addition, BHDP was shown to inhibit the binding of [^{3}H]pentazocine, a prototype ligand for σ1 receptors. This prompted us to analyze the binding properties of BHDP. In the present study, we characterized the binding properties of [^{3}H]BHDP to σ receptors first, in mitochondria purified from rat liver, and then in rat brain synaptosomes, and compared these properties with those of typical σ receptor ligands. In addition, BHDP was tested for its ability to protect cultured astrocytes against hypoxia-induced adenosine triphosphate (ATP) depletion.

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Abbreviations: BHDP, *N*-benzyl-*N*′-(2-hydroxy-3,4-dimethoxybenzyl)-piperazine; DTG, 1,3-di(2-tolyl)guanidine; 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine

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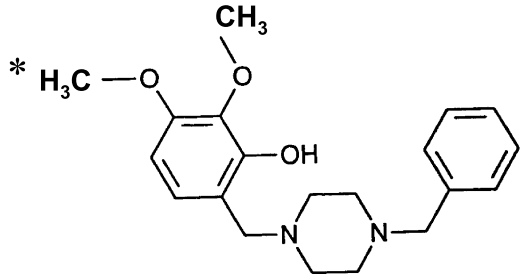

Fig. 1. Chemical structure of BHDP. *Labelling position.

2. Materials and methods

2.1. Drugs

[^{3}H]BHDP (84 Ci/mmol) was obtained from Amersham. [^{3}H](+)-pentazocine and [^{3}H]DTG were purchased from New England Nuclear (Paris, France). NE-100 was synthesized according to [12]. Other drugs and chemicals were obtained from Sigma (St Quentin Fallavier, France) or Merck (Nogent-sur-Marne, France) and were of the highest purity available.

2.2. Preparation of synaptosomal membranes and purified rat liver mitochondria

Purified rat liver mitochondria and synaptosomal membranes were obtained as previously described [7]. Protein contents were determined by the method of Lowry et al. [13].

2.3. Receptor binding assay

Binding of [^{3}H]BHDP to rat liver and brain synaptosomes was measured as follows: mitochondria or synaptic membranes (0.5 mg/ml) were incubated with [^{3}H]BHDP in 250 μl of 50 mM Tris–HCl buffer (pH = 8) for 150 min at 25°C. For saturation experiments the range of concentrations of [^{3}H]BHDP was 0.1–20 nM. For inhibition experiments 0.5–1 nM [^{3}H]BHDP was incubated in the absence or in the presence of increasing concentrations (15) of the competing drug. Non-specific binding was defined using 1 μM haloperidol. Inhibition of [^{3}H](+)-pentazocine and [^{3}H]DTG binding to rat brain membranes was measured as previously described [7,11]. Briefly, each assay tube contained 2 nM [^{3}H](+)-pentazocine or 5 nM [^{3}H]DTG (in the presence of 0.4 μM (±)pentazocine to mask σ1 binding), brain membranes (1 mg/ml) and various concentrations of BHDP in a final volume of 500 μl. Incubations were carried out at 37°C for 150 min in the [^{3}H](+)-pentazocine assay and at 25°C for 90 min in the [^{3}H]DTG assay.

For competition binding studies performed at other receptor types, assay conditions are summarized in Table 2.

Binding was stopped by the addition of ice-cold binding buffer, and bound and free ligands were separated by rapid filtration through Whatman GF/B glass fiber filters (presoaked in 0.1% polyethylenimine). Each filter was washed twice with an additional 5 ml of ice-cold Tris buffer (50 mM) and counted in a liquid scintillation counter Packard 1600 TR with an efficiency of 45%.

2.4. Astrocytes in tissue culture

Astrocyte cultures were obtained from neonate rats using a technique described by Booher and Sensenbrenner [14]. After the meninges had been cleaned off, the brain tissue was forced gently through a nylon sieve (180 μm). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Hyclone, USA), 2 mM glutamine and 50 μg/ml of gentamicin was used for the dissociation of cerebral tissue and development of astrocytes. Cultures were used for 3 weeks after preparation. The oxygen-free culture medium (DMEM) was added and different concentrations of drug were then added. Hypoxia was carried out in an anaerobic chamber (98% N_2, 2% O_2) for 24 h. ATP was extracted and the ATP concentration, expressed in pmol of ATP/mg of cell proteins, was measured by a luciferin-luciferase method using a Sigma kit. The decrease in ATP in the cells (expressed as a percentage) after 24 h hypoxia gave a measurement of the severity of the insult. As demonstrated by the LDH test (Sigma), no cell lysis was observed after the 24 h experiments (data not shown).

2.5. Data analysis

Equilibrium (K_d (dissociation constant) and B_{max} values (maximal density of binding sites)), and inhibition (IC_{50}) binding parameters were calculated by means of a non-linear regression method using a commercially available software (Microparm, INSERM 1990; [15]) by modeling the data according to Hill equations as described previously [16].

All data are presented as the mean ± S.E.M. of three or more individual experiments. They were compared by one-way analysis of variance (ANOVA); a statistically significant difference was accepted if $P < 0.05$.

3. Results

3.1. Characterization of [^{3}H]BHDP binding to liver mitochondria

Binding of low concentrations of [^{3}H]BHDP to liver mito-

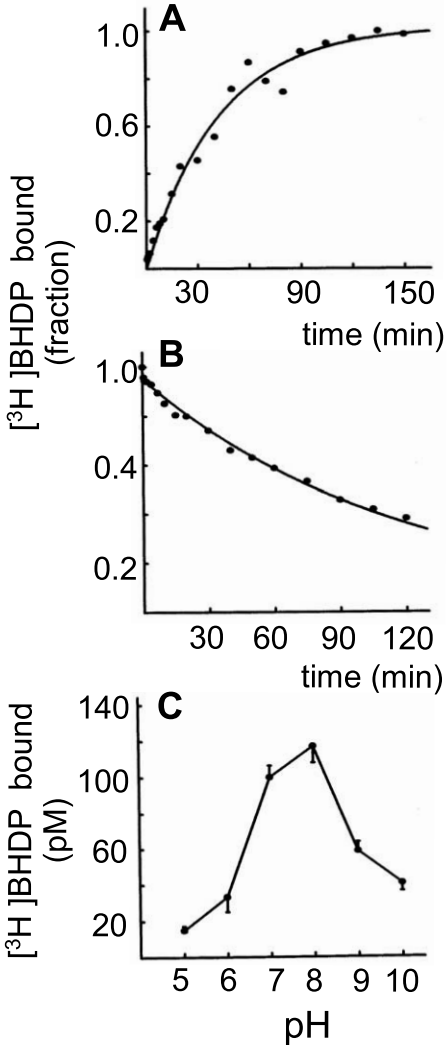

Fig. 2. Kinetics of [^{3}H]BHDP binding to rat liver mitochondria and effect of pH. Association experiment (A): Liver mitochondria (0.5 mg/ml) were incubated with 0.5 nM [^{3}H]BHDP at 25°C. Dissociation experiment (B): After equilibrium was reached, dissociation was induced by dilution and specific binding monitored for 120 min. Data shown are typical experiments plotted as fractions of the maximal binding value ([^{3}H]BHDP bound = 1). Effect of pH (C): Liver mitochondria (0.5 mg/ml) were incubated with 0.5 nM [^{3}H]BHDP at 25°C. Each point represents the mean ± S.E.M. of three experiments performed in triplicate. Non-specific binding was defined in the presence of 1 μM haloperidol.

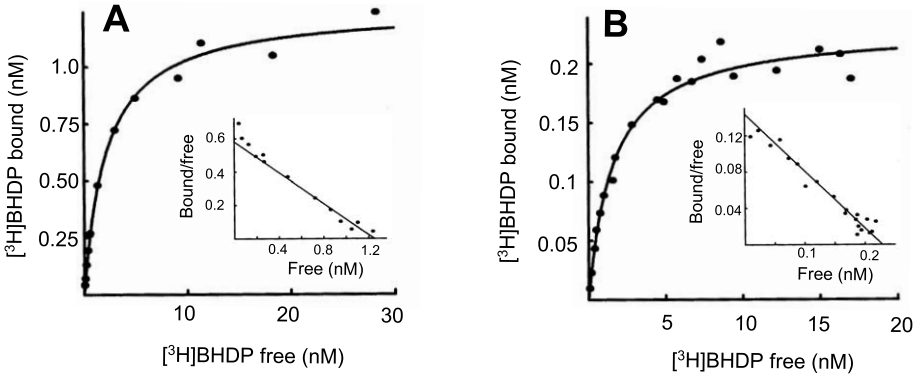

Fig. 3. Representative equilibrium binding curve of [^{3}H]BHDP binding to liver mitochondria (A) and brain membranes (B). Concentration of [^{3}H]BHDP (0.1–30 nM) was incubated with liver mitochondria or brain synaptosomes (0.5 mg/ml) for 150 min at 25°C and non-specific binding was defined using 1 μM haloperidol. Equilibrium parameters were estimated by a non-linear regression analysis. In these particular experiments, K_d and B_{max} values were 2.15 nM and 2.5 pmol/mg protein and 1.59 nM and 0.46 pmol/mg protein in liver mitochondria and brain membranes, respectively. Inset: Scatchard plot.

chondria is saturable and time dependent, reaching equilibrium after 120 min of incubation at 25°C (Fig. 2A). The same pattern was obtained with broken mitochondria, i.e. when they were subjected to hypoosmotic shock and sonication, indicating that the radioligand did not accumulate within mitochondria without binding. After equilibrium was reached, dilution of the suspension (1/100) induced a slow dissociation of [^{3}H]BHDP from its binding sites, demonstrating that the binding was reversible (Fig. 2B). BHDP binding was also pH dependent. Maximum binding occurred at pH = 8, so all experiments were performed at this pH (Fig. 2C).

Equilibrium binding experiments with [^{3}H]BHDP concentrations ranging from 0.1 to 30 nM revealed that the specific binding was saturable. The non-linear regression analysis revealed that [^{3}H]BHDP recognized a single class of binding sites ($n_H = 1$) with mean K_d and B_{max} values of 3.18 ± 0.55 nM and 2.17 ± 0.17 pmol/mg, respectively. The linear Scatchard plot confirmed this analysis of the data. A representative binding curve is shown on Fig. 3A.

In order to verify that the [^{3}H]BHDP binding sites in liver mitochondria correspond to a σ binding site, inhibitory effects of several reference σ ligands on [^{3}H]BHDP binding were studied (Table 1).

BHDP and the antipsychotic dopamine receptor antagonist haloperidol, which is commonly used as a σ receptor ligand, were the most potent inhibitors of [^{3}H]BHDP binding. NE-100, (±)pentazocine, ifenprodil, carbetapentane and 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine (3-PPP) showed good affinity for these sites ($IC_{50} < 100$ nM), whereas progesterone and dextrometorphan were less active. Rimcazole was the lowest inhibitor of the well-identified σ receptor ligands tested with an IC_{50} value of 1550 nM. It should be noted that all these compounds, except (±)pentazocine, displaced [^{3}H]BHDP binding with a Hill coefficient (n_H) value close to unity, additional evidence for the presence of a single population of binding sites. The result obtained with (±)pentazocine ($n_H < 1$) was probably due to the use of the racemic form of the drug as the two enantiomers have been shown to have different affinities for σ receptors [2,17].

3.2. Characterization of [^{3}H]BHDP binding to rat brain synaptosomal membranes

σ Receptors are highly concentrated in brain and most of the studies designed to analyze the binding properties of radiolabelled σ ligands have been performed on rat or guinea pig brain membranes. In order to confirm the binding of

Table 1
Inhibition of [^{3}H]BHDP binding by various σ reference ligands

σ Ligands	Liver mitochondria		Brain synaptosomes	
	IC_{50} (nM)	n_H	IC_{50} (nM)	n_H
Haloperidol	7.93 ± 1.25	0.95 ± 0.02	8.96 ± 1.09	0.95 ± 0.22
(±)Pentazocine	40.97 ± 1.76	0.68 ± 0.07[a]	38.5 ± 6.37	0.75 ± 0.07[a]
BHDP	5.69 ± 0.82	0.93 ± 0.08	3.64 ± 0.76	0.95 ± 0.21
NE-100	10.1 ± 2.50	0.85 ± 0.17	9.62 ± 2.10	0.83 ± 0.19
DTG	115 ± 13.5	1.14 ± 0.07	94.3 ± 32.4	1.13 ± 0.07
Ifenprodil	19.3 ± 5.79	0.97 ± 0.12	8.50 ± 1.63	0.91 ± 0.14
Carbetapentane	50.6 ± 6.21	1.30 ± 0.20	25.5 ± 2.95	1.13 ± 0.20
(±)3-PPP	93.3 ± 8.11	0.93 ± 0.55	97 ± 11	0.94 ± 0.08
Rimcazole	1550 ± 144	1.12 ± 0.05	1733 ± 285	1.02 ± 0.09
Dextrometorphan	476 ± 4.88	0.97 ± 0.05	580 ± 63.8	0.98 ± 0.07
Progesterone	188 ± 31.6	0.94 ± 0.04	121 ± 9.75	0.62 ± 0.22
DHEA	2450 ± 86.6	1.05 ± 0.09	1800 ± 115	0.73 ± 0.22

Data shown are the mean ± S.E.M. of three to four experiments. [^{3}H]BHDP concentration was 0.5–1 nM. IC_{50} values were estimated by a non-linear regression analysis by a Hill model. A Hill coefficient (n_H) value equal to 1 indicates that the interaction is competitive. All drugs displaced 100% of [^{3}H]BHDP specific binding.
DHEA, dehydroepiandrosterone.
[a]Statistically smaller than 1.

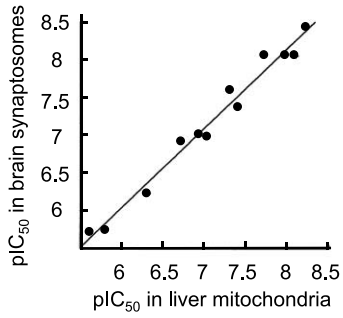

Fig. 4. Relationship between the affinity of σ reference compounds for [^{3}H]BHDP binding to liver mitochondria and brain synaptosomes. Plotted are IC_{50} values listed in Table 1. Regression line was obtained by least squares fit analysis of the data points.

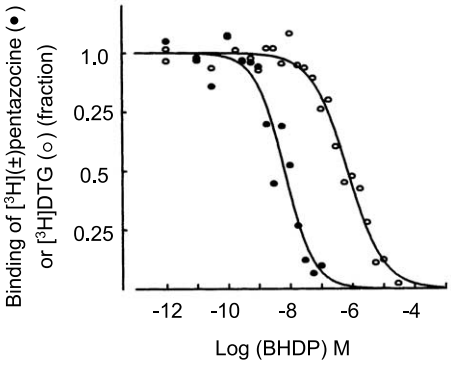

Fig. 5. Inhibition of [^{3}H]pentazocine and [^{3}H]DTG binding to rat brain synaptosomes (0.5 mg/ml) by increasing concentrations of BHDP. Data shown are from typical experiments and are plotted as fractions of the control [^{3}H]pentazocine or [^{3}H]DTG binding values (in the absence of BHDP [^{3}H]radioligand bound = 1). [^{3}H]pentazocine and [^{3}H]DTG concentrations were 2 and 5 nM, respectively.

[^{3}H]BHDP to σ receptors, we investigated the binding properties of [^{3}H]BHDP in rat brain. Here again, [^{3}H]BHDP binding was saturable and labelled a single class of sites (K_d = 2.08 ± 0.28 nM, B_{max} = 0.42 ± 0.11 pmol/mg) as suggested by the linear Scatchard plot (Fig. 3B). Pharmacological characterization using reference σ compounds confirmed the σ receptor profile of the radioligand (Table 1). The rank order of potency was identical to that observed in liver mitochondria, showing a strong correlation ($r = 0.988$; $P < 0.0001$; Fig. 4). In addition, BHDP was able to displace the specific binding of [^{3}H](+)-pentazocine and [^{3}H]DTG (Fig. 5) which are now commonly used to specifically label σ1 and σ2 receptors, respectively.

BHDP had a high affinity for [^{3}H](+)-pentazocine binding sites in rat brain synaptosomal membranes with an IC_{50} value of 6.62 ± 1.79 nM. It is similar to the affinity values obtained with [^{3}H]BHDP in saturation and inhibition experiments. On the other hand, BHDP has a lower affinity for the σ2 receptor

Table 2
Radioligand binding conditions and effects of BHDP on several receptors, transporters and ionic channels

Target	Radioligand (nM)	Tissue	Non-specific (μM)	Incubation	BHDP (IC_{50}, μM)
Receptors					
Adenosine A_1	[^{3}H]DPCPX (1)	CHO cells	DPCPX (1)	60 min/22°C	> 10
Adenosine A_2	[^{3}H]CGS 21680 (6)	rat striatum	NECA (100)	90 min/22°C	> 10
α_1-adrenergic	[^{3}H]prazosin (0.25)	rat cortex	prazosin (0.5)	60 min/22°C	> 10
α_{2A}-adrenergic	[^{3}H]RX821002 (0.8)	CHO cells	phentolamine (10)	45 min/25°C	8.5
α_{2B}-adrenergic	[^{3}H]RX821002 (1)	CHO cells	phentolamine (10)	45 min/25°C	4.0
α_{2C}-adrenergic	[^{3}H]RX821002 (2)	CHO cells	phentolamine (10)	45 min/25°C	1.8
β-adrenergic	[^{3}H]CGP 12177 (0.5)	rat cortex	isoproterenol (100)	60 min/25°C	> 10
Dopaminergic D_1	[^{3}H]SCH 23390 (0.3)	rat striatum	SCH 23390 (10)	45 min/22°C	> 10
Dopaminergic D_2	[^{3}H]YM-09151-2 (0.1)	rat striatum	(+)-butaclamol (10)	60 min/22°C	≈ 10
Histamine H_1	[^{3}H]pyrilamine (0.5)	g.p. cerebellum	triprolidine (100)	10 min/22°C	> 10
Histamine H_2	[^{125}I]APT (0.1)	g.p. striatum	tiotidine (10)	150 min/22°C	> 10
5-HT_{1A}	[^{3}H]8-OH-DPAT (0.4)	rat hippocampus	5-HT (10)	15 min/37°C	1.6
5-HT_{2A}	[^{3}H]ketanserin (0.5)	rat cortex	ketanserine (1)	15 min/37°C	≈ 10
5-HT_{2B}	[^{3}H]5-HT (0.4)	Cos-7 cells	5-HT (10)	120 min/4°C	0.19
5-HT_{2C}	[^{3}H]mesulergine (1)	CHO cells	mianserin (10)	30 min/37°C	> 10
5-HT_3	[^{3}H]BRL 43694 (1)	N1E-115 cells	METO (100)	180 min/4°C	> 10
Cannabinoides	[^{3}H]WIN 55212-2 (0.5)	rat cerebellum	WIN 55212-2 (1)	90 min/37°C	> 10
Endothelin A	[^{125}I]endothelin-1 (0.01)	CHO cells	endothelin-1 (0.1)	120 min/37°C	> 10
GABA	[^{3}H]GABA (10)	rat cortex	GABA (1000)	20 min/22°C	> 10
AMPA	[^{3}H]AMPA (5)	rat cortex	L-GLU (1000)	60 min/4°C	> 10
NMDA	[^{3}H]CGP 39653 (5)	rat cortex	L-GLU (100)	60 min/4°C	> 10
Muscarinic	[^{3}H]QNB (0.05)	rat cortex	atropine (1)	120 min/22°C	> 10
Nicotinic	[^{3}H]cytisine (1.5)	rat cortex	nicotine (10)	75 min/4°C	> 10
Neuropeptide Y	[^{3}H]neuropeptide Y (0.5)	rat cortex	neuropeptide Y (1)	90 min/22°C	> 10
Opioids	[^{3}H]naloxone (1)	rat cortex	naloxone (1)	40 min/22°C	> 10
TXA_2/PGH_2	[^{3}H]SQ 29,548 (5)	human platelets	U44069 (0.05)	30 min/22°C	> 10
Amine uptake sites					
Dopamine	[^{3}H]BTCP (0.5)	rat striatum	BTCP (10 μM)	90 min/4°C	≈ 10
5-HT	[^{3}H]paroxetine (0.05)	rat cortex	serotonine (100)	15 min/37°C	> 10
Noradrenaline	[^{3}H]nisoxetine (1)	rat cortex	desipramine (1)	240 min/4°C	> 10
Ion channels					
Ca^{2+}, L type	[^{3}H]diltiazem (5)	rat cortex	diltiazem (10)	120 min/22°C	> 10
K^+, ATP sensitive	[^{3}H]glibenclamide (0.1)	rat cortex	glibenclamide (1)	60 min/22°C	> 10
K^+, volt. dependent	[^{125}I]CHBT (0.05)	rat cortex	CHBT (0.01)	15 min/22°C)	> 10

g.p., guinea pig; METO, metoclopramide; L-GLU, L-glutamate; CHBT, charybdotoxin.

Table 3
Effects of BHDP on the decrease in ATP levels in astrocytes maintained in hypoxic conditions for 24 h

	ATP (pmol/mg protein)
Hypoxia (48%)	
Normoxia	450 ± 62
Hypoxia	232 ± 35††
Hypoxia	
+BHDP 0.1 μM	248 ± 20
+BHDP 1 μM	313 ± 32*
+BHDP 10 μM	699 ± 58**
Hypoxia (62%)	
Normoxia	826 ± 45
Hypoxia	316 ± 25††
Hypoxia	
+BHDP 0.1 μM	455 ± 33**
+BHDP 1 μM	615 ± 40**
+BHDP 10 μM	1105 ± 234**

Cultured astrocytes were maintained in hypoxic conditions for 24 h in the absence or in the presence of increasing concentrations of BHDP. Then, ATP concentrations were measured by luminescence.
††$P < 0.01$ versus normoxia.
*$P < 0.05$ versus hypoxia.
**$P < 0.01$ versus hypoxia.

subtype with an IC_{50} value of 713 ± 95 nM. These data reinforced our hypothesis that BHDP preferentially labelled σ1 receptors.

3.3. Selectivity of BHDP for σ receptors

The affinity of BHDP for a number of receptors, ionic channels or amine transporters was studied. Binding assays were performed according to the references listed in Table 2. BHDP showed low affinity ($IC_{50} \geq 10$ μM) for most of the receptors examined (Table 2). Exceptions were 5-HT_{1A} and 5-HT_{2B} receptors, exhibiting an IC_{50} value of 1.6 and 0.19 μM, respectively. Several α-adrenergic receptors also showed IC_{50} values on the micromolar range but it was 300–500 times lower than that for the σ1 receptor subtype. This underlines the selectivity of the ligand for σ1 receptors.

3.4. Effect of BHDP on hypoxia-induced ATP depletion in cultured astrocytes

Several σ receptor agonists and antagonists have been reported to have neuroprotective actions in both in vitro and in vivo models [18–21]. This led us to study the protective effects of BHDP against 24 h hypoxia-induced ATP depletion in cultures of astrocytes. Two degrees of hypoxia were studied. In a first series of experiments, a 48% decrease in ATP was observed following a 24 h hypoxia. BHDP prevented the fall in ATP levels in a concentration-dependent manner. The prevention was significant at 1 μM and at 10 μM the protective effect was complete (Table 3). When a more severe hypoxia was performed (decrease in ATP level of 62%), the preventing effect of BHDP still persisted (Table 3).

4. Discussion

This study was undertaken to characterize a novel σ receptor ligand, BHDP, in receptor binding studies. This was performed on rat brain synaptosomal membranes and liver mitochondria as we identified recently the existence of σ receptors on the outer membranes of liver mitochondria [7].

We demonstrated that [^{3}H]BHDP combines both high affinity and high selectivity for σ receptors. [^{3}H]BHDP labelled one class of high affinity binding sites in liver mitochondria and in synaptic membranes with similar K_d values of 2–3 nM. σ Reference compounds such as haloperidol, NE-100, (±)pentazocine, ifenprodil, carbetapentane, (±)3-PPP exhibited high affinity for [^{3}H]BHDP binding sites, haloperidol being the most potent. The rank order of potency of these σ reference compounds was identical in the two preparations and similar to that observed for σ1 receptors in previous studies [3–5].

On the other hand, [^{3}H]BHDP binding was inhibited by progesterone which is considered as a putative endogenous ligand for σ1 receptors. Taken together, these data suggested that [^{3}H]BHDP preferentially bound to the σ1 receptor subtype. This hypothesis was confirmed by means of displacement experiments where σ1 and σ2 receptors were labelled with [^{3}H](+)-pentazocine and [^{3}H]DTG, respectively. Indeed, the inhibitory potency of BHDP for the σ1 receptor subtype was about 100 times higher than that for the σ2 receptor subtype.

Numerous drugs were shown to label σ receptors but only a few of them (e.g. SA4503, [11]) display a real selectivity. The receptor profile of BHDP suggests that this compound has a similar behavior. Indeed, BHDP did not show any significant affinity for a number of receptors, ion channels and transporters tested. Thus, BHDP appears to be a potent and selective σ1 receptor ligand in binding experiments.

There is increasing evidence that σ receptors represent an interesting target for neuroprotection since various σ receptor ligands, specifically the σ1 subtype, have demonstrated robust neuroprotective properties in in vitro and animal models of cerebral ischemia. Several mechanisms have been postulated to explain their neuroprotective effects and, up today, they remained controversial. These mechanisms include inhibition of presynaptic glutamate release [22], buffering of postsynaptic glutamate-evoked Ca^{2+} influx [23], prevention of the increase in nitric oxide synthase activity [18,24] and inhibition of dopamine release [25]. Given that BHDP demonstrated both high affinity and selectivity for σ1 receptors in binding experiments, we postulated that this drug might possess cytoprotective properties. Therefore, we tested this hypothesis in a model of cultured astrocytes subjected to an hypoxia which induced a severe depletion of ATP. This in vitro test was choosen first because ATP level is a good index of the ability of a cell to recover and secondly because we previously demonstrated the presence of σ receptor in mitochondria [7] which is the major provider of ATP in the cell. The results indicate that BHDP prevents ATP depletion in a concentration-dependent manner and that a complete recovery is obtained at 10 μM. These results can be compared to those obtained with the σ1 receptor agonists (+)-pentazocine and SA4503 which suppressed hypoxia/hypoglycemia neurotoxicity in rat primary neuronal cultures [20].

Although the mechanism of action of BHDP cannot be defined from this particular experiment, these data reinforce the hypothesis that σ ligands are able to mediate cytoprotective effects through σ receptors.

In conclusion, BHDP binds specifically with high affinity to a single σ1 binding site in liver mitochondria and rat brain membranes. The preventing effect of the drug against hypoxia-induced ATP depletion suggests that BHDP is a potential cytoprotective agent which appears a promising tool to study σ1 receptors located in mitochondrial membranes.

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References

[1] Bowen, W.D. (2000) Pharm. Acta Helv. 74, 211–218.
[2] Quirion, R., Bowen, W.D., Itzahk, Y., Junien, J.L., Musacchio, J.M., Rothman, R.B., Su, T.P., Tam, S.W. and Taylor, D.C. (1992) Trends Pharmacol. Sci. 13, 85–86.
[3] DeHaven-Hudkins, D.L., Lanyon, L.F., Ford-Rice, F.Y. and Ator, M.A. (1994) Biochem. Pharmacol. 47, 1231–1239.
[4] Cagnotto, A., Bastone, A. and Mennini, T. (1994) Eur. J. Pharmacol. 266, 131–138.
[5] Mccann, D.J., Weissman, A.D. and Su, T.P. (1994) Synapse 17, 182–189.
[6] Alonso, G., Phan, V., Guillemain, I., Saunier, M., Legrand, A., Anoal, M. and Maurice, T. (2000) Neuroscience 97, 155–170.
[7] Klouz, A., Sapena, R., Liu, J., Maurice, T., Tillement, J.P., Papadopoulos, V. and Morin, D. (2002) Br. J. Pharmacol. 135, 1607–1615.
[8] Balijepalli, S., Kenchappa, R.S., Boyd, M.R. and Ravindranath, V. (2001) Neurochem. Int. 38, 425–435.
[9] Karbon, E.W., Patch, R.J., Pontecorvo, M.J. and Ferkany, J.W. (1990) Eur. J. Pharmacol. 176, 247–248.
[10] Whittemore, E.R., Ilyin, V.I. and Woodward, R.M. (1997) J. Pharmacol. Exp. Ther. 282, 326–338.
[11] Matsuno, K., Nakazawa, M., Okamoto, K., Kawashima, Y.A. and Mita, S. (1996) Eur. J. Pharmacol. 306, 271–279.
[12] Berardi, F., Ferorelli, S., Colabufo, N.A., Leopoldo, M., Perrone, R. and Tortorella, V. (2001) Bioorg. Med. Chem. 9, 1325–1335.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 93, 265–275.
[14] Booher, J. and Sensenbrenner, M. (1972) Neurobiology 1972, 97–105.
[15] Urien, S. (1995) Pharm. Res. 12, 1225–1230.
[16] Morin, D., Zini, R., Urien, S., Sapena, R. and Tillement, J.P. (1992) J. Receptor Res. 12, 369–387.
[17] Bowen, W.D., Hellewell, S.B. and McGarry, K.A. (1989) Eur. J. Pharmacol. 163, 309–318.
[18] O'Neill, M., Caldwell, M., Earley, B., Canney, M., O'Halloran, A., Kelly, J., Leonard, B.E. and Junien, J.L. (1995) Eur. J. Pharmacol. 283, 217–225.
[19] Maurice, T. and Lockhart, B.P. (1997) Prog. Neuropsychopharmacol. Biol. Psychiat. 21, 69–102.
[20] Nakazawa, M., Matsuno, K. and Mita, S. (1998) Neurochem. Int. 32, 337–343.
[21] Harukuni, I., Bhardwaj, A., Shaivitz, A.B., DeVries, A.C., London, E.D., Hurn, P.D., Traystman, R.J., Kirsch, J.R. and Faraci, F.M. (2000) Stroke 31, 976–982.
[22] Lobner, D. and Lipton, P. (1990) Neurosci. Lett. 117, 169–174.
[23] DeCoster, M.A., Klette, K.L., Knight, E.S. and Tortella, F.C. (1995) Brain Res. 671, 45–53.
[24] Lesage, A.S., De Loore, K.L., Peeters, L. and Leysen, J.E. (1995) Synapse 20, 156–164.
[25] Gonzalez-Alvear, G.M. and Werling, L.L. (1994) J. Pharmacol. Exp. Ther. 271, 212–219.