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Synthesis and characterization of [^{125}I]3′-(−)-iodopentazocine, a selective σ_1 receptor ligand

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Abstract

Pentazocine is a potent ligand at both opioid and σ receptors, but with opposite stereoselectivities. Whereas (−)-pentazocine has high affinity for a number of opioid receptors, (+)-pentazocine labels σ_1 receptors. Iodination of (−)-pentazocine at the 3′-position reverses its selectivity for opioid and σ_1 receptors. 3′-(−)-Iodopentazocine competes at σ_1 receptor binding sites with a K_i value of 8 nM, compared to approximately 40 nM for (−)-pentazocine. 3′-(−)-Iodopentazocine also has lost its affinity for opioid receptors. In contrast, iodination of (+)-pentazocine lowers its affinity at σ_1 receptors. Synthesis of [^{125}I]3′-(−)-iodopentazocine is readily performed with incorporations of up to 80%. Binding is of high affinity and shows the selectivity anticipated for a σ_1 receptor-selective ligand. Exposing membranes prebound with [^{125}I]3′-(−)-iodopentazocine to ultraviolet light can covalently couple the ligand into the membranes. Polyacrylamide gel electrophoresis reveals a major band at about 25 kDa and a minor one at about 20 kDa, indicating photolabeling of σ_1 receptors with minor incorporation into σ_2 sites.

Keywords: σ Receptor; Benzomorphan; Affinity label

1. Introduction

σ Receptors are an unusual class of receptors found in the brain and other tissues (Gundlach et al., 1986; Roman et al., 1988, 1989; Su and Wu, 1990; Dumont and Lemaire, 1991; Rogers and Lemaire, 1992; Walker et al., 1992; DeHaven-Hudkins et al., 1994; Hellewell et al., 1994; Su, 1991). Over the years a number of novel ligands have been developed which have enabled the classification of σ receptor subtypes (Bowen et al., 1989, 1990, 1992, 1993; Musacchio et al., 1989; Hellewell and Bowen, 1990; Itzhak et al., 1990, 1991; Musacchio, 1990; Itzhak and Stein, 1991; Rothman et al., 1991; Su, 1991; Wu et al., 1991; Zhou and Musacchio, 1991; Quirion et al., 1992). In addition to their presence in brain and peripheral tissues, σ receptor subtypes are expressed in a number of cell lines (Hellewell and Bowen, 1990; Wu et al., 1991; Vilner et al., 1995a,b). Their functional significance is only now being uncovered (Chavkin, 1990; Walker et al., 1990; Carr et al., 1992; Yoneda et al., 1992; Paul et al., 1993; Gonzalez-Alvear and Werling, 1994; Bastianetto et al., 1995; DeCoster et al., 1995; Hayashi et al., 1995; Kreeger et al., 1995; Liu et al., 1995; Okuyama et al., 1995; Shibata et al., 1995; Vilner et al., 1995a,b; Yamamoto et al., 1995). One of the more intriguing areas is the modulation of opioid analgesia by the σ_1 receptor (Chien and Pasternak, 1993, 1994, 1995a). In these studies, the selective σ_1 receptor drug (+)-pentazocine effectively blocks opioid analgesia without influencing morphine-induced inhibition of gastrointestinal transit. In contrast to (+)-pentazocine, which labels σ_1 receptors with high affinity, its (−)-isomer is a potent opioid which labels μ-opioid and

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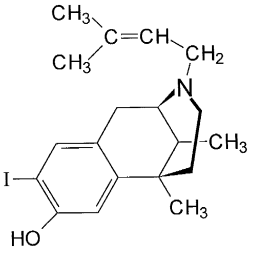

Fig. 1. Structure of 3′-iodopentazocine.

κ_1-opioid receptor binding sites up to 10-fold more potently than σ receptors (Chien and Pasternak, 1995b). A study of the effect of halogen substitution in the phenolic ring of pentazocine isomers revealed that iodine could be incorporated at the 3′-position of the (−)-isomer with retention of σ_1 receptor affinity (Danso-Danquah et al., 1995). We now present evidence for the development of a highly selective σ_1 ^{125}I radioligand based on (−)-pentazocine. While other radioiodinated σ ligands have been described previously (John et al., 1994a,b; Kimes et al., 1992; Kahoun and Ruoho, 1992), this is, to our knowledge, the first radioiodinated benzomorphan-based probe for σ sites (Fig. 1).

2. Materials and methods

2.1. Materials

[^{125}I]NaI (1680 Ci/mmol) was purchased from Dupont-NEN (Boston, MA, USA). (+)-Pentazocine and (−)-pentazocine were generous gifts from Sanofi-Winthrop (New York, NY, USA). (±)-SKF10,047, (+)-SKF10,047, (−)-SKF10,047, (+)-cyclazocine and (−)-cyclazocine were generous gifts from the Research Technology Branch of the National Institute on Drug Abuse. Haloperidol, (−)-sulpiride, sodium metabisulfite and chloramine T were purchased from Sigma (St. Louis, MO, USA). 1,3-Di(2-tolyl)-guanidine (DTG) was purchased from Research Biochemicals International (Natick, MA, USA).

2.2. Synthesis of [^{125}I]3′-(−)-iodopentazocine

(−)-Pentazocine was reacted with an equimolar amount of [^{125}I]NaI at room temperature by adding a 10-fold excess of chloramine T and the iodination was terminated with 25-fold excess of sodium metabisulfite. The product was separated from unreacted [^{125}I]NaI using a reverse phase (C_{18})-Sep-Pak. After putting the aqueous reaction mixture over the Sep-Pak and washing with distilled water, the product was eluted with ethanol. [^{125}I]3′-(−)-Iodopentazocine was then purified using silica gel thin layer chromatography with chloroform/methanol/ammonium hydroxide (90:10:0.5).

2.3. Tissue preparation

BE(2)-C neuroblastoma cells (Ciccarone et al., 1989) were maintained in tissue culture flasks in a 1:1 mixture of Eagle's minimum essential medium with nonessential amino acids and Ham's nutrient mixture F-12 and supplemented with 10% fetal bovine serum. Cells were grown in a 6% CO_2-94% air humidified atmosphere at 37°C. Plates of cells were used at 75–95% confluence. Cells were harvested and membranes were prepared as previously described (Standifer et al., 1994). Rat liver membranes were prepared as previously described (Hellewell et al., 1994).

2.4. [^{3}H](+)-Pentazocine and [^{125}I]3′-(−)-iodopentazocine binding

All assays were performed at 37°C in 1 ml potassium phosphate buffer (10 mM; pH 7.2) using BE(2)-C cell or rat liver membrane preparations (0.1–0.2 mg protein/ml) with [^{3}H](+)-pentazocine (0.5 nM) or [^{125}I]3′-iodopen-

Table 1
Competition of σ and opioid receptor binding by pentazocine and iodopentazocine stereoisomers

Radioligand	K_i value (nM)			
	(−)-Pentazocine	3′-(−)-Iodopentazocine	(+)-Pentazocine	3′-(+)-Iodopentazocine
[^{3}H](+)-Pentazocine (σ_1)	37.6 ± 4.6	8.2 ± 1.8	1.8 ± 0.5	28.9 ± 3.0
[^{125}I]3′-(−)-Iodopentazocine (σ_1)	47.3 ± 20.9	8.2 ± 1.7	4.1 ± 2.9	32.8 ± 2.1
[^{3}H]DTG (σ_2)	36.5 ± 3.6	94.1 ± 14.4	> 1000	233 ± 3
[^{3}H]DADLE (μ_1)	4.0 ± 0.1	> 1000	760 ± 200	> 1000
[^{3}H]DAMGO (μ_2)	12.4 ± 0.6	> 1000	> 1000	> 1000
[^{3}H]DPDPE (δ)	58.7 ± 7.8	> 1000	> 10000	> 10000
[^{3}H]U50,488H (κ_1)	3.4 ± 0.1	485 ± 210	75.5 ± 9.9	746 ± 21
[^{3}H]NalBzoH (κ_3)	31.6 ± 1.5	> 1000	> 1000	> 1000

Binding was performed with the indicated radioligand and the IC_{50} values determined. K_i values were then calculated and presented as the means ± S.E.M. of a minimum of three determinations. σ Receptor binding was determined in the BE(2)-C cell line; μ-opioid receptor binding in calf thalamus; δ-opioid receptor binding in calf frontal cortex; κ_1-opioid receptor binding in guinea pig cerebellum; and κ_3-opioid receptor binding in calf striatum.

tazocine (2 nM) for 2 h, unless otherwise stated. Non-specific binding was determined in the presence of haloperidol (1 μM). After the incubation, tissue was diluted with 3 ml ice-cold Tris buffer (5 mM; pH 7.7) and filtered with a Brandel cell harvester (Cambridge, MA, USA) over No. 32 glass-fiber filters (Schleicher & Schuell, Keene, NH, USA) which has been soaked in 0.3% polyethyleneimine solution for 30 min at 25°C beforehand. Filters were washed with 5 ml of buffer twice and placed in counting tubes for radioactivity determination. Specific binding was determined by subtracting the binding in the presence of haloperidol from the binding in its absence. All determinations within an experiment were performed in triplicate and replicated three times. All points within each experiment were performed in triplicate, with a typical variability of less than 5%, and each experiment replicated at least three times, unless otherwise noted.

2.5. Opioid receptor binding assays

Opioid receptor binding was performed at 25°C, as reported previously (Clark et al., 1989). Calf thalamic membranes were used for μ_1- and μ_2-opioid receptor binding assays, calf striatal membranes for κ_3-opioid receptor binding assays, calf frontal cortical membranes for δ-opioid receptor binding assays and guinea pig cerebellar membranes for κ_1-opioid receptor binding. For μ_1-opioid receptor binding assays, [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADLE, 0.7 nM) was used in the presence of $MgSO_4$ (5 mM) and [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 10 nM) to block δ binding. μ_2-Opioid receptor binding studies utilized [^{3}H][D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO, 1.5 nM) with $MgSO_4$ (5 mM) and [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET, 5 nM) to block μ_1-opioid receptor binding. δ-Opioid receptor binding was measured with [^{3}H]DPDPE (1.5 nM) and κ_1-opioid receptor binding with [^{3}H]U69,593 (1.5 nM). Finally, [^{3}H]naloxone benzoylhydrazone (NalBzoH, 1 nM) in the presence of K_2EDTA (5 mM) was used to measure κ_3-opioid receptor binding.

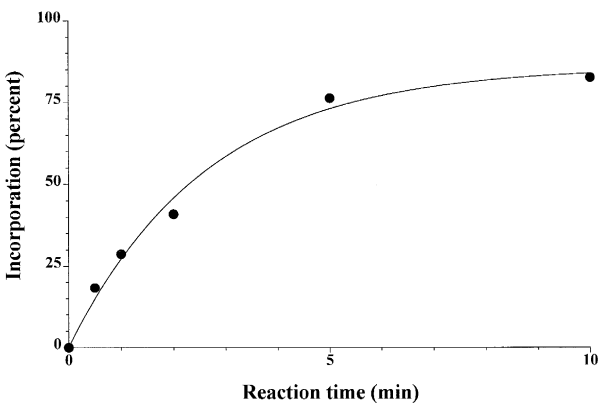

Fig. 2. Kinetics of ^{125}I incorporation into (−)-pentazocine. (−)-Pentazocine was reacted with Na^{125}I for the indicated time and the amount of incorporation determined.

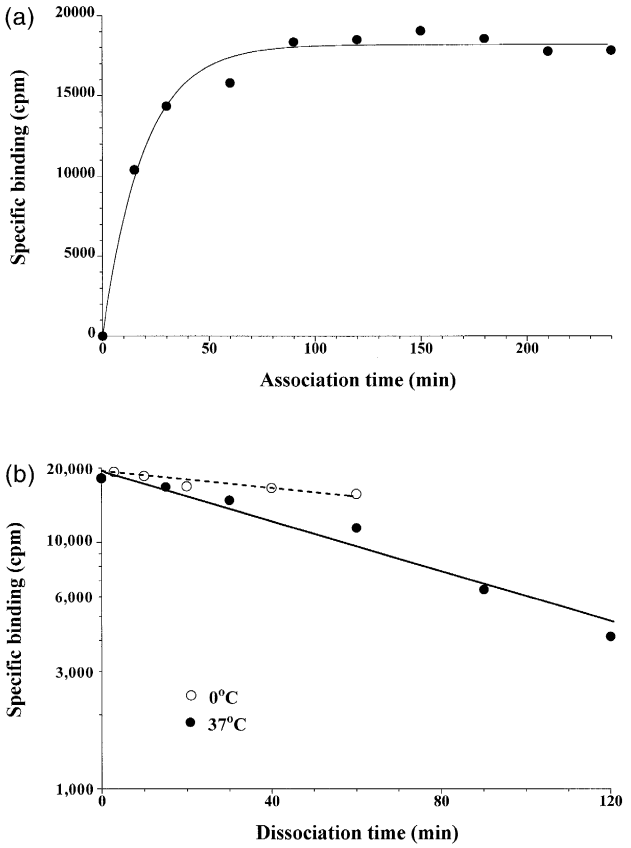

Fig. 3. Kinetics of [^{125}I]3′-(−)-iodopentazocine binding. (a) The association rate for [^{125}I]3′-(−)-iodopentazocine was determined by incubating the radioligand (2 nM) with tissue for the indicated time at 37°C. Only specific binding is reported. (b) Dissociation of [^{125}I]3′-(−)-iodopentazocine was evaluated by prebinding the ligand at either 0 or 37°C and adding haloperidol (1 μM) at time 0. Only specific binding is reported.

Nonspecific binding was determined in the presence of levallorphan (1 μM). All determinations were performed in triplicate and replicated three times.

2.6. Photoaffinity labeling

BE(2)C cell membranes or rat liver membranes (2 mg/ml) were incubated with 0.5 nM [^{125}I]3′-(−)-iodopentazocine at 37°C in 10 mM potassium phosphate buffer (pH 7.2) for 2 h. Non-specific binding was determined in the presence of haloperidol (1 μM). At the end of incubation, the membranes were centrifuged (49 000 × g for 20 min). Tissues were resuspended in 40% of their original volume potassium phosphate buffer (10 mM; pH 7.2) and exposed to ultraviolet radiation (254 nm; 1×10^6 microjoules/cm^2) in a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA, USA) in a shallow dish. The treated membranes were collected by centrifugation and the pellets were washed by resuspending them in 20% of the original volume of Tris buffer (50 mM; pH 7.7) with haloperidol (10 μM) to prevent reassociation of free radioligand and by centrifuging them again. The pellets then were resus-

pended in 50 mM Tris (pH 7.7, 30 mg wet weight tissue/ml). After determining the protein concentration (Lowry et al., 1951) an aliquot was boiled for 5 min with concentrated sample buffer (50 mM Tris, pH 6.8), containing SDS (3%), β-mercaptoethanol (4%) and bromphenol blue (0.01%) before diluted 2-fold before running on a polyacrylamide 5–20% gradient gel. After electrophoresis, the gel was exposed to Kodak film for 24 h and the apparent molecular mass was calculated.

3. Results

3.1. Binding selectivity of 3′-iodopentazocine

First, we examined the stereospecificity of pentazocine and 3′-iodopentazocine (Table 1). (+)-Pentazocine is a potent σ receptor ligand, competing [^{3}H](+)-pentazocine binding with a K_i value of approximately 2 nM while lacking appreciable affinity at opioid receptors. In contrast,

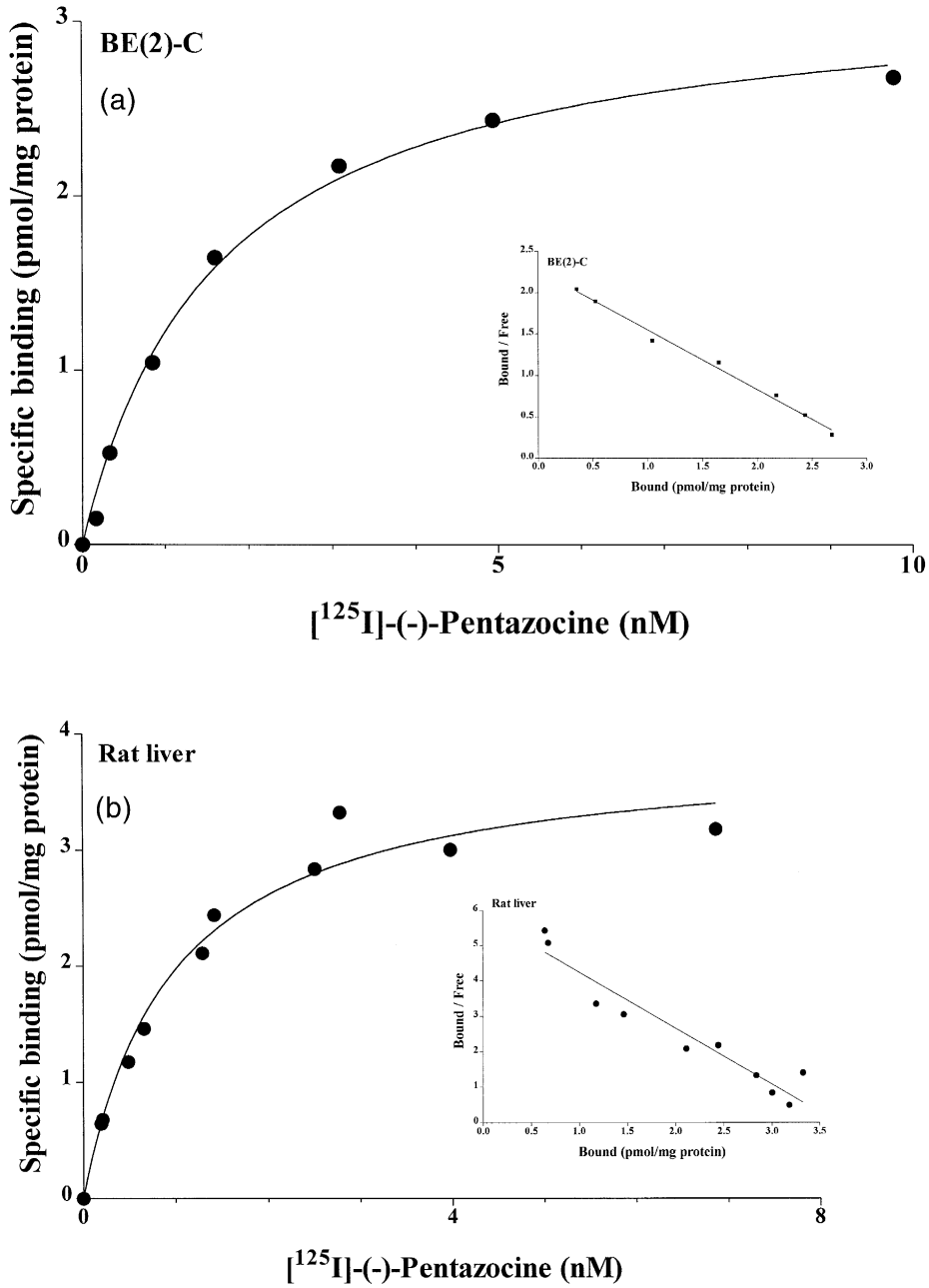

Fig. 4. Saturation analysis of [^{125}I]3′-(−)-iodopentazocine binding in rat liver and BE(2)-C membranes. (a) Rat liver membranes were incubated with a range of concentrations of [^{125}I]3′-(−)-iodopentazocine, as shown. Only specific binding is shown. Nonlinear regression analysis fit the data best to a straight line, yielding a K_d of 0.67 ± 0.12 nM and B_{max} of 3.85 ± 42 pmol/mg membrane protein. The Scatchard plot of the saturation curve is given in the inset. (b) BE(2)-C membranes were incubated with a range of concentrations of [^{125}I]3′-(−)-iodopentazocine, as shown. Only specific binding is shown. Nonlinear regression analysis fit the data best to a straight line, yielding a K_d of 2.12 ± 0.55 nM and B_{max} of 2.52 ± 0.42 pmol/mg membrane protein. The Scatchard plot of the saturation curve is given in the inset.

(−)-pentazocine lowers μ- and κ_1-opioid receptor binding quite potently while having far lower affinity at σ receptors (K_i value approximately 40 nM). After iodination, the stereoselectivity of (−)-pentazocine is reversed. The σ_1 receptor affinity of (−)-iodopentazocine is increased 5-fold while its ability to compete -opioid receptor binding is dramatically lowered. (−)-Iodopentazocine also has lower affinity for σ_2 sites than (−)-pentazocine (F.I. Carroll and W. Bowen, personal communication). In contrast, iodination of (+)-pentazocine lowers its affinity for σ_1 receptors. Thus, iodination reverses the stereoselectivity of pentazocine for σ receptors.

3.2. Synthesis of [^{125}I]3′-(−)-iodopentazocine

Pentazocine is readily iodinated using chloramine T. The rate of incorporation is relatively rapid, reaching a plateau of approximately 80% within 5 min at room temperature (Fig. 2). Extending the time to 10 min does not significantly enhance the incorporation. The reaction mixture after the iodination contains both [^{125}I]3′-(−)-iodopentazocine and unreacted (−)-pentazocine in a 4:1 ratio. The far lower concentration of (−)-pentazocine coupled with its lower affinity for σ receptors compared to [^{125}I]3′-(−)-iodopentazocine suggests that its presence should not interfere with [^{125}I]3′-(−)-iodopentazocine binding. When examined, the binding of [^{125}I]3′-(−)-iodopentazocine eluted from the Sep-Pak with ethanol is the same as purified [^{125}I]3′-(−)-iodopentazocine (data not shown). [^{125}I]3′-(+)-Iodopentazocine shows no specific binding in these assays (data not shown).

3.3. [^{125}I]3′-(−)-Iodopentazocine binding

Specific [^{125}I]3′-(−)-iodopentazocine binding is rapid at 37°C, reaching steady-state levels within 90 min in both BE(2)-C cell membranes (Fig. 3a) and liver membranes (data not shown). Dissociation of the bound radioligand from the BE(2)-C membranes is slow (Fig. 3b), with a loss of only 20% of binding after 1 h at 0°C (Fig. 3b). Saturation studies with [^{125}I]3′-(−)-iodopentazocine (Fig. 4) reveal linear Scatchard plots using either BE(2)-C cell membranes (K_d of 2.1 ± 0.55 nM; B_{max} 2.52 ± 0.42 pmol/mg protein) or rat liver membranes (K_d 0.67 ± 0.12 nM; B_{max} 3.85 ± 0.42 pmol/mg protein).

The competition studies against [^{3}H](+)-pentazocine indicate that (−)-iodopentazocine has high affinity for σ_1 receptors. However, (−)-iodopentazocine still might label additional sites other than σ receptors. Competition studies with [^{125}I]3′-(−)-iodopentazocine confirm the selectivity of this ligand for σ_1 sites (Table 2). The K_i values seen in liver membranes are quite similar to those reported in the literature for σ_1 receptors (Ross, 1991; Hellewell et al., 1994) and to those observed with [^{3}H](+)-pentazocine by our group (J. Ryan-Moro, C.-C. Chien and G.W. Pasternak, unpublished observations). We also see significant σ_1 receptor binding in BE(2)-C cells. The selectivity profile from the competition studies is consistent with σ_1 receptors, but the affinities in this human neuroblastoma cell line are somewhat lower than those seen in rat liver membranes. Hill coefficients for these competition studies do not vary significantly from unity, consistent with a single site. Equally important, the σ receptor ligands compete all specific binding.

Table 2
K_i values of a variety of compounds against [^{125}I]3′-(−)-iodopentazocine binding

Competitor	K_i value (nM)	
	BE(2)-C	Rat liver
(+)-Pentazocine	5.37 ± 1.9	1.47 ± 0.12
(−)-Pentazocine	56.4 ± 13.8	10.5 ± 0.9
(+)-SKF10,047	128 ± 4.4	37.4 ± 3.5
(−)-SKF10,047	2400 ± 164	293 ± 8.9
(±)-Cyclazocine	227 ± 19	
(+)-Cyclazocine	32.1 ± 2.4	12.9 ± 1.9
(−)-Cyclazocine	753 ± 3	108 ± 41
DTG	57.4 ± 11.6	9.36 ± 1.9
Haloperidol	1.38 ± 0.5	0.42 ± 0.03
(+)-3-PPP	82.4 ± 15.3	54.6 ± 4.3
MK801	> 10000	> 10000
Diprenorphine	> 10000	> 10000
(−)-Sulpiride	> 10000	> 10000

Each of the indicated compounds was tested against [^{125}I]3′-(−)-iodopentazocine binding in competition studies using either BE(2)-C cells or rat liver and their K_i values determined. Results are the means ± S.E.M. of at least three determinations.

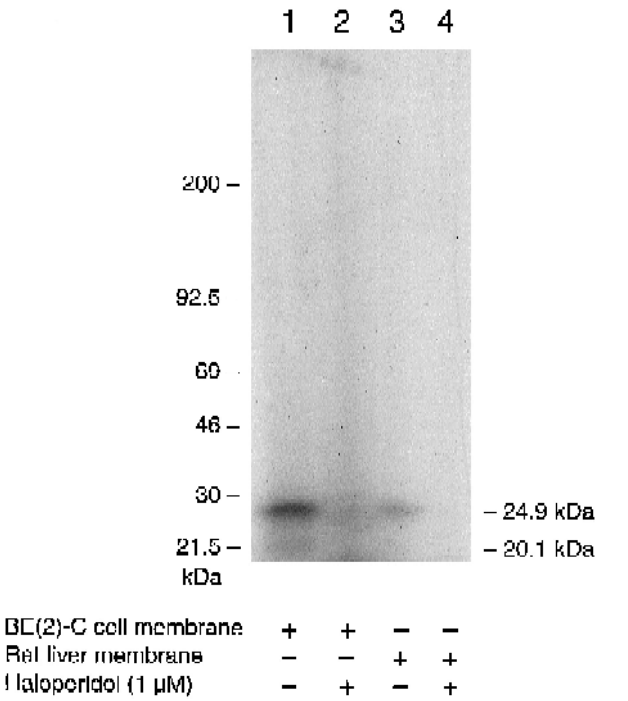

Fig. 5. Affinity-labeled σ receptors from BE(2)-C and rat liver membranes. Tissue was affinity labeled with [^{125}I]3′-(−)-iodopentazocine in the absence and presence of haloperidol (1 μM), as described in Section 2.

3.4. Affinity labeling σ_1 receptors with [^{125}I]3′-(−)-iodopentazocine

Exposing BE(2)-C membranes prebound with [^{125}I]3′-(−)-iodopentazocine to ultraviolet light can covalently couple the ligand to membrane proteins. Typically, up to 35% of the specifically labeled material is coupled. When run on a sodium docecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and exposed to film, we see two bands (Fig. 5). A major band is observed at approximately 25 kDa, similar to previous reports (Kavanaugh et al., 1988; Hellewell and Bowen, 1990; Kahoun and Ruoho, 1992; Schuster et al., 1995; Hellewell et al., 1994), while a less intense band of approximately 20 kDa also is seen on the gel. The 25 kDa band is consistent with photolabelling of σ_1 sites, whereas the 20 kDa band probably represents some labeling of σ_2 sites (Hellewell and Bowen, 1990).

4. Discussion

Although many drugs display high affinity for both σ and opioid receptors, they often demonstrate opposite stereoselectivities for the two families of receptors. For example, (+)-pentazocine potently labels σ_1 sites without appreciable affinity at any classes of opioid receptors while its (−)-isomer is a potent opioid with far lower affinity at σ_1 receptors. Placement of an iodine at the 3′-position of pentazocine reverses its stereoselectivity for σ_1 receptors. This substitution increases the affinity of the (−)-isomer approximately 5-fold while decreasing the affinity of the (+)-isomer approximately 10-fold. The placement of the iodine at this position also dramatically lowers the affinity of (−)-pentazocine for opioid receptors in competition studies.

[^{125}I]3′-(−)-Iodopentazocine demonstrates high affinity and selectivity for σ_1 receptors and gives K_i values similar to those observed with [^{3}H](+)-pentazocine. Competition studies also suggest that it labels only a single site. The binding in the BE(2)-C cells is interesting. This neuroblastoma cell line has a wide variety of neurotransmitter receptors, including μ-, δ- and κ_3-opioid receptors. We now document the presence of σ_1 receptors in this cell line as well. The affinities of many of the agents examined are similar in the BE(2)-C and rat liver membranes. However, several ligands appear to differ by as much as 10-fold, particularly the (−)-isomers of the benzomorphans. It will be interesting to assess whether the σ receptors in these two tissues have other distinguishing characteristics as well.

[^{125}I]3′-(−)-Iodopentazocine also has utility as an affinity ligand. Exposure to strong ultraviolet light enhances the covalent coupling of the agent to membranes. SDS-PAGE yields molecular weights similar to those previously observed for σ_1 sites (Kavanaugh et al., 1988; Hellewell and Bowen, 1990; Kahoun and Ruoho, 1992; Schuster et al., 1995; Hellewell et al., 1994). However, [^{125}I]3′-(−)-iodopentazocine has many advantages over traditional affinity labels. First, the high specific activity and the ability to detect the radioactivity without scintillation fluors has a number of technical advantages. Second, the compound is readily synthesized at a high yield. The use of [^{125}I]3′-(−)-iodopentazocine may facilitate the purification and characterization of σ receptors.

In conclusion, we have developed a simple and rapid synthesis of a potent benzomorphan-based σ_1 receptor ^{125}I radioligand with a high yield. ^{125}I ligands have significant advantages, such as their high specific activity and the ability to detect the isotope directly without scintillation counting. Together, these properties offer significant advantages in the biochemical characterization of σ binding sites.

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