Is the σ_2 Receptor a Histone Binding Protein?

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Received January 18, 2006

Starting from the high affinity σ_2 receptor ligand **2**, (PB28), we synthesized amino derivative **4** and coupled it to an NHS-ester activated sepharose stationary phase column to elute a crude protein prepared by lysed human SK-N-SH neuroblastoma cells. We characterized the SDS−PAGE gel electrophoresis stained bands by MALDI-MS and LC-MS-MS analysis. The MASCOT MS-MS ion search program led to the identification of the protein components. The six eluted proteins had a molecular weight ranging from 13 kDa to 26 kDa and were human histone proteins. A human 40S ribosomal protein S3 (SwissProt accession number: P23396) was also identified as a comigrated band. The human histone proteins that were characterized were H3.3A histone (NCBI accession number: 51859376), H2B histone (NCBI accession number: 1568557), H2A.5 histone (NCBI accession number: 70686), H1 (NCBI accession number: 22770677), and H2.1 histone (SwissProt accession number: P16403). These results disclosed a dual hypothesis about the σ_2 receptor, that is, that it is formed by histones or that the σ_2 ligands also bind histone proteins.

Introduction

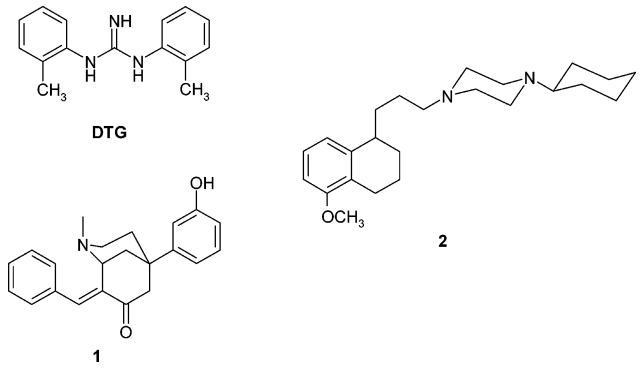
Sigma (σ) receptors are classified in σ_1 and σ_2 subtypes[1] and are localized in different tissues, including the central (CNS) and peripheral nervous systems.[2] In the CNS, these receptors are involved in the modulation of neurotransmitter release, in memory and cognitive processes, and in locomotor activity, whereas their role in the peripheral nervous system and their signal transduction have to be clarified.[3−5] Moreover, σ_1 and σ_2 receptor protein expression in normal tissues is lower than that in the corresponding tumor tissues.[6,7] To date, the σ_1 receptor has been cloned,[8] whereas σ_2 subtype isolation and characterization have not been carried out yet.

Considering σ_2 receptor overexpression in several tumor tissues,[9,10] potent and selective σ_2 ligands could be employed as radiotracers to label tumor cells by diagnostic imaging techniques such as PET[11,12] or SPECT.[13,14] Although the diagnostic potential of σ_2 receptor agents has been suggested, the physiological role of this receptor and the mechanism of overexpression induction in tumor cells need to be elucidated.

Even though several questions need to be clarified, σ_2 receptor ligands can be considered a tool for cancer diagnosis[15] and a novel pharmacological perspective in cancer therapy.[16,17] To date, the endogenous ligand for the σ_2 receptor, if it exists, is unknown, and the signal pathway involved in receptor activation has to be discovered. Furthermore, the best known σ_2 receptor ligands, such as DTG (1,3-di-2-tolylguanidine) and (+)-(1*R*,5*R*)-(*E*)-8-benzilidene-5-(3-hydroxyphenyl)-2azabiciclo[3.3.1]nonan-7-one (**1**, CB64 D)[18] (Chart 1), display poor selectivity toward the σ_1 subtype. A great contribution to better understand the σ_2 physiological role and the corresponding biochemical pathway could be made by protein isolation and characterization.

Recently, we developed several σ ligands,[19−21] and among them, 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine (**2**, PB28) (Chart 1) displayed high σ_2 receptor affinity and potent agonist activity.[22] We also demonstrated that compound **2** induced caspase and p53 independent apoptosis and modulated P-glycoprotein expression in the MCF-7/Adr adriamycin-resistant cell line partially reverting the MDR level.[23] Similarly, compound **2** determined the same effect in human SK-N-SH neuroblastoma cell line. As previously demonstrated, in this cell line, the σ_2 receptors were overexpressed, whereas the σ_1 receptors were found in low-affinity state so that the human SK-N-SH neuroblastoma could be considered a specific in vitro model to evaluate drug activity at the σ_2 receptor.[17]

Chart 1. Potent and Selective σ_2 Receptor Ligands



Considering that the human SK-N-SH neuroblastoma is a selective biological matrix for σ_2 receptors and compound **2** is the most potent and selective σ_2 receptor agonist studied we developed an affinity chromatography method to isolate the σ_2 receptor protein by an interaction with a σ_2 specific selector linked to the stationary phase. Because compound **2** did not have the structural requirements to couple the stationary phase, we remodeled it to compound **4**, introducing an amino group on the aromatic ring to link the column-packed stationary phase by covalent bond (Scheme 1). The amino group was inserted in the position where, as demonstrated by previous SAFIR (structure−affinity relationship) studies,[21] a substituent introduction on the tetralin moiety should not affect σ_2 receptor affinity and activity. The linkage to the stationary phase was monitored by UV analysis through the production of *N*-hydroxysuccinimide (NHS) as the leaving group, and the human SK-N-SH neuroblastoma protein suspension was eluted across the supported stationary phase. The proteins separated by elution through the column were monitored by SDS−PAGE gel electrophoresis, and the resultant bands were characterized using MALDI-MS and LC-MS-MS analyses.

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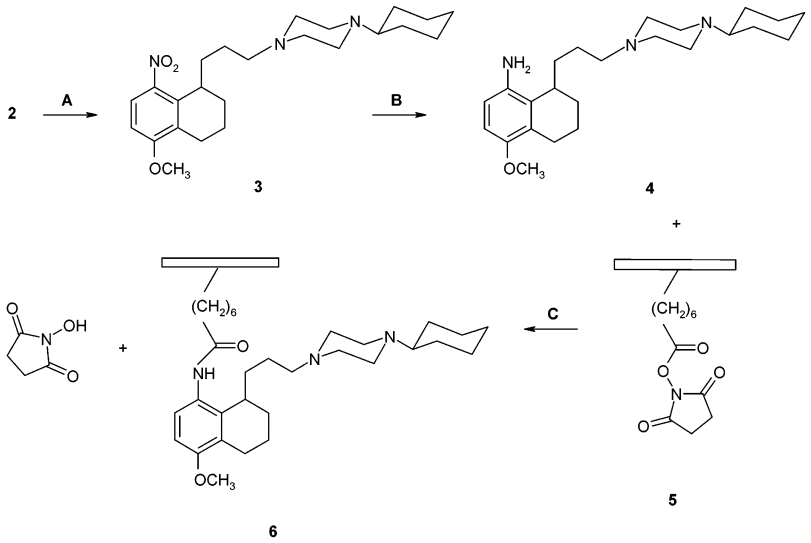
10.1021/jm0600592 CCC: $33.50
Published on Web 06/10/2006

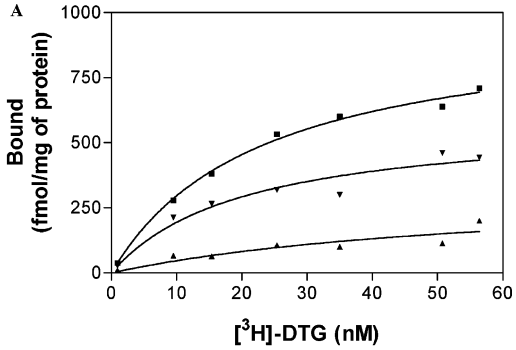
Scheme 1[a]



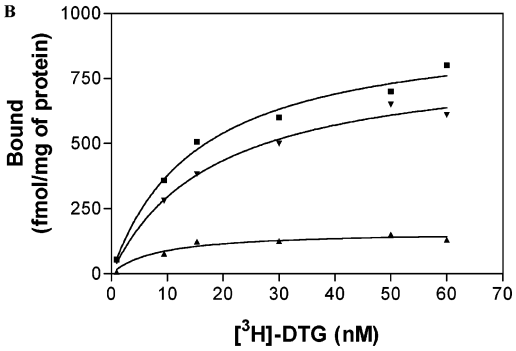
[a] Reagents: (A) NO_2BF_4; (B) $SnCl_2$, HCl; (C) 0.2 M $NaHCO_3$ and 0.5 M NaCl buffer at pH 8.3.

Chemistry. Compound **4** was synthesized as depicted in Scheme 1. The nitration of compound **2** by nitronium tetrafluoroborate (NO_2BF_4) afforded a mixture containing 1-cyclohexyl-4-[3-(5-methoxy-8-nitro-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]-piperazine (**3**), along with the 6-nitro- and 6,8-dinitro analogues.[24] Flash chromatography of the mixture afforded purified compound **3**, which was reduced by $SnCl_2$ in HCl to give the corresponding amine derivative **4**. This latter compound was used for the coupling with the activated sepharose column **5**. The stationary phase **5** was perfused with compound **4** dissolved in a specific coupling buffer. The nucleofilic substitution afforded the functionalized stationary phase **6**, producing NHS as a leaving group, which was eluted and monitored by UV spectroscopy. The coupling yield calculated was 80%. The concentration of compound **4** remaining in the column corresponded to a 10% excess of the stationary phase active sites described in the technical sheet. For compound **4**, the UV spectroscopic properties were $\lambda_{max} = 290$ nm, $\epsilon = 2140$ in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl at pH 8.3), and $\epsilon = 420$ in ethanol.

Biological Materials and Methods. RPMI 1640 medium, trypsin-EDTA, penicillin (10 000 U/mL) streptomycin (10 mg/mL), L-glutamine solution (100×), fetal calf serum, non essential amino acid (100×), and sodium pyruvate (100 mM) culture flasks and petri dishes were from Corning Glassworks (Corning, NY). The radioligand [^{3}H]-DTG (58.10 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Zavantem, Belgium). Compound **2** dihydrochloride was synthesized in our laboratories. DTG was purchased from Tocris Cookson Ltd., U.K. The NHS HiTrap column was purchased from Amersham Bioscience (Freiburg, Germany). The human SK-N-SH neuroblastoma cells were lysed,[6] and a protein membrane preparation was obtained. This biological sample was injected into the affinity chromatography column, and the proteins were eluted using an appropriate buffer. The eluted proteins were alkalinized at pH 8.0 and monitored by SDS−PAGE gel electrophoresis (Figure 2). The SDS−PAGE fractionation gave rise to a protein pattern from which several bands with an apparent mobility ranging from 13 to15 kDa and from 30 to35 kDa were excised. In detail, three bands at 15 kDa (15A, 15B, and 15C, from higher to lower molecular weight, respectively), and two at 32 kDa (32A and 32B, from higher to lower molecular weight, respectively) were digested by an in situ hydrolysis procedure. Each peptide mixture was then directly analyzed by MALDI-MS.



[a]$K_d = 20.3 \pm 2.5$ nM; $B_{max} = 588 \pm 50$ fmol/mg of protein



[a]$K_d = 18.2 \pm 2.4$ nM; $B_{max} = 830 \pm 75$ fmol/mg of protein

Figure 1. Representative saturation curves for [^{3}H]-DTG at σ_2 receptors in eluted membrane preparation (A) and in protein nuclear fraction (B**)** of the SK-N-SH cells; (■) total binding; (▼) specific binding; (▲) nonspecific binding. [a]K_d (nM) and B_{max} (fmol/mg of protein) were obtained by specific binding curves ($P < 0.0001$). K_d (nM) and B_{max} values are the means ± sem from three experiments in triplicate. Groups of data were compared with the analysis of variance followed by Tukey's multiple comparison tests (total binding vs specific binding, total binding vs non specific binding, non specific vs specific binding; $P < 0.001$).

Results

The measured molecular mass values were used for searching in the nonredundant NCBI and/or SwissProt protein database

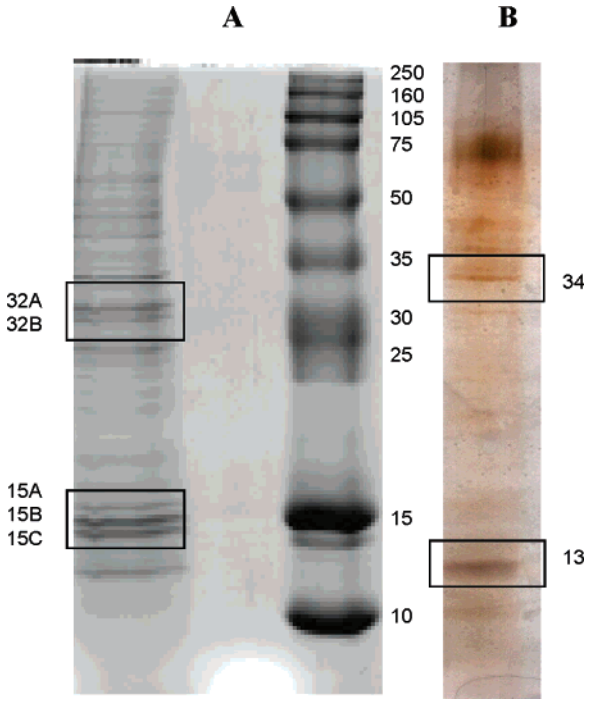


Figure 2. (A) SDS−PAGE gel electrophoresis of eluted proteins from coupled stationary phase (left) and biomarker proteins (right). (B) SDS−PAGE gel electrophoresis silver staining of eluted proteins from the uncoupled stationary phase at pH 3.0. The values refer to molecular weight expressed in kDa.

through MASCOT peptide mass fingerprint for protein identification. The peptide mass fingerprint strategy was useful for the identification of the 15 kDa proteins. Indeed, the protein named 15A was identified as human H3 histone (NCBI accession number: 51859316), 15B as human H2B histone (NCBI accession number: 1568557), and 15C as human H2A.5 histone (NCBI accession number: 70686). This approach was not useful for the 32 kDa protein identification because of several factors such as the comigration of several proteins. To characterize the unassigned protein gel bands, these samples were analyzed by LC-MS-MS. The eluate of the C_{18} reverse-phase chromatography was directly analyzed using the Q-ToF mass spectrometer, which was set to fragment on-line up to 3, coeluting the most intense peptides producing daughter ion spectra from which the sequence information on individual peptides was obtained. This information was then used to search for protein databases by the MASCOT MS-MS ion search program, leading to the identification of the protein components. In particular, the main component of the 32A protein band was histone H1 (NCBI accession number: 22770677), identified on the basis of the highest number of peptides matched (12 peptides sequenced). Similarly, protein band 32B showed as the main protein component the human 40S ribosomal protein S3 (SwissProt accession number: P23396) (12 peptides sequenced) and as the second component, on the basis of the number of matched peptides (6 peptides sequenced), human H2.1 histone (SwissProt accession number: P16403). The mass and amino acid composition for each analyzed peptide are listed in Table 1.

The eluted target proteins were desalted, lyophilized, and suspended in an incubation buffer for determining the specific binding by saturation analysis with [^{3}H]-DTG. As reported in Figure 1A, K_d (20.3 ± 2.5 nM) and B_{max} (588 ± 50 fmol/mg of protein) values were determined, and these findings were consistent with the corresponding values previously determined in SK-N-SH cell membrane preparation (K_d = 21.0 ± 2.0 nM and B_{max} = 656 ± 25 fmol/mg of protein).[17] Because histones constitute the protein nuclear fraction, we prepared a crude SK-N-SH protein nuclear fraction by centrifugation to verify the specific binding by saturation analysis with [^{3}H]-DTG. The curves are depicted in Figure 1B, and the K_d (18.2 ± 2.4 nM) and B_{max} (830 ± 75 fmol/mg of protein) values were consistent with the corresponding results found both in eluted proteins and in the crude SK-N-SH cell membrane preparation.

Table 1. Identification of Proteins Binding Activated Stationary Phase **6**

band	protein	mass (Da)	a.a.	NCBI identification	SwissProt accession number
15A[a]	histone H3.3A	15285	136	51859376	Q66I33
15B[a]	histone H2B	13775	125	1568557	Q93080
15C[a]	histone H2A.5	14053	129	70686	
32A[b]	histone H1	22565	226	22770677	P16401
32B[b]	histone H2.1	21234	212		P16403
	40S ribosomal protein S3	26688	243		P23396

[a] Analyzed by MALDI-MS. [b] Analyzed by LC-MS-MS.

Discussion

The affinity chromatography of the crude biological sample by neuroblastoma cells afforded the same stained proteins, as displayed in SDS−PAGE gel electrophoresis (Figure 2). To estimate the possible interaction between the uncoupled stationary phase and the proteins, we previously eluted the crude proteins through an uncoupled column using buffers with different pH (7.4 and 3.0). At pH 3.0, the bands with apparent molecular weight 13 kDa and 34 kDa were eluted by the uncoupled stationary phase (Figure 2B). Consequently, these aspecific binding proteins were also eluted together with specific binding proteins by the coupled stationary phase. For this reason, the proteins having an apparent mobility of 13 kDa and 34 kDa were considered aspecific bands.

Therefore, we considered for characterization only the stained proteins termed 15A, 15B, 15C, 32A, and 32B (Figure 2A) eluted by the coupled stationary phase. The 15A, 15B, and 15C MALDI-MS characterization revealed that the analyzed proteins are H3.3A, H2B, and H2A.5 human histones, respectively. The 32A and 32B stained proteins were analyzed by LC-MS-MS because of the comigration of several proteins, and 32B was constituted by two different proteins, human H2.1 histone and 40S ribosomal protein S3.

Saturation analysis with the radioligand displayed that the K_d value of [^{3}H]-DTG was the same as that determined using the crude sample protein of neuroblastoma cells (20.3 and 21.0 nM, respectively). To confirm these findings, we prepared the nuclear fraction by lysing neuroblastoma cells because the histones are the major protein component of chromatin. Also, in this case, the K_d value of [^{3}H]-DTG (18.2 nM) was comparable to the corresponding values found in the same analysis using eluted or crude proteins.

Using these findings, we disclosed two different hypotheses. The first was that σ_2 receptors are histone proteins, and the second was that σ_2 ligands also bind histone proteins, besides the σ_2 receptors.

The human histones are basic proteins classified in H2A, H2B, H3, H4, and H1 subtypes. The last one is not involved in the octameric structure of nucleosoma, which is constituted by two units of each histone. The human H1 histone is interposed between two nucleosoma, easing their packing.

It has been found that the nucleosoma histones undergo acetylation and deacetylation mediated by histone acetylases

(HATs) and deacetylases (HDACs). These processes influence the DNA accessibility to factors regulating replication, repair, and transcription. Furthermore, the inhibition of histone deacetylation constitutes a novel strategy in cancer therapy.[25,26] The evidence that σ_2 receptors are overexpressed in several tumor tissues[6] could be justified by histone deacetylation activity, which is higher in tumor cells that produces new lysine−NH_2 groups providing more sites for σ_2 ligand binding.[27] Some reported effects mediated by σ_2 receptor agonists, such as apoptosis[28,29] and P-glycoprotein downregulation,[23] are already explained with histone activity modulation.[30] Therefore, the present results led us to consider σ_2 receptors as histone proteins. However, σ_2 receptors could be demonstrated to be different from histones so that histone proteins would be just another binding site for σ_2 ligands. In fact, according to another hypothesis, σ_2 receptors would be localized in membrane lipid rafts and not in the nucleus of hepatocytes using [^{3}H]-DTG as a specific binding control in rat liver.[31]

The human 40S ribosomal protein S3 (rpS3) in the 32B band protein as comigrating with the human H2.1 histone poorly contributed to the corroboration of one of the two hypotheses mentioned above. The presence of rpS3 eluted in an affinity chromatography fraction, could be explained as originating from an aspecific bound, even if its involvement in a DNA damage processing was reported.[32,33] The enigma related to σ_2 receptor isolation and characterization could be elucidated using σ_2 selector ligands having different structural properties from that of our compound **4**. In this way, the histones that we isolated and identified could be confirmed to be actually σ_2 receptor proteins.

Experimental Section

Chemistry. Flash column chromatography was performed with 1:40 ICN silica gel at 60 Å (15−40 μm) as the stationary phase. The melting point was determined in an open capillary on a Gallenkamp electrothermal apparatus. Elemental analysis (C, H, N) was performed on an Eurovector Euro EA 3000 analyzer; the analytical results were within ±0.4% of the theoretical values for the formula given. ^{1}H NMR spectra were recorded at 300 MHz on a Mercury Varian spectrometer with $CDCl_3$ as the solvent. All values are reported in ppm (δ). The attribution of ^{1}H NMR signals for compound **3** was confirmed by ^{1}H NMR NOESY. The recording of mass spectra was done on an Agilent 6890-5973 MSD gas chromatograph/mass spectrometer and on an Agilent 1100 series LC-MSD trap system VL; only significant *m*/*z* peaks with the percentage of relative intensity in parentheses are reported. All spectra were in accordance with the assigned structures. The chemicals were from Aldrich or Acros and were used without further purification.

1-Cyclohexyl-4-[3-(5-methoxy-8-nitro-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine (3). Nitronium tetrafluoroborate NO_2BF_4 (5.8 mmol, 0.76 g) was added to a solution of **2** (4.78 mmol, 1.77 g) in CH_3CN (15 mL). The mixture was stirred at room temperature for 8 h. The solvent was removed under reduced pressure, and the crude residue was taken up with CH_2Cl_2 and washed with brine. The organic solution was dried (Na_2SO_4) and evaporated under reduced pressure to afford a mixture of the corresponding 8-nitro derivative **3** together with the 6-nitro- and 6,8-dinitro- derivatives. Purification through flash chromatography with AcOEt, MeOH (9:1), and NH_4OH (0.1%) as the eluent afforded the title compound in a 20% yield as a yellow semisolid: ^{1}H NMR δ 1.00−1.38 [m, 5H, cyclohexyl (C*H*H)$_5$], 1.40−2.00 [m, 13H, (C*H*$_2$)$_2$CH(C*H*$_2$)$_2$, cyclohexyl (CH*H*)$_5$], 2.40−2.50 (m, 3H, CHN and CH_2N), 2.62−2.98 (m, 11H, piperazine, benzyl CH and CH_2), 3.92 (s, 3H, OCH_3), 6.68 (d, 1H, J = 9.0 Hz, aromatic), 7.79 (d, 1H, J = 9.0 Hz, aromatic); GC-MS *m*/*z* 415 (M^+, 3), 398 (34), 202 (100), 181 (55).

8-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-4-methoxy-5,6,7,8-tetrahydronaphthalen-1-yl-amine (4). 1-Cyclohexyl-4-[3-(5-methoxy-8-nitro-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine (**3**) (0.81 mmol, 0.34 g) was dissolved in 95% EtOH (10 mL) and added in a dropwise manner to a solution of $SnCl_2$ (3.2 mmol, 0.61 g) in HCl (1.8 mL). Then the mixture was heated to reflux for 3 h. After cooling, 5 N NaOH was added and the alkaline solution was extracted with Et_2O (3 × 15 mL). The organic phase was washed with H_2O, dried (Na_2SO_4), and evaporated under reduced pressure to afford a brown oil, which was purified by flash chromatography with AcOEt/MeOH (4:1) as the eluent, affording compound **4** in 50% yield;^{1}H NMR δ 1.20−1.40 [m, 6H, cyclohexyl (CH$_2$)$_3$], 1.40−2.00 [m, 12H, (C*H*$_2$)$_2$CH(C*H*$_2$)$_2$, cyclohexyl (CH$_2$)$_2$], 2.20−2.40 (m, 3H, CHN and CH_2N), 2.54−2.76 (m, 11H, piperazine, benzyl CH and CH_2), 3.63−3.76 (s+m, 5H, OCH_3 and NH_2, D_2O exchanged), 6.57 (d, 1H, J = 8.2 Hz, aromatic), 6.75 (d, 1H, J = 8.2 Hz, aromatic); GC-MS *m*/*z* 387 (M^+ + 2, 3), 386 (M^+ + 1, 17), 385 (M^+, 61), 181 (100); ESI-MS *m*/*z* 386.3 (MH^+).

Stationary Phase Coupling Procedure. HiTrap NHS-activated column bears the *N*-hydroxy-succinimide (NHS) ester (**5**) attached to sepharose high performance via a six atom spacer arm. The active esters are stable in the absence of water. The HiTrap NHS-activated column is supplied in 100% 2-propanol to preserve its activity prior to coupling. A HiTrap NHS-activated 5 mL column was washed by a syringe with ice-cold 1 mM HCl (3 × 10 mL) at a flow rate of 1 mL/min. Compound **4** (50 μmol) was dissolved in a standard coupling buffer (5 mL) containing 0.2 M $NaHCO_3$ and 0.5 M NaCl at pH 8.3. The column was sealed, keeping it at 25 °C for 30 min. Then the excess groups, not coupled to compound **4**, were deactivated, and the nonspecifically bound ligand **4** was washed out using buffer A (0.5 M ethanolamine, 0.5 M NaCl at pH 8.3) and buffer B (0.1 M acetate, 0.5 M NaCl at pH 4.0). First, buffer A (3 × 10 mL), then buffer B (3 × 10 mL), and finally buffer A (3 × 10 mL) were injected. The column was stored at room temperature for 30 min and then washed with buffer B (3 × 10 mL), buffer A (3 × 10 mL), and finally with buffer B (3 × 10 mL). In this way, the column was made ready for affinity chromatography. After use, it was sealed in 0.05 M Na_2HPO_4 and 0.1% NaN_3 at pH 7.0 until the next experiment.

Measuring of Coupling Efficiency. After coupling, the coupling solution was washed out from the column with a coupling buffer (3 mL). Then a part of the resulting solution (1 mL) was mixed with 2 M glycine at pH 2.0 (1 mL). The absorbance of the obtained solution was determined at 280 nm. The coupling yield calculation was determined by $(A - B)/A \times 100$, where $A = A_{280}$ (coupling solution) × V (volume of NHS-activated column), and $B = A_{280}$ (post coupling wash after acidification) × V (volume postcolumn wash) × 2 (dilution when acidified).

Biology. Cell culture. The human SK-N-SH neuroblastoma cells were grown in RPMI 1640 medium with 10% heat inactivated fetal calf serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 at 37 °C.

Membrane Preparation. The membrane preparations from SK-N-SH cell lines were carried out as described by Vilner et al. with minor modifications.[6] Briefly, SK-N-SH cells were cultured to 80% confluence, the medium was removed, and the cells were rinsed in phosphate buffer saline (PBS). After detaching, the cells were suspended in ice-cold 10 mM Tris-HCl at pH 7.4 containing 0.32 M sucrose and homogenized in a Potter-Elvehjem homogenizer (Teflon pestle). The homogenate was centrifuged at 31 000*g* for 15 min at 4 °C, and the supernatant was discarded. The final pellet was resuspended in ice-cold 10 mM Tris-HCl at pH 7.4 and stored at −80 °C until use. The protein content was determined by the Lowry method.[34]

Nuclear Fraction Preparation from SK-N-SH Cells. The nuclear fraction was prepared as reported by Cagnotto et al.[35] Briefly, the SK-N-SH membranes were resuspended in ice-cold 10 mM Tris-HCl (pH 7.4) and centrifuged at 1000*g* for 10 min at 4 °C to obtain the nuclear fraction (NP1). The final pellet (NP1) and the supernatant were resuspended in ice-cold 10 mM Tris-HCl at

pH 7.4 and stored at −80 °C until use. The protein determination in NP1 and in the supernatant was carried out by the Lowry method.[34]

Saturation Binding Assay. The saturation binding experiments were carried out as described by Vilner et al. with minor modifications.[6] The σ_2 receptors in the SK-N-SH membranes, in the nuclear fraction (NP1), and in the NP1 supernatant were radiolabeled using [^{3}H]-DTG concentrations ranging from 1.0 to 150 nM. The samples containing 400 mg of membrane protein, radioligand, and 10 mM DTG to determine nonspecific binding and 1 mM (+)-pentazocine to mask σ_1 receptors were equilibrated in a final volume of 500 mL of 50 mM Tris-HCl at pH 8.0 at 25 °C for 120 min. Incubations were stopped by the addition of 5 mL of ice-cold buffer (50 mM Tris-HCl at pH 7.4). The samples were filtered through glass fiber filters (GF/C) presoaked in 0.5% polyethylenimine for at least 30 min prior to use. The filters were washed twice with ice-cold buffer (5 mL). The radioactivity bound to the filters was measured by liquid scintillation spectrometry. The saturation results (K_d and B_{max}) were analyzed by nonlinear curve fitting utilizing the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA) and are reported in Figure 1. The values are the means ± sem from three experiments in triplicate. One-way ANOVA analysis of variance was used to estimate the significance of difference. A difference with $P < 0.05$ was considered statistically significant.

Affinity Chromatography Experiment. A suspension of the SK-N-SH membrane preparation (4 mg in 5 mL) containing 50 mM Tris at pH 8.0 was injected in activated column **6**. The column was sealed, keeping it at room temperature for 1 h. Then it was washed with 50 mM Tris at pH 8.0 (3 × 5 mL) to remove the biological fraction that was not bound to the selector. The protein bound to the selector was eluted with 20 mM glycine at pH 3.0 (3 × 5 mL). The solution was lyophilized at −52 °C and 0.061 mbar and analyzed in SDS−PAGE gel electrophoresis revealing four distinct bands having an apparent molecular weight from 13 to 15 kDa and three bands having an apparent molecular weight from 30 to 35 kDa.

Electrophoresis Fractionation and in Situ Digestion. The protein mixture was fractionated by SDS−PAGE on a 12% bis-acrylamide gel. The protein bands stained by Colloidal Comassie (Pierce) and containing the proteins to be identified were excised, cut into small pieces, and washed in 50 mM NH_4HCO_3 at pH 8.0 in 50% acetonitrile to completely destain them. The gel pieces were resuspended in 50 mM NH_4HCO_3 at pH 8.0 reduced with 10 mM dithiothreitol (DTT) at 56 °C for 45 min and alkylated with a 55 mM solution of iodoacetamide in the same buffer for 30 min at room temperature in the dark. The excess reagent was descarded, and the gel pieces were washed several times with the buffer, resuspended in 50 mM NH_4HCO_3, and incubated with 100 ng of trypsin for 2 h at 4 °C and overnight at 37 °C. The supernatant containing peptides was removed, and the remaining gel pieces were washed with acetonitrile to extract the peptides still present in the gel. These two fractions were then collected and freeze dried.

MALDI-MS Analysis. MALDI mass spectra were recorded on an Applied Biosystem Voyager DE-PRO mass spectrometer equipped with a reflectron analyzer and used in delayed extraction mode. Then 1 μL of the peptide sample was mixed with an equal volume of the α-cyano-4-hydroxycynnamic acid as thematrix (10 mg/mL in 0.2% trifluoroacetate in 70% acetonitrile), applied to the metallic sample plate, and air dried. Mass calibration was performed by using the standard mixture provided by the manufacturer. Raw data, reported as monoisotopic masses, were then introduced into the MASCOT peptide fingerprinting search program (Matrix Science, Boston, MA), available on the Internet and used for protein identification.

LC-MS-MS Analysis. When the identity of the present proteins could not be established by MALDI analysis alone, the peptide mixtures were further analyzed by LC-MS-MS using a Q-ToF hybrid mass spectrometer (Micromass, Waters) equipped with a Z-spray source and coupled on-line with a capillary chromatography system CapLC (Waters). After loading, the peptide mixture (10 μL) was first concentrated and washed at 10 μL/min onto a C_{18} reverse-phase precolumn (Waters) using 0.2% formic acid as the eluent. The sample was then fractionated onto a C_{18} reverse-phase capillary column (75 μm × 20 mm) at a flow rate of 280 nL/min using a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 5% acetonitrile) from 7% to 60% in 50 min. The mass spectrometer was set up in a data-dependent MS/MS mode, where a full scan spectrum (*m*/*z* acquisition range from 400 to 1600 Da/e) was followed by a tandem mass spectrum (*m*/*z* acquisition range from 100 to 2000 Da/e). The precursor ions were selected as the three most intense peaks of the previous scan. A suitable collision energy was applied depending on the mass and charge of the precursor ion. ProteinLynx software, provided by the manufacturers, was used to analyze raw MS and MS/MS spectra and to generate a peak list, which was introduced into the MASCOT MS/MS ion search software for protein identification.

Acknowledgment. This study was supported by Research Grant No. 2003034413_007 (2004-2005) from Università degli Studi di Bari and MIUR, Italy. We thank CEINGE Biotecnologie Avanzate s.c. a r.l., Via Comunale Margherita 482, 80145, Naples, Italy, for supporting the protein characterization by MALDI-MS and LC-MS-MS analyses.

Supporting Information Available: Elemental analysis of compound **4**, MALDI mass spectrum of the bands 15A, 15B, and 15C, and the parameters for protein identification. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0600592