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Short communication

Binding of [^{3}H]U-101958 to σ_1 receptor-like sites in human cerebellum and neuroblastoma cells

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Abstract

1-Benzyl-4-[*N*-(3-isopropoxy-2-pyridinyl)-*N*-methyl]-amino-piperidine ([^{3}H]U-101958), a dopamine D_4 receptor ligand, was found to bind to a large σ_1 receptor-like component in human cerebellum and SK-N-MC neuroblastoma cells with high affinity (2–4 nM K_d). By contrast, binding to dopamine D_4 receptors represented 10% or less of the σ_1 receptor-like site. Considering that U-101958 has been characterized as either a dopamine D_4 receptor agonist or antagonist, depending on the system under study, the observation that U-101958 also binds to σ_1 receptor-like sites is important for accurate interpretation of the pharmacological actions of this compound. [^{3}H]U-101958 may be a useful radioligand for σ_1 rather than dopamine D_4 receptor sites.

Keywords: σ_1 receptor-like site; Dopamine D_4 receptor; U-101958; Cerebellum, human; SK-N-MC neuroblastoma

1. Introduction

The dopamine D_4 receptor has been implicated in a number of psychiatric disorders, including schizophrenia. However, the low levels of dopamine D_4 receptor expression and lack of specific ligands for this receptor have hindered analysis of dopamine D_4 receptor function. [^{3}H]Nemonapride has been previously used but is not selective for dopamine D_4 receptor sites when used in a subtraction method with [^{3}H]raclopride (Helmeste et al., 1996, 1997; Tang et al., 1997). 1-Benzyl-4-[*N*-(3-isopropoxy-2-pyridinyl)-*N*-methyl]-amino-piperidine ([^{3}H]U-101958) is one of several new compounds developed as selective dopamine D_4 receptor ligands. The selectivity and specificity of dopamine D_4 receptor ligands are especially important in pharmacological evaluation of dopamine D_4 receptor function due to the small receptor quantities present in brain and often overlapping regional distributions with other dopamine D_2 receptor family members. This paper presents data showing that [^{3}H]U-101958 binding in human cerebellum and SK-N-MC neuroblastoma cells is not selective for dopamine D_4 receptors. Rather, it revealed labeling of an abundantly expressed σ_1 receptor-like site. These data may be important for understanding the pharmacological actions of U-101958, which has presented conflicting pharmacological profiles depending on the system under study (Schlachter et al., 1997; Gazi et al., 1998; Wilke et al., 1998).

2. Materials and methods

2.1. Tissue preparation and radioligand binding assay

Post-mortem human brain tissue was obtained from the National Neurological Research Specimen Bank (VAMC, Los Angeles, c/o Dr. W.W. Tourtellotte), which is supported by NINDS/NIMH, National Multiple Sclerosis Society, Hereditary Disease Foundation, Comprehensive Epilepsy program, Tourette Syndrome Association, Dystonia Medical Research Foundation, and Veterans Health

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Services and Research Administration, Department of Veterans Affairs. Human SK-N-MC neuroblastoma cells (metastasis to supra-orbital area, ATCC HTB 10) were obtained from American Type Culture Collection (Rockville, MD). SK-N-MC cells were grown in Minimum Essential Medium (Gibco BRL, Gaithersburg, MD), containing 10% fetal bovine serum (Gibco BRL), 1% 10 mM non-essential amino acids, 1% 100 mM sodium pyruvate, 1% 200 mM L-glutamate, and 2% penicillin–streptomycin (Gibco BRL), under 5% CO_2, 95% air, 95% humidity conditions.

Tissue was homogenized (glass/Teflon, 10 up–down strokes) in D_4 buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 120 mM NaCl) and frozen at −80°C until use. For radioligand binding assays, the tissue was washed twice by centrifugation at 30,996 × *g* and resuspended in D_4 buffer to give 1.2 mg original wet weight per tube (final assay volume of 0.3 ml). All radioligand-binding assays were done in D_4 buffer in triplicate or quadruplicate in polypropylene tubes and incubated with [*N-methyl*-^{3}H]U-101958 (79.5 Ci/mmol; Research Biochemicals International, Chemical Synthesis Program, Natick, MA) for 3 h at room temperature (22°C). Three hours was determined to be the time necessary to reach equilibrium for the binding experiments (data not shown). Competition experiments were done using 5 nM of [^{3}H]U-101958. K_i values were calculated from IC_{50} values using the Cheng–Prusoff equation (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1 + L^*/K_d)$ where L^* is the concentration of [^{3}H]U-101958, and K_d is obtained from Scatchard analysis (2.7 nM). Separation of bound from free ligand was done by vacuum filtration through GF/B glass fiber filters (Whatman, UK), which were presoaked in polyethyleneimine (2%) to minimize non-specific binding to filters. Chemicals and reagents were from Research Biochemicals International, and Sigma (St. Louis, MO). Analysis of binding data was done using the LIGAND program (Biosoft, Milltown, NJ). The Bio-Rad Protein assay (Bio-Rad, Hercules, CA) method using bovine serum albumin standard was used to measure protein.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared by the acid guanidine–phenol–chloroform method (Chomczynski and Sacchi, 1987). In brief, 10^7–10^8 SK-N-MC cells were suspended in 2 ml of guanidine isothiocyanate solution containing 20 mM sodium acetate, 0.1 M dithiothreitol, 5 g/l sarkosyl (*N*-lauroylsarcosine), and 0.1 M 2-mercaptoethanol, and then homogenized with a Polytron homogenizer (Brinkmann, Los Angeles, CA) for 1 min. Cell lysates were then mixed with 200 μl of 2 M sodium acetate, 2 ml of water-saturated phenol (pH 5.0), and 1 ml of chloroform:isoamyl alcohol (49:1 vol.), and incubated on ice for 15 min. Lysates were centrifuged at 9700 × *g* for 20 min at 4°C. After centrifugation, supernatant solutions were transferred to new tubes, mixed with equal volumes of isopropanol, and incubated for 1 h at −20°C. RNA materials were pelleted by centrifugation at 9700 × *g* for 20 min at 4°C. The precipitate was washed once with 75% ethanol and dried by centrifugation under reduced pressure. Total RNA was then denatured for 10 min at 65°C and cooled to 4°C. Reverse transcription (RT) was performed in solution containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM each of dATP, dGTP, dCTP and dTTP, 40 units of Rnasin, and 200 units of Superscript II reverse transcriptase (Gibco BRL) at 37°C for 1.5 h with oligo(dT) primers.

One microliter of RT products was used in the polymerase chain reaction (PCR) mixture, with PCR-buffer (5 × PCR buffer, 1.0 mM $MgCl_2$, 100 μM of dNTPs, 0.25 unit of Taq polymerase (Perkin Elmer, Foster City, CA), 0.5 μM each of upstream sense and downstream antisense primer in a final volume of 10 μl. Each PCR was conducted in a thermal cycler (Model 480, Perkin Elmer, Norwalk, CT) with 35 cycles of 30 s at 94°C, 30 s at 57°C and 1 min at 72°C for σ_1 receptor; and 35 cycles of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C for dopamine D_4 receptor. Five microliters of amplified PCR products were analyzed in 1 or 2% agarose gel electrophoresis. The expected sizes of PCR products were 209 and 170 bp for σ_1 receptor and dopamine D_4 receptor, respectively. These PCR products were further confirmed by the appropriate restriction enzyme digestions using *Bst*X1 (New England Biolabs, Beverly, MA) for σ_1 mRNA and *Bgl*1 (Promega, Madison, WI) for D_4 mRNA.

Primer sequences were determined by using the HYB simulatorTM computer program (AGCT, Irvine, CA) (Mitsuhashi et al., 1994; Hyndman et al., 1996) and appropriate design strategy (Mitsuhashi, 1996a,b). Criteria for primer design were: (1) the same length for both sense and antisense primers; (2) difference of T_m between sense and antisense primers within 2°C; (3) stacking region between sense and antisense primers is no more than 3 bases long; (4) stacking region within sense and antisense primers is no more than 3 bases long; (5) identical sequence among various entries of target gene; and (6) the least homology against non-target genes published in the Gene Bank primate and rodent databases. Resultant oligonucleotides (sense 5′-CACCACCAAAAGTGAGGTCTTCTACC-3′ and antisense 5′-CCCGAGCATAGGAGCGAAGAGTAT-3′ for the σ_1 receptor; sense 5′-GAGGGTCCTGCCG-GTGGTGGTC-3′ and antisense 5′-CAGTGTAGAT-GACGGGGTTGAG-3′ for dopamine D_4 receptor) were then synthesized by the DNA synthesizer 380 B type (Applied Biosystems, San Jose, CA). After treatment with ammonium hydroxide at 55°C overnight, synthesized oligonucleotides were dried, and resuspended in water at 0.1 μg/μl, and stored at −20°C until use.

3. Results

Competition experiments for [^{3}H]U-101958 (5 nM) binding to human cerebellum showed a σ_1-receptor like binding profile. Table 1 shows K_i values for competing drugs in order of decreasing potencies (Table 1). Dopamine receptor ligands (+)butaclamol, spiperone and (−)sulpiride did not compete for binding at the highest concentrations used [10 μM for (−)sulpiride and (+)butaclamol, 3 μM for spiperone]. σ receptor ligands [(−)butaclamol, PPAP (*R*(−)-*N*-(3-phenyl-*n*-propyl)-1-phenyl-2-aminopropane hydrochloride), (±)pentazocine, progesterone, DTG (1,3-Di(2-tolyl)guanidine) and U-50488 ((−)-(1*S*,2*R*)-*cis*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl-benzeneacetamide)) did compete for binding with σ_1 receptor-like affinities. Haloperidol and nemonapride (mixed σ and dopaminergic ligands) had high affinities for this site in a range characteristic for σ_1 receptor binding. Clorgyline (monoamine oxidase inhibitor), sertraline and imipramine (serotonin uptake inhibitors), and neurosteroids DHEA and DHEA-SO_4, had affinities consistent with a σ_1-like binding site. A non-dopamine receptor-like profile was also seen in SK-N-MC neuroblastoma cells. Haloperidol, PPAP, (±)pentazocine, and DTG demonstrated K_i values of 5.3 ± 0.4, 26 ± 1, 133 ± 19 and 340 ± 30 nM, respectively ($n = 3$). (−)Butaclamol gave a K_i of 411 ± 140 nM while (+)butaclamol did not compete for binding at concentrations up to 10 μM. Both spiperone and (−)sulpiride competed for less than 10% of total binding at the highest concentrations used (1 μM and 10 μM, respectively).

Table 1
[^{3}H]U-101958 (5 nM) binding in human cerebellum

Competing drug	K_i (nM)
Clorgyline	3.4 ± 0.1
Sertraline	4.6 ± 2.5
Haloperidol	10 ± 1
(−)Nemonapride	15 ± 1
(+)Nemonapride	16 ± 6
(−)Butaclamol	79 ($n = 2$)
PPAP	99 ± 33
(±)Pentazocine	171 ± 34
Progesterone	203 ± 7
DTG	315 ± 9
U-50488	407 ± 114
Imipramine	491 ($n = 2$)
Spiperone	minimal competition at 3 μM
Dehydroepiandrosterone (DHEA)	$3{,}612 \pm 806$
(+)Butaclamol	minimal competition at 10 μM ($n = 2$)
(−)Sulpiride	minimal competition at 10 μM
Dehydroepiandrosterone-sulfate (DHEA-SO_4)	minimal competition at 100 μM

$n = 3$–7, except where indicated.

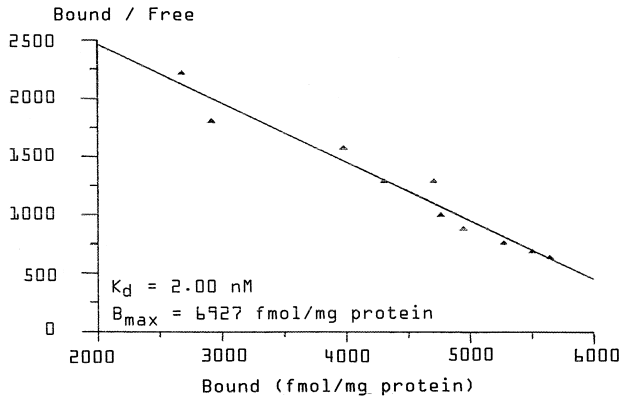

Fig. 1. Scatchard plot of [^{3}H]U-101958 binding to SK-N-MC neuroblastoma cells. Specific binding was defined with 10 μM (±)pentazocine (representative assay).

Specific binding for the competition experiments represented 90% of total binding for SK-N-MC cells and 75% of total binding for cerebellum (total binding being the sum of specific and non-specific binding as defined with 10 μM (±)pentazocine). A small dopamine D_4 receptor component was seen in SK-N-MC cells but was difficult to quantify since it represented 10% or less of the σ_1 receptor-like component.

To confirm that both σ_1 and dopamine D_4 receptor mRNAs were expressed in SK-N-MC cells under our cell culture conditions, RT-PCR was performed. PCR products of the appropriate sizes (before and after restriction enzyme digestion) confirmed the presence of both mRNAs in this cell line (data not shown).

Scatchard analysis (1 to 15 nM [^{3}H]U-101958) revealed a single site with K_d of 2–4 nM when (±)pentazocine (10 μM) was used to define specific binding. In human SK-N-MC neuroblastoma cells, binding density (B_{max}) averaged 7685 ± 744 fmol/mg protein with K_d of 2.7 ± 0.5 nM ($n = 4$). A representative Scatchard analysis is shown in Fig. 1. In human cerebellum, analysis revealed a single site with B_{max} of 1545 fmol/mg protein and K_d of 2.6 nM ($n = 1$ due to limited tissue availability).

4. Discussion

U-101958 is one of several selective dopamine D_4 receptor ligands that have recently become commercially available after the original cloning of this receptor (Van Tol et al., 1991). It has been shown to bind to dopamine D_4 receptors with nanomolar affinity (Kula et al., 1997; Schlachter et al., 1997) and was originally classified as a dopamine D_4 antagonist in a quinpirole-induced mitogenesis test in Chinese hamster ovary cells (Schlachter et al., 1997). However, recent studies on dopamine D_4 receptor-mediated inhibition of K+ currents have suggested that U-101958 does not always behave as a typical dopamine D_4 receptor antagonist (Wilke et al., 1998). This has led to

the suggestion that U-101958 may be a dopamine D_4 receptor agonist, not antagonist (Wilke et al., 1998). Therefore, it is of interest that a large proportion of high affinity [^{3}H]U-101958 binding in human tissues is σ_1 receptor-like in its binding properties. Considering that σ receptor ligands have been reported to inhibit K + conductance in other studies (Soriani et al., 1998), an inhibition of K + current by U-101958 could indicate a σ rather than a dopamine D_4 agonist property of this compound.

Analysis of the dopamine D_4 component of [^{3}H]U-101958 binding indicated that it represents 10% or less of the σ_1 receptor-like binding population in SK-N-MC cells. This is when dopamine D_4 binding is defined by either spiperone, (+)butaclamol or sulpiride baselines. Although dopamine D_4 receptor mRNA is present in SK-N-MC cells as determined by RT-PCR, the level of dopamine D_4 receptor protein expression appears to be low compared to the σ_1 receptor-like binding population. In post-mortem human cerebellum, no significant binding to dopamine D_4 receptor sites (as defined by either spiperone or sulpiride) could be seen. This is in agreement with the study of Primus et al. (1997) which found no evidence of high-affinity [^{3}H]NGD 94-1 binding to dopamine D_4 receptors in human cerebellum.

The major component of [^{3}H]U-101958 binding is classified as σ_1 receptor-like based on a number of different characteristics. Firstly, (−)butaclamol has higher affinity than (+)butaclamol for this site. This is characteristic of σ receptor binding. Dopamine $D_2/D_3/D_4$ type receptors, by contrast, have high affinity for (+)butaclamol but low affinity for (−)butaclamol. Secondly, clorgyline has higher affinity than DTG for this site. This is characteristic of σ_1 receptor binding and of the recently cloned rat brain σ_1 receptor (Hanner et al., 1996; Seth et al., 1998). Additionally, σ receptor ligands (pentazocine, PPAP, progesterone, DTG, U-50488) compete for this site while dopaminergic ligands (spiperone, sulpiride) do not. DTG appears to have relatively low affinity under our assay conditions. This is similar to previous studies of [^{3}H](+)pentazocine binding (σ_1 receptor selective ligand) in human cerebellum which demonstrated an IC_{50} of 257 nM for DTG (Zabetian et al., 1994). Other σ_1 receptor binding studies have reported DTG K_i values of 307 nM in rat cerebellum and 1386 nM in human peripheral blood leukocytes (Wolfe et al., 1988). Our own characterization of [^{3}H]nemonapride binding to σ_1 receptor-like sites gave a K_i of 260 nM for DTG in cow striatum (Helmeste et al., 1997). Nemonapride (mixed σ and dopamine receptor ligand) binds to our [^{3}H]U-101958 labelled site with high affinity. The large B_{max} (defined with pentazocine) is characteristic of σ_1 receptor type binding in both human brain and tumor cell lines. Study of high affinity binding of [^{3}H]U-101958 to cells with a pure population of σ_1 receptors, such as recombinant cell lines (Pan et al., 1998; Seth et al., 1998) would be useful to confirm that U-101958 could serve as a suitable σ_1 receptor ligand.

Given the high affinity (K_d 2–4 nM) and low levels of nonspecific binding for [^{3}H]U-101958 under our assay conditions, this ligand appears to be a very suitable label for σ_1 receptor-like binding sites. Dopamine D_4 receptor binding should be feasible in tissues which have a higher ratio of dopamine D_4 to σ-like receptor sites.

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