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Expression of σ receptors of human urinary bladder tumor cells (RT-4 cells) and development of a competitive receptor binding assay for the determination of ligand affinity to human σ_2 receptors

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ABSTRACT

A selective competitive binding assay for the determination of the affinity of compounds to the human σ_2 receptor using 96-well multiplates and a solid state scintillator was developed. In the assay system, [^{3}H]ditolylguanidine (DTG) was used as radioligand and membrane homogenates from human RT-4 cells physiologically expressing σ_2 receptors served as receptor material. In order to block the interaction of the unselective radioligand [^{3}H]DTG with σ_1 receptors, all experiments were performed in the presence of the σ_1 selective ligand (+)-pentazocine. The density of σ_2 receptors of the cells was analyzed by a saturation experiment with [^{3}H]DTG. The radioligand [^{3}H]DTG was bound to a single, saturable site on human σ_2 receptors, resulting in a B_{max} value of 2108 ± 162 fmol/mg protein and K_d-value of 8.3 ± 2.0 nM. The expression of competing σ_1 receptors was evaluated by performing a saturation experiment using the σ_1 selective radioligand [^{3}H](+)-pentazocine, which resulted in a B_{max} value of 279 ± 40 fmol/mg protein and K_d value of 13.4 ± 1.6 nM. For validation of the σ_2 binding assay, the K_i-values of four σ_2 ligands (ditolylguanidine, haloperidol, rimcazole and BMY-14802) were determined with RT-4 cell membrane preparations. The K_i values obtained from these experiments are in good accordance with the K_i-values obtained with rat liver membrane preparations as receptor material and with K_i values given in the literature.

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1. Introduction

The σ receptors were first postulated in 1976 by William Martin. Originally, they were erroneously classified as a subtype of opioid receptors [1]. Meanwhile, the σ receptors are recognized as unique class of receptors and are further divided into at least two receptor subtypes, termed σ_1 and σ_2 receptor [2]. The existence of a third subtype, the σ_3 receptor, has been proposed, but further experiments have shown that it rather belongs to the histamine receptor family [3,4]. Among the σ receptors, more details are known about the σ_1 receptor subtype. It has been cloned from various tissues from guinea pigs, rats, and humans [5–7]. In contrast to G-Protein coupled receptors with seven transmembrane helices, the σ_1 receptor consists of 223 amino acids, which pass the membrane in two transmembrane domains [6]. There is no structural relationship to other mammalian proteins. The gene for the σ_1 receptor resides on chromosome 9 of humans and also contains a cytokine and steroid responsive element [8]. The amino acid sequence of the human σ_1 receptor is 93% identical with the σ_1 receptor of guinea pigs. As endogenous ligands for the σ_1 receptor certain neuroactive steroids, in particular dehydroepiandrosterone and progesterone have been described. Testosterone also has a moderate affinity to σ_1 receptors [9,10]. More recently, N,N-dimethyltryptamine, an endogenous hallucinogenic compound, has been found to bind to σ_1 receptors [11].

In contrast to the σ_1 receptor, there is considerably less knowledge about the σ_2 receptor subtype. The molecular weight of the σ_2 receptor is estimated to be 21.5 kDa [12]. The existence of the σ_2 receptors was proved by the specific binding of unselective σ receptor ligands in σ_1 receptor knock-out mice [13,14]. The σ_2 receptors are ubiquitously expressed in the body with exception of the central nervous system (CNS) and they are found in many peripheral tissues including liver, kidney, lung and heart [15].

Both σ receptor subtypes are expressed in high density in different human tumor cells, including breast, lung, colon, ovary and prostate cancer [16–21]. Recently, we detected that the human urinary bladder cell line RT-4 [22] has a high density of σ receptors, in particular σ_2 receptors. This encouraged us to characterize the σ_1 and σ_2 receptor expression of these human tumor cells in more detail.

Due to the postulated involvement of σ_2 receptors in tumor cell proliferation, the development of σ_2 selective ligands is a very

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promising field of tumor research. On the one hand, σ_2 selective PET ligands can be used to image and monitor the progression of many tumor cells, [23,24] on the other hand the development of σ_2 selective ligands could lead to a new class of anti-cancer drugs. However, the development of novel ligands with high affinity and selectivity to the human σ_2 receptor requires selective binding assays.

Generally, σ_2 receptor binding assays are based on membrane preparations from rat liver [12]. A method for evaluating the activity of σ_2 ligands using preparations from guinea pig bladder has also been described [25]. However, the human σ_2 receptor has not been cloned or functionally expressed in cells yet. These issues provided the rationale to establish a radioligand based competitive binding assay for the human σ_2 receptor using cell membrane preparations of a commercially available human tumor cell line with a high density of σ_2 receptors.

Herein, we report on the development of a selective, filtration-based receptor binding assay on 96-well-multiplates for the human σ_2 receptor using the human tumor cell line RT-4 as receptor material and [^{3}H]ditolylguanidine as radioligand. While the σ_2 receptor expression in tissues of human bladder cancer has already been characterized [17], the σ_1 and σ_2 receptor expression of the RT-4 tumor cell line has not been investigated in full detail and had to be characterized for the purpose of our assay system.

2. Materials and methods

2.1. Cell culture and preparation of membrane homogenates from RT-4 cells

RT-4 cells [26] were commercially available (DSMZ, Braunschweig, Germany). The cells were grown in RPMI 1640 medium containing 10% of standardized FCS (Biochrom AG, Berlin, Germany). The cells were split in a ratio of 1:3 using trypsine/EDTA solution (Biochrom AG, Berlin, Germany) when the cell density of the adherent growing cells had reached approximately 90% of confluency. The cells were harvested mechanically by scraping off from the bottom of the cell culture flasks and pelleted (10 min, 5000 × *g*, Hettich Rotina 35R centrifuge, Tuttlingen, Germany).

For the binding assay, the cell pellet was resuspended in phosphate buffered saline solution (PBS; Biochrom AG) and the number of cells was determined using an improved Neubauer's counting chamber (VWR, Darmstadt, Germany). Subsequently, the cells were lysed by sonication (4 °C, 6 × 10 s cycles with breaks of 10 s, device: Soniprep 150, MSE, London, UK). The resulting cell fragments were centrifuged with a high performance cooling centrifuge (20,000 × *g*, 4 °C, Sorvall RC-5 plus, Thermo Scientific). The supernatant was discarded and the pellet resuspended in a defined volume of PBS yielding cell fragments from approximately 4,000,000 cells/mL. The suspension of membrane homogenates was sonicated again (4 °C, 2 × 10 s cycles with a break of 10 min) and stored at −80 °C.

2.2. Preparation of membrane homogenates from rat liver [27,28]

Two rat livers were cut into small pieces and homogenized with a potter (500–800 rpm, 10 up-and-down strokes, device: Elvehjem Potter, B. Braun Biotech International, Melsungen, Germany) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200 × *g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 × *g* for 20 min at 4 °C. The pellet was resuspended in 5–6 volumes of buffer (50 mM TRIS, pH 8.0) and incubated at room temperature for 30 min. After the incubation, the suspension was centrifuged again at 31,000 × *g* for 20 min at 4 °C. The final pellet was resuspended in 5–6 volumes of buffer and stored at −80 °C in 1.5 mL portions containing about 2 mg protein/mL (refer to chapter "Quantitative protein concentration analysis").

2.3. Analysis of protein concentration

The protein concentration was determined by the method of Bradford [29], modified by Stoscheck [30]. The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL of EtOH (95%, v/v). 10 mL deionized H_2O and 5 mL phosphoric acid (85%, m/v) were added to this solution, the mixture was stirred and filled to a total volume of 50.0 mL with deionized water. The calibration was carried out using bovine serum albumin as a standard in 9 concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 mg/mL). In a 96-well standard multiplate, 10 μL of the calibration solution or 10 μL of the membrane receptor preparation were mixed with 190 μL of the Bradford solution, respectively. After 5 min, the UV absorption of the protein-dye complex at λ = 595 nm was measured with a platereader (Tecan Genios, Tecan, Crailsheim, Germany).

2.4. σ_2 receptor binding assay

The competitive binding assays were performed with the radioligand [^{3}H]DTG (specific activity 50 Ci/mmol; ARC, St. Louis, MO, USA) using standard 96-well-multiplates (Diagonal, Muenster, Germany). 50 μL of the thawed membrane preparations (either membrane fragments prepared from approximately 200,000 RT-4 cells containing 150 μg protein or rat liver preparation containing 100 μg protein) were incubated with 50 μL of test compound (six different concentrations, usually 10 μM–0.1 nM final assay concentration), 50 μL radioligand (12 nM [^{3}H]DTG in 50 mM TRIS, final assay concentration 3 nM), and buffer containing (+)-pentazocine (2 μM (+)-pentazocine in 50 mM TRIS, pH 8.0, final assay concentration 500 nM) in a total volume of 200 μL for 120 min at 37 °C (RT-4 cell fragments) or room temperature (rat liver membranes). Generally, the receptor preparation was added last. All experiments were carried out in triplicates. The incubation was terminated by rapid filtration through filtermats using a cell harvester (MicroBeta FilterMate-96 Harvester, Perkin Elmer). Prior to harvesting, the filtermats were presoaked in 0.5% aqueous polyethylenimine for 2 h at room temperature. After washing each well five times with 300 μL of water, the filtermats were dried at 95 °C. Subsequently, the solid scintillator was placed on the filtermat and melted at 95 °C. After 5 min, the solid scintillator was allowed to solidify at room temperature. The bound radioactivity trapped on the filters was counted in the scintillation analyzer (Microbeta Counter, Perkin Elmer). The overall counting efficiency was 20%. The nonspecific binding was determined with 10 μM non-labeled DTG.

2.5. Saturation experiments for σ_2 receptors

The saturation analysis was performed by incubating increasing concentrations of [^{3}H]DTG (final assay concentrations 0.1 nM, 0.25 nM, 0.5 nM, 1.0 nM, 2.5 nM, 5.0 nM, 10 nM and 20 nM) together with cell fragments obtained from 200,000 cells (containing 150 μg protein) in buffer containing (+)-pentazocine (final assay concentration 500 nM (+)-pentazocine in 50 mM TRIS, pH 8.0) for 2 h at 37 °C. For each concentration, the nonspecific binding was determined with an excess of non-labeled DTG (10 μM). All experiments were carried out in triplicates. The filtration and scintillation counting was performed as described above. K_d and B_{max} were calculated as described in Section 2.7.

2.6. Saturation experiments for σ_1 receptors

The saturation analysis was performed by incubating increasing concentrations of [^{3}H](+)-pentazocine (specific activity 22 Ci/mmol, Perkin Elmer) final assay concentration (0.1 nM,

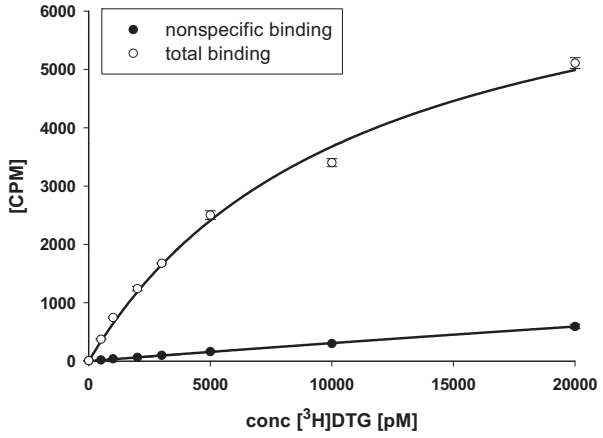

Fig. 1. σ_2 saturation experiment using RT-4 cell line preparations and [^{3}H]DTG as radioligand; total binding = recorded counts per minute using increasing concentrations of [^{3}H]DTG; nonspecific binding = counts per minute using increasing amounts of [^{3}H]DTG in the presence of large amount of non-labeled DTG.

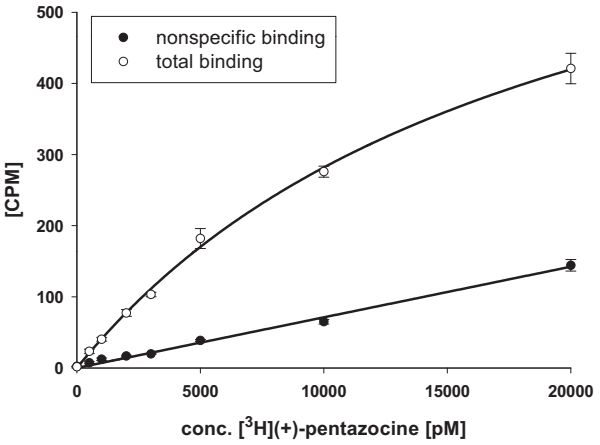

Fig. 2. σ_1 saturation experiment using RT-4 cell line preparations and [^{3}H](+)-pentazocine as radioligand; total binding = recorded counts per minute using increasing concentrations of [^{3}H](+)-pentazocine; nonspecific binding = counts per minute using increasing amounts of [^{3}H](+)-pentazocine in the presence of large amount of non-labeled (+)-pentazocine.

0.25 nM, 0.5 nM, 1.0 nM, 2.5 nM, 5 nM, 10 nM and 20 nM) together with cell fragments obtained from 200,000 cells (containing 150 μg protein) in TRIS-buffer (50 mM, pH 7.4) for 2 h at 37 °C. For each concentration, the nonspecific binding was determined with an excess of non-labeled (+)-pentazocine (10 μM). All experiments were carried out in triplicates. The filtration and scintillation counting was performed as described above. K_d and B_{max} were calculated as described in Section 2.7.

2.7. Data analysis

Data analysis was performed with Graph Pad Prism® Software, Version 3.0 (Graph Pad Software Inc., San Diego, CA, USA). Saturation analyses were made by nonlinear regression using the "one-site-saturation" calculation method. The K_d and B_{max} values are given as mean values from three independent experiments. The Scatchard (Rosenthal) plot was generated by linear regression using the least squares method from one representative saturation experiment. The IC_{50}-values of the reference compounds used in the competitive binding experiments were determined by nonlinear regression using the "one-site-competition" calculation method. Subsequently, the K_i-values of the reference compounds were calculated according to the equation of Cheng and Prusoff [31]. The K_i values are given as mean values from three independent experiments ± Standard Error of the Mean (SEM).

3. Results and discussion

The density of σ_1 and σ_2 receptors of RT-4 cells was determined by saturation experiments. For the determination of the amount of σ_1 receptors the highly σ_1 selective radioligand [^{3}H](+)-pentazocine was used. Since a selective radioligand for labeling of σ_2 binding sites is not commercially available, the σ_2 saturation experiments were performed with the σ unselective radioligand [^{3}H]DTG in the presence of σ_1 selective (+)-pentazocine masking σ_1 receptors.

The σ_2 saturation experiment is shown in Fig. 1. Comparison of the curves resulting without inhibitor (total binding) and in the presence of a large excess of inhibitor ditolylguanidine (nonspecific binding) clearly indicates that the RT-4 cell membrane preparation contained a significant amount of σ_2 receptors. Nonlinear regression analysis led to a B_{max}-value of 2108 fmol σ_2 receptors per mg protein (Table 1).

The σ_1 saturation experiments performed with the same receptor preparation from RT-4 cell lines using the radioligand [^{3}H](+)-pentazocine also resulted in a specific σ_1 receptor binding (Fig. 2). However, the amount of σ_1 receptors (B_{max} = 279 fmol per mg protein) is around 7-fold lower compared with the amount of σ_2 receptors in this preparation.

The alternative Scatchard (Rosenthal) Plot analysis of the saturation experiments resulted in somewhat different σ_1 and σ_2 receptor concentrations (Table 1). However the ratio of σ_2: σ_1 receptors is almost the same, proving that the RT-4 cells predominantly produce σ_2 receptors. These results are in good accordance with the results from Vilner et al. [21] who reported high expression rates of σ_2 receptors in various proliferating solid tumor cells. Moreover, the linearity of the Scatchard Plot demonstrates that a single saturable binding site was labeled by [^{3}H]DTG in the presence of (+)-pentazocine (Fig. 3).

Since the saturation experiments were carried out with cell fragments from approximately 200,000 cells, the calculated B_{max} values of 1581 pM and 209 pM protein are corresponding to approxi-

Table 1
Characterization of the RT-4 cell membrane preparation with respect to σ_2 and σ_1 receptor expression using [^{3}H]DTG and [^{3}H](+)-pentazocine as radioligands. B_{max} and K_d values calculated by nonlinear regression are expressed as mean values ± SEM (n = 3), the results from the Scatchard analysis were calculated from one representative experiment.

	B_{max} ± SEM [fmol/mg protein]		B_{max} ± SEM [pM]		K_d ± SEM [nM]	
	Nonlinear regression analysis	Scatchard analysis	Nonlinear regression analysis	Scatchard analysis	Nonlinear regression analysis	Scatchard analysis
σ_1 receptors ([^{3}H](+)-pentazocine)	279 ± 40	257	209 ± 30	193	13.4 ± 1.6	16.5
σ_2 receptors ([^{3}H]DTG)	2108 ± 162	1717	1581 ± 121	1288	8.3 ± 2.0	6.3

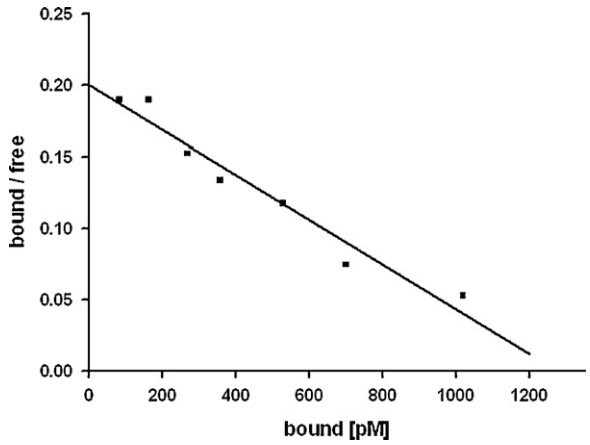

Fig. 3. Scatchard plot analysis of the σ_2 saturation experiment using RT-4 cell line preparations and [^{3}H]DTG as radioligand.

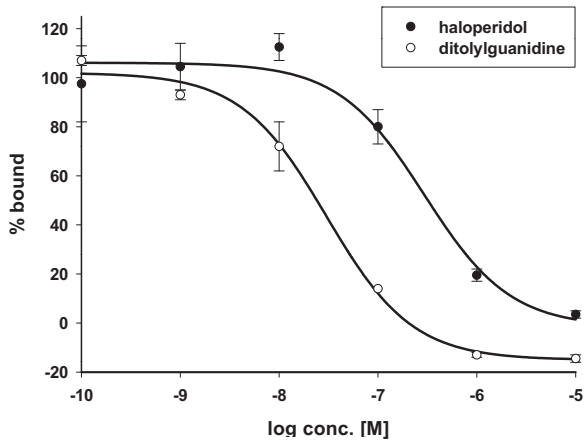

Fig. 5. Competition curves of DTG and haloperidol with [^{3}H]DTG in the σ_2 assay using RT-4 cell preparations, data from one representative experiment.

mately 948,000 copies of σ_2 receptors/cell and 125,000 σ_1 binding sites/cell. The high σ_2 receptor density rendered the RT-4 cell line attractive for the development of a competitive σ_2 binding assay. However, the RT-4 cells still produce σ_1 receptors and therefore selective masking of the detected σ_1 binding sites with (+)-pentazocine is required to gain a selective assay with [^{3}H]DTG.

The nonlinear regression analysis and the Scatchard analysis of the σ_2 saturation experiment resulted in K_d-values for ditolylguanidine of 8.3 and 6.3 nM, respectively (Table 1). These K_d-values are quite similar to the reported K_d-value of ditolylguanidine with rat liver membrane preparations (K_d = 17.9 nM) [32]. The slightly reduced K_d value might indicate a higher affinity of ditolylguanidine to the human σ_2 receptor. The K_d-value from the nonlinear regression analysis (8.3 nM) was used for the calculation of K_i-values of reference compounds, because the data transformation used in Scatchard plot analysis violates the assumptions of linear regression. Therefore, the data obtained by nonlinear regression analysis are generally considered to be more accurate. The calculated K_d-values for (+)-pentazocine by non-linear regression and Scatchard analysis are very similar (13.4 and 16.5 nM, respectively). But for the same reason as mentioned above, all further calculations were performed with the K_d-value obtained by nonlinear regression.

3.1. Competition experiments

In order to validate the RT-4 cell membrane preparation, K_i-values of known σ_2 ligands were recorded in competition experiments by incubation of RT-4 cell membrane preparations with [^{3}H]DTG as radioligand and different concentrations of the respective σ_2 ligands. The IC_{50}-values were determined by nonlinear regression analysis, transformed into K_i-values by the Cheng–Prusoff equation and the resulting data were compared with the K_i-values recorded with standard rat liver membrane preparations. For this purpose, the unselective σ ligands di-*o*-tolylguanidine and haloperidol and, moreover, the σ_2 preferential ligands BMY-14802 and rimcazole were selected (Fig. 4). With these ligands, a broad affinity spectrum ranging from 49 nM to 1 μM is covered.

In Table 2 the σ_2 receptor affinities of the four reference compounds are summarized. Generally the K_i-values generated with the RT-4 cell preparation are in the same range as the K_i-values generated with rat liver preparations. For ditolylguanidine K_i-values

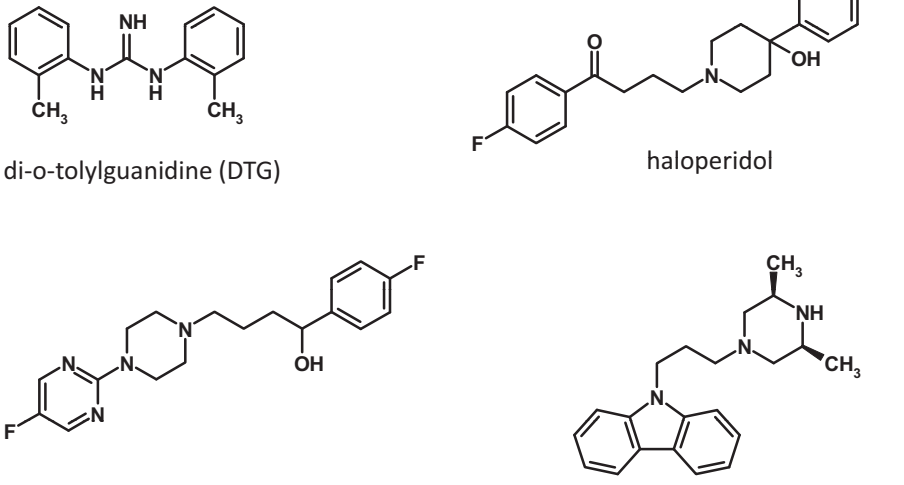

Fig. 4. Structures of four selected reference compounds.

Table 2
Comparison of K_i-values and Hill slopes (n_H) of four reference compounds using RT-4 cell line preparations and rat liver preparations; comparison with literature data. All mean values ± SEM are based on 3 independent experiments ($n = 3$).

	Human σ_2 receptor (RT-4)		Rat σ_2 receptor (liver)		Literature (rat liver)
	$K_i \pm$ SEM [nM]	$n_H \pm$ SEM	$K_i \pm$ SEM [nM]	$n_H \pm$ SEM	$K_i \pm$ SEM [nM]
Di-o-tolylguanidine (DTG)	20 ± 5.8	−0.98 ± 0.08	58 ± 18	−0.99 ± 0.09	43 ± 9 [33]
Haloperidol	200 ± 33	−1.19 ± 0.09	78 ± 2.3	−1.22 ± 0.10	54 ± 10 [33]
BMY-14802	232 ± 48	−1.21 ± 0.02	266 ± 45	−1.09 ± 0.08	230[34]
Rimcazole	571 ± 155	−1.12 ± 0.08	852 ± 51	−1.13 ± 0.10	1162 ± 160 [33]

of 20 nM (RT-4 cells) and 58 nM (rat liver) were determined. This slight difference might be explained by the usage of receptor material from different species. For haloperidol, K_i-values of 200 nM (RT-4 cells) and 78 nM (rat liver) were recorded. This is in good accordance to the observations of Vilner, John and Bowen, who also observed a significant lower affinity of haloperidol to σ_2 binding sites of human tumor cell lines [21]. Also, the Hill slopes of the competition curves are very similar in both assay systems and the values close to −1.0 indicate that no positive or negative cooperativity is involved. This confirms the results from the saturation experiment in which one single, saturable binding site was observed. The competition curves of DTG and haloperidol from one representative experiment using membrane fragments from RT-4 cells as receptor material are shown in Fig. 5.

4. Conclusion

The high σ_2 receptor density of human urinary bladder tumor cell line RT-4 was exploited to develop a binding assay for the determination of σ_2 receptor affinity. In a saturation experiment using the radioligand [^{3}H]DTG a σ_2 receptor amount of 2108 fmol/mg protein and a K_d-value of 8.3 nM were determined. Although the σ_1 receptor density of the RT-4 cells is considerably lower than the σ_2 receptor density, (+)-pentazocine has to be added to the equilibration mixture to render the assay completely σ_1 selective. The σ_2 affinities of reference compounds are in good accordance with the σ_2 affinities recorded with standard rat liver membrane preparations and previously reported data. For the first time a binding assay for the σ_2 receptor is described using a defined, commercially available human tumor cell line as receptor material instead of tissue preparations from tumor patients. This leads to a greater reproducibility of the σ_2 assay, since the cell material is more homogenous than the inhomogeneous native material. But even more important, the assay based on RT-4 cells allows the routine determination of affinity of test compounds to the human σ_2 receptor. Moreover, using cell lines as receptor material in the first screening reduces the amount of animals required for drug development. Another advantage of the assay system is the performance and validation at 37 °C instead of room temperature which simplifies the standardization of the assay conditions.

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