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The Existence of Stable Enantiomers of Telenzepine and Their Stereoselective Interaction with Muscarinic Receptor Subtypes

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SUMMARY

[^{3}H]Telenzepine has been shown to bind with high affinity (3×10^9 M^{-1}) to a subpopulation of muscarinic binding sites in rat cerebral cortex, which have a high affinity for pirenzepine. The binding kinetics were very slow at 30°. Only 50% of the [^{3}H] telenzepine was found to be capable of binding to the receptors with high affinity. This suggested the presence of optical isomers of telenzepine. These were partially resolved on the picomole scale by using cortical muscarinic receptors to selectively bind the active isomer. It was then possible to measure the temperature and time dependence of the racemization of the inactive to the active enantiomer. The energy barrier for the inversion was 35 kcal/mol, and racemization was very slow even at 90°. The affinity and selectivity of the unlabeled enantiomers for the different muscarinic receptor subtypes present on membranes from rat cerebral cortex, heart, and lacrimal gland was measured. The selectivity of active (+)-isomer was considerably greater than that of the (−)-isomer. As a consequence, the stereoselectivity of the enantiomers varied from 500 (M_1 receptors in cerebral cortex) to 75 (cardiac receptors).

Tz (4,9-dihydro-3-methyl-4-[(4-methyl-1-piperazinyl)acetyl]-10*H*-thieno[3,4-b][1,5]benzodiazepin-10-one) is a new selective muscarinic antagonist developed for the treatment of peptic ulcer disease. It exhibits a selectivity in functional tests that is comparable to that shown by Pz (1, 2) but it is at least 10 times more potent than Pz. [^{3}H]Pz is extensively used as a radioligand for M_1 receptors (e.g., Refs. 3–5). However, [^{3}H]Tz, by virtue of its selectivity and higher affinity, might be a superior radioligand. Indeed, preliminary binding studies have shown that [^{3}H]Tz binds with high affinity and a high specific:nonspecific binding ratio to a subpopulation of muscarinic receptors on calf cerebral cortex membranes (6). In our studies using [^{3}H]Tz, we discovered the existence of optical isomers of Tz. This paper describes the binding properties of the isomers and their very slow rate of isomerization. An abstract of some of the findings has been published (7).

Materials and Methods

Membranes from rat cerebral cortex, myocardium, and extraorbital lacrimal gland were prepared as described previously (8). Pellets could be stored at −20° for up to 2 months without loss of binding activity. The incubation buffer for the binding assays was 100 mM NaCl, 10 mM $MgCl_2$, 20 mM Na^+/HEPES, pH 7.4. Routine saturation and inhibition experiments were carried out at 30° for 2.5–3 hr using a microcentrifugation assay (9). Membrane pellets, after superficial washing, were allowed to dry and were dissolved in Soluene (100 μl). Scintillant (nonaqueous, 1.2 ml) was added, and the microcentrifuge tube plus contents were counted in a wide-necked plastic scintillation vial. This procedure has some advantages, as follows: (i) all manipulations are carried out in the microcentrifuge tube, (ii) low volumes of scintillant are used, (iii) the plastic scintillation vials are reused, and (iv) disposal of radioactive waste is relatively easy. In several assays there was a significant depletion of the total radioligand concentration, caused by its binding to muscarinic receptors. In those instances, the free concentration of the radioligand was directly measured by sampling 0.5 ml of the supernatant after centrifugation of the membranes. Nonspecific binding was defined as the radioactivity measured in the pellet when the radioligand was coincubated with 1 μM 3-quinuclidinylbenzilate.

Isomerization of [^{3}H]Tz was accomplished by heating supernatants from membrane incubations in screw-capped glass vials at an appropriate temperature (±1°) in a thermostated oven, for different lengths of time. Care was taken to ensure that there was no excessive build-up of air pressure.

All data were analyzed by nonlinear least squares analysis, taking depletion of radioligand or competitor into account where necessary.

In one series of experiments, the binding of a constant concentration of [^{3}H]Tz was examined as a function of increasing receptor (or protein) concentration. The equation for the simple binding of a radioligand (L) to a receptor (R) with an affinity constant K can be rearranged to give:

ABBREVIATIONS: M_1, muscarinic receptors having a high affinity for pirenzepine; Tz, telenzepine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NMS, *N*-methylscopolamine; non-M_1, muscarinic receptor binding sites in the cerebral cortex that do not have a high affinity for pirenzepine; Pz, pirenzepine; PrBCh, *N,N*-dimethyl-*N*-propyl-2-aminoethylbenzilate.

$$\frac{1}{[R_{tot}] - [RL]} = \frac{K[L_{tot}]}{[RL]} - K$$

where $[R_{tot}]$ and $(L_{tot}]$ are the total concentrations of receptor and ligand, respectively, and $[RL)$ is the concentration of bound radioligand. For $[R_{tot}] \gg [RL)$, this simplifies to:

$$\frac{1}{[R_{tot}]} = \frac{K[L_{tot}]}{[RL]} - K$$

A plot of $[R_{tot}]^{-1}$ versus $[RL]^{-1}$ at constant $[L_{tot}]$ is thus a straight line, gradient $K[L_{tot}]$. When $R_{tot} \rightarrow \infty$, $[RL] \rightarrow [L_{tot}]$ where $[L_{tot}]$ is the total concentration of radioligand capable of binding to the receptor. This estimate can be compared with the total concentration inferred from radioactivity measurements. As $[R_{tot}] \propto$ [protein] for any given preparation, an equivalent plot of [protein]$^{-1}$ versus [RL]$^{-1}$, as shown in Fig. 2, will also be linear with the same intercept, $[L_{tot}]$, on the x axis.

[^{3}H]Tz was analyzed for radiochemical purity by thin layer chromatography (silica gel; $CHCl_3/CH_3OH$, 5:3; toluene/dioxan/CH_3OH/0.88 NH_4OH, 20:40:40:2). The purity and specific radioactivity of [^{3}H]Tz was analyzed by mass spectrometry and high performance liquid chromatography.

[^{3}H]NMS (72 Ci/mmol) was obtained from Amersham International (Little Chalfont, UK) and [^{3}H]Pz (84 Ci/mmol) from New England Nuclear (Boston, MA). [^{3}H]Tz (85 Ci/mmol) was synthesized by Dr. S. Hurt, New England Nuclear. [^{3}H]PrBCh (40 Ci/mmol), synthesized by previously described methods (9), was a kind gift from Dr. S. Lazareno, Glaxo Group Research (UK). (+)-Tz ($[\alpha]_D^{25} = +40.8°$ ($c = 1$, CH_2Cl_2/methanol, 1:1) and (−)-Tz ($[\alpha]_D^{25} = -41.0°$ ($c = 1$, CH_2Cl_2/methanol, 1:1) were synthesized by Dr. Grundler (Byk-Gulden). Small quantities of the isomers are available on request.

Results

Kinetics of [^{3}H]Tz binding to cortical muscarinic receptors. The rate of approach to equilibrium of the binding of low concentration of [^{3}H]Tz (0.40 nM) is quite slow at 30° (Fig. 1A). The data could be fitted reasonably well by a monoexponential function [$k = (5.3 \pm 0.3) \times 10^{-4}$ sec^{-1}; $t_{½} = 23 \pm 1$ min) and the binding was stable for up to 6.5 hr. Nonspecific binding, which was less than 5% of the equilibrium total binding of [^{3}H]Tz, was constant over the time range examined. The dissociation rate of [^{3}H]Tz was determined after preincubation of the cortical membranes with [^{3}H]Tz for 150 min by the addition of quinuclidinyebenzilate (1 μM) to prevent rebinding of [^{3}H]Tz (Fig. 1B). The dissociation data could be described by a monoexponential function with a half-time of about 70 min [$k_{-1} = (1.8 \pm 0.2) \times 10^{-4}$ sec^{-1}).

Binding of [^{3}H]Tz as a function of increasing receptor concentration. A low concentration of [^{3}H]Tz (68 pM) was incubated with increasing concentrations of cortical membranes (0.5–4.0 mg/ml) and, hence, muscarinic receptors (0.8–6 nM). The percentage of total [^{3}H]Tz specifically bound to the receptors after 2-hr incubation at 30° was estimated by counting the pellets and supernatants after centrifugation. The two estimates of bound [^{3}H]Tz agreed with each other. Somewhat to our surprise, the percentage of added [^{3}H]Tz bound to muscarinic receptors tended to asymptote at 50% at high receptor concentrations (Fig. 2A). For comparison, over 95% of added [^{3}H]NMS (0.1 nM) was bound at 4 mg/ml membrane protein under comparable conditions (Fig. 2A).

A double reciprocal plot of [protein]$^{-1}$ versus [[^{3}H]Tz bound]$^{-1}$ (see Materials and Methods) was linear and extrapolated to 50% of the added radioactivity at [protein]$^{-1} = 0$, i.e., [receptor]$\rightarrow\infty$ (Fig. 2B).

This result suggested that either the [^{3}H]Tz was radiochemically impure or that there were optical isomers of [^{3}H]Tz, with only one isomer binding with high affinity. The former possibility was disfavored by the fact that the [^{3}H]Tz appeared to be radiochemically and chemically pure by thin layer chromatography, high performance liquid chromatography, and mass spectrometry (data not shown).

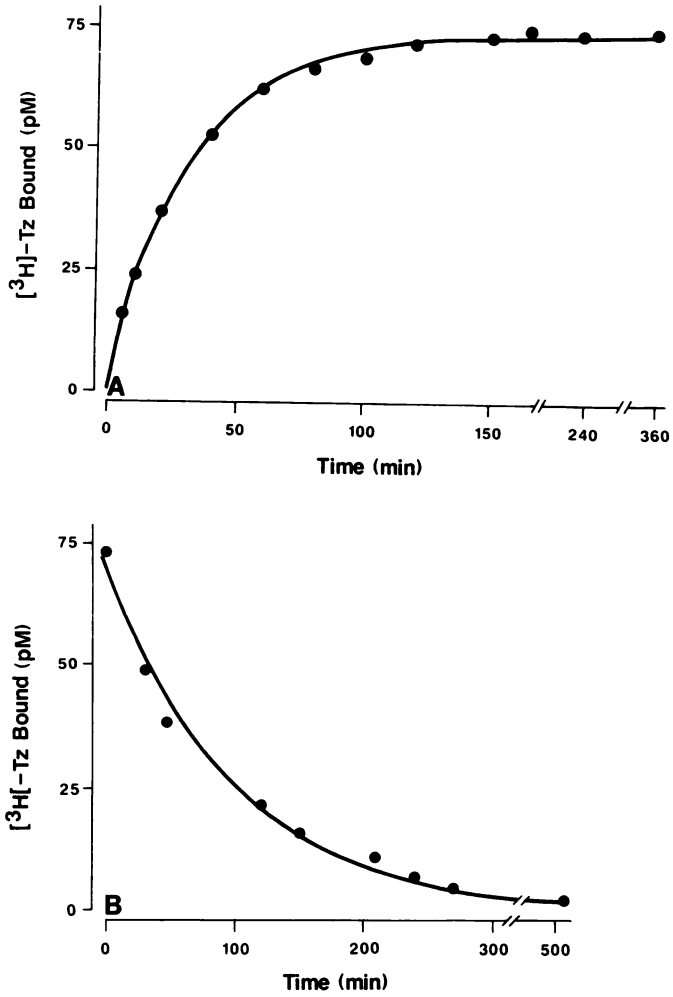

Fig. 1. Kinetics of [^{3}H]Tz binding to rat cortical muscarinic receptors. A, Rat cortical membranes were incubated at 30° with [^{3}H]Tz (0.40 nM) for the times indicated. The *curve* through the *data points* is a best fit single exponential time course, with a rate constant of $(4.3 \pm 0.3) \times 10^{-4}$ sec^{-1} ($t_{½} = 23$ min). The depletion of total [^{3}H]Tz at equilibrium in this experiment was 28%. The mean rate constant was $(5.5 \pm 0.7) \times 10^{-4}$ sec^{-1} (mean ± SE; five experiments). B, After preequilibrating rat cortical membranes with [^{3}H]Tz (0.4 nM) for 150 min, the dissociation rate of [^{3}H]Tz at 30° was measured by adding 3-quinuclidinylbenzilate (1 μM) to the samples and measuring the specifically bound [^{3}H]Tz at the indicated times. The *curve* through the data *points* is a best fit single exponential with a rate constant of $(1.8 \pm 0.2) \times 10^{-4}$ sec^{-1}. The mean value k_{-1} was $(1.8 \pm 0.2) \times 10^{-4}$ sec^{-1} (mean ± SE; four experiments). In fact, the dissociation rate profile appeared to be biphasic in all experiments, with approximately 90% of the [^{3}H]Tz at low concentrations dissociating with a $t_{½}$ of 87 ± 2 min (mean ± SE; four experiments). The residual off-rate was much faster ($t_{½} < 5$ min) and may correspond to the binding of [^{3}H]Tz to non-M_1 sites, inasmuch as at higher concentrations of [^{3}H]Tz the proportion of this fast dissociating component increased towards the value (30–40%) found for the low affinity binding component in equilibrium binding experiments.

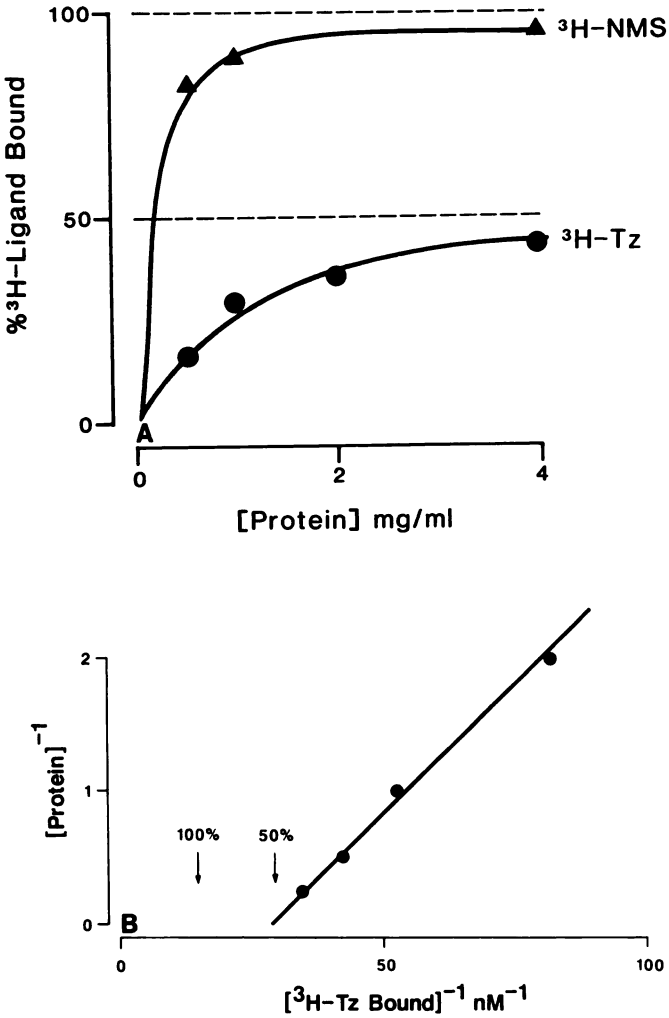

Fig. 2. Receptor-bound [³H]Tz and [³H]NMS as a function of increasing receptor concentration. Membranes from rat cerebral cortex (0.5–4.0 mg of protein/ml) were incubated with [³H]Tz (68 pM) or [³H]NMS (100 pM) for 2 hr at 30°. The percentage of total added radioactivity that was specifically bound to muscarinic receptors was estimated by counting the membrane pellets and the supernatants (0.5 ml) after centrifugation. These two estimates agreed to within ±2%. There was no indication of 'true' nonspecific binding of [³H]Tz to membranes, as shown by the fact that the radioactivity measured in the 0.5-ml supernatants from estimates of nonspecific binding did not vary with protein concentration. The data shown are from one of four experiments, all of which gave the same result. A, Plot of percentage of ³H-ligand bound as a function of protein concentration. The *dashed lines* at 100% and 50% emphasize the asymptotic approach of the percentage of [³H]NMS and [³H]Tz bound at high protein concentrations to 100% and 50%, respectively. B, Double-reciprocal plot of the data for [³H]Tz shown in A. The intercept of the *straight line* on the *x* axis (extrapolation to infinite protein concentration) is at 50% of the added [³H]Tz. The position of the intercept if 100% of the [³H]Tz were binding is shown for comparison.

Racemization studies. If optical isomers of [³H]Tz were present, we thought it might be possible to confirm their existence by racemization. To this end, a scheme was devised by which the conversion of the putative inactive isomer to the active isomer could be tested. In this protocol, a low concentration of [³H]Tz (0.17 nM) was treated batchwise with cortical membranes (2 mg/ml). The unbound [³H]Tz (59% of the original activity) was collected as the supernatant after centrifugation of the receptor-bound [³H]Tz. The unbound "depleted" [³H]Tz was incubated with fresh membranes (0.5 mg/ml) and the percentage of the radioactivity bound to muscarinic receptors was compared with the percentage of the radioactivity bound when the same concentration of an untreated batch of [³H]Tz was used.

Only 4% of the depleted [³H]Tz bound to muscarinic receptors, whereas 21% of the control untreated [³H]Tz at the same concentration was bound to the receptors. In a converse experiment, [³H]Tz (0.5 nM) was treated with membranes (2 mg/ml) and the bound [³H]Tz (29% of the added [³H]Tz) was released from the receptors by incubation at 85° for 10 min. Exposure of the released [³H]Tz to fresh membranes (2 mg/ml) resulted in the binding of 74% of the [³H]Tz to the receptors. These experiments demonstrated the lower propensity of the depleted [³H]Tz to bind to muscarinic receptors and the increased ability of receptor-bound [³H]Tz to rebind to the receptors.

The depleted [³H]Tz was heated at a given temperature for increasing lengths of time and the ability of the heat-treated depleted [³H]Tz to bind to muscarinic receptors was examined. As shown in Fig. 3, the effect of heat treatment was to increase the percentage of [³H]Tz bound to the receptors from 4% towards the control value of 21%. This is the behavior expected if racemization were occurring. Control experiments showed that untreated [³H]Tz was radiochemically and chemically stable (<15% decomposition after 24 hr at 110°) and the receptors bound the same percentage of the radioactivity after such treatment.

The rate of racemization was very temperature sensitive, with half-times for racemization varying from approximately 2.5 hr at 120° to 48 hr at 100° and >200 hr at 90°C. The estimated rate constants gave a linear Arrhenius plot (Fig. 3B) over the temperature range examined, with a calculated energy of activation for the racemization process of 35 kcal/mol.

Binding of [³H]Tz to cerebral cortical muscarinic receptors. The equilibrium binding of [³H]Tz (5 pM to 40 nM) to rat cortical muscarinic receptors was examined at 30°. Nonspecific binding was linear with increasing [³H]Tz concentration (data not shown). The ratio of specific to nonspecific binding was high and varied from about 30:1 for concentrations below 0.5 nM total [³H]Tz to approximately 2:1 at 40 nM [³H]Tz. A representative Scatchard plot of one binding curve is shown in Fig. 4. The curvilinear nature is apparent. After correction for depletion, including the fact that only 50% of the [³H]Tz was capable of binding, nonlinear least squares analysis of the data according to a two-site model gave 70 ± 5% of a high affinity site, log K_A = 9.42 ± 0.03, and 30 ± 5% of a low affinity site, log K_A = 8.48 ± 0.13 (both log K_A values refer to that of the active isomer), with the estimate of the total concentration of binding sites being 513 ± 5 pM. In a parallel experiment, the concentration of [³H]NMS binding sites was estimated to be 490 ± 10 pM.

It, therefore, appears that the binding of [³H]Tz to all the muscarinic receptor sites found in membranes from rat cerebral cortex can be measured. These sites are heterogeneous with regard to Tz binding.

Binding studies of (+)-Tz, (−)-Tz, and (±)-Tz. As a consequence of the prediction of the existence of optical isomers of Tz, efforts were made to obtain the pure isomers. The binding properties of those isomers and their selectivity for muscarinic receptors in different rat tissues were examined.

In the rat cerebral cortex, competition studies were performed using [³H]Pz (1 nM), [³H]PrBCh (1 nM), and [³H]NMS

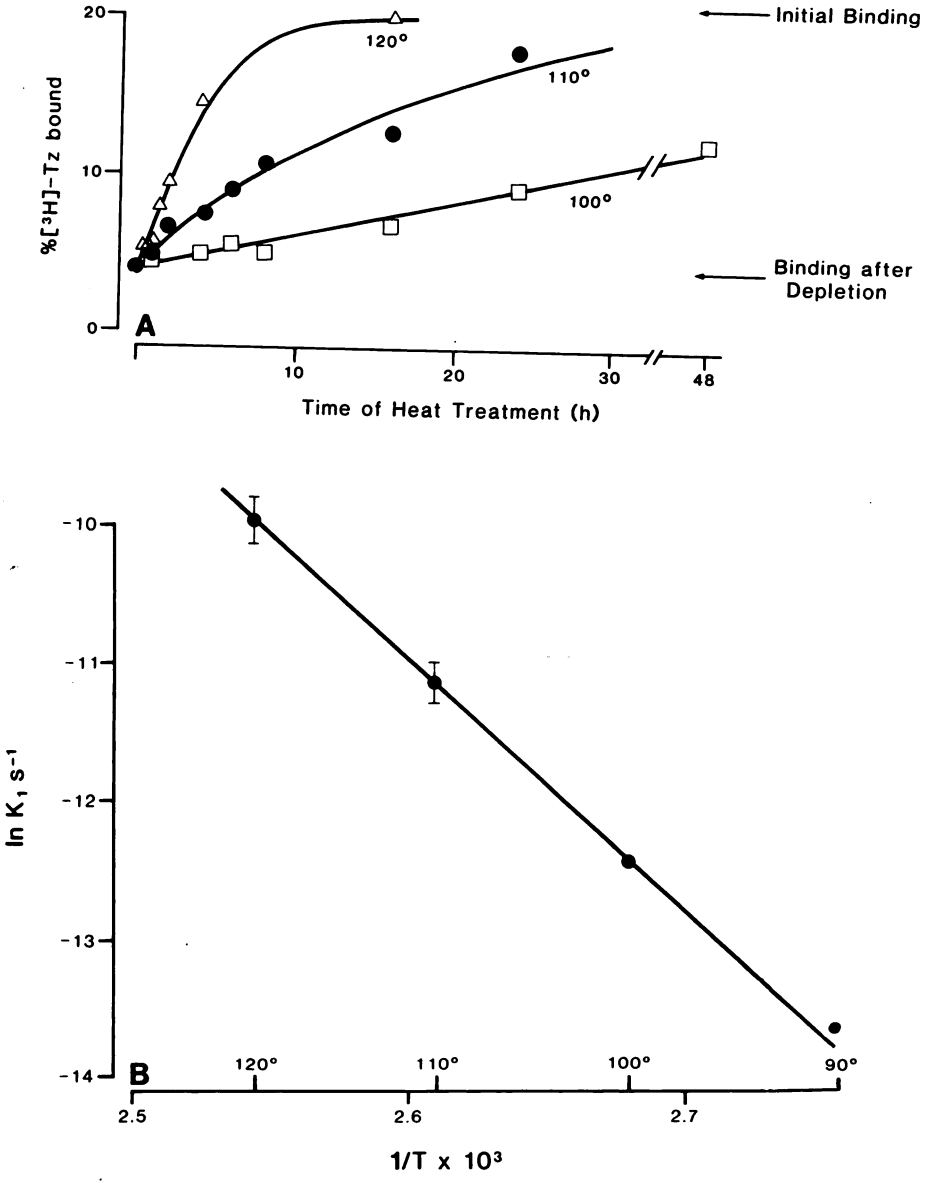

Fig. 3. Time course of racemization of [^{3}H]Tz at different temperatures. Rat cortical membranes (2 mg of protein/ml) were treated with [^{3}H]Tz (0.17 nM) for 2.5 hr at 30°. Of the added radioactivity, 41% bound to the muscarinic receptors. The supernatant obtained after centrifugation was heated for the stated times at 100° (□), 110° (●), or 120° (△). The ability of the heated supernatant (25 pM) to bind to muscarinic receptors on fresh membranes (0.5 mg of protein/ml; 30°; 2.5 hr) was examined (A). Binding increased from the original value of 4% and approached the value (21%) found for 25 pM fresh [^{3}H]Tz or [^{3}H]Tz that had been heated at 110° for 24 hr. B, Arrhenius plot of the rate constants for the racemization of [^{3}H]Tz at different temperatures. The data from Fig. 4A together with additional data obtained for separate experiments at 90°, 110°, and 120° were fitted to a simple exponential function. In the case of the data at 90° and 100°, the fit was constrained so that the value at $t \rightarrow \infty$ was 20% . In the case of the data at 120°, the best fit value was not significantly different from 20% (20.8 ± 1.2%). The free energy of activation calculated from the Arrhenius plot is 35 kcal/mol.

(1.3 nM) as tracers. The experiments were carried out at low protein concentrations (0.3 mg/ml) to reduce (but not abolish) depletion of the active isomer of Tz. [^{3}H]Pz was used as a monitor for those sites with a high affinity for Pz (M_1 sites), whereas [^{3}H]PrBCh and [^{3}H]NMS were used as monitors of all the muscarinic receptor sites, that is, M_1 sites and non-M_1 sites. Whereas the concentrations of [^{3}H]Pz and [^{3}H]PrBCh were well below their K_D values (10^{-8} M) and, thus, the inhibition curves provided a relatively unperturbed description of the binding curves of the competing ligands, the inhibition curves for [^{3}H]NMS were displaced to the right from the intrinsic occupancy curves because the [^{3}H]NMS concentration was considerably above its dissociation constant. However, this latter assay minimizes the problems of depletion of the total concentration of Tz resulting from its binding to the receptors.

The occupancy concentration curves for (+)- and (−)-Tz, derived from competition experiments with [^{3}H]Pz, [^{3}H]PrBCh, and [^{3}H]NMS are shown in Fig. 5. It is clear that (+)-Tz is over 100-fold more potent than (−)-Tz. In addition, (−)-Tz shows a considerably lower ability than (+)-Tz to discriminate between subpopulations of muscarinic receptors in rat cerebral cortex. This is manifest by the fact that the (−)-Tz/[^{3}H]PrBCh and (−)-Tz/[^{3}H]Pz curves both approximate to simple mass action curves and are much closer together than the corresponding curves for (+)-Tz. The (+)-Tz/[^{3}H]PrBCh competition curve is a 'flat' curve (Hill coefficient = 0.70) and the affinity constants found by analysis of this curve [and the (+)-Tz/[^{3}H]NMS competition curve] according to a two-site model agree well, as would be expected, with the parameters obtained by analysis of the direct binding curve of [^{3}H]Tz (Table 1). Furthermore, the affinity constant for the high affinity site agrees with the affinity calculated from the (+)-Tz/[^{3}H]Pz competition curve, where only the binding to the receptor subtype having a high affinity for Pz is monitored.

These studies show that, in the resolved sample of (−)-Tz, it is (−)-Tz and not a residual impurity of (+)-Tz that is binding to muscarinic receptors, because the selectivity of the (−)-Tz sample is not the same as that of (+)-Tz. In addition, it is shown that the muscarinic receptor subtype that binds Pz with high affinity is also the subtype that binds Tz with high affinity. Fig. 5 also shows that, as would be predicted, (+)-Tz is a factor of 2 (0.3 log unit) weaker than (+)-Tz.

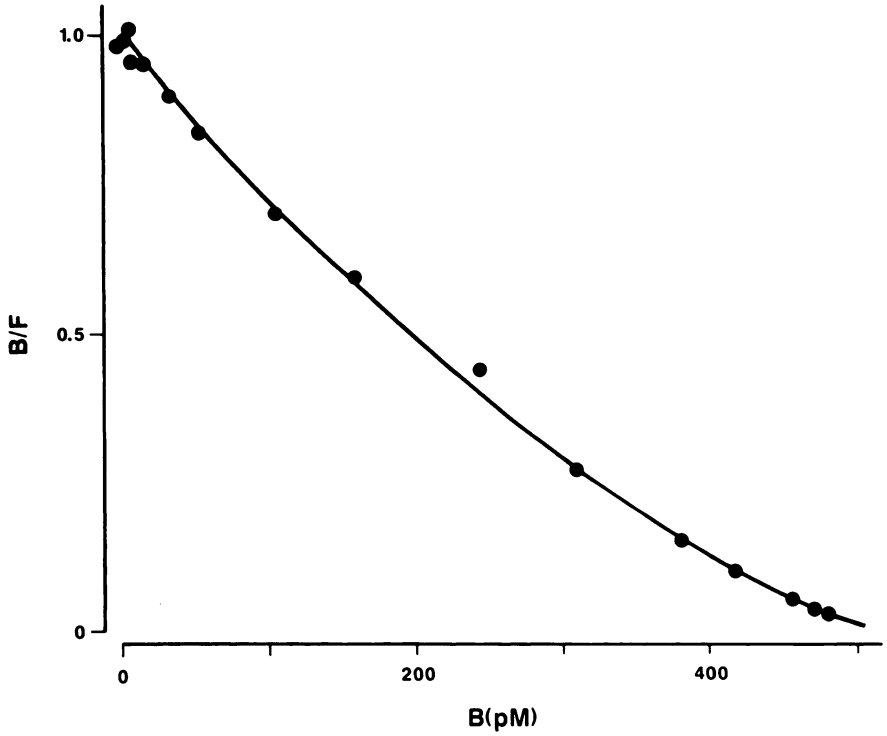

Fig. 4. Scatchard plot of the binding of [^{3}H]-Tz to muscarinic receptors on rat cortical membranes. Rat cortical membranes (0.3 mg/ml) were incubated with [^{3}H]Tz (6 pM to 35 nM) for 2.5 hr at 30°. Nonlinear least squares analysis of the data using a two-site model gave 70 ± 5% high affinity sites (log K_A = 9.42 ± 0.03) and 30 ± 5% low affinity sites (log K_A = 8.48 ± 0.13), the affinity constants being those for the active (+)-enantiomer. These data are taken from one of four independent experiments, which yield very similar results (Table 1).

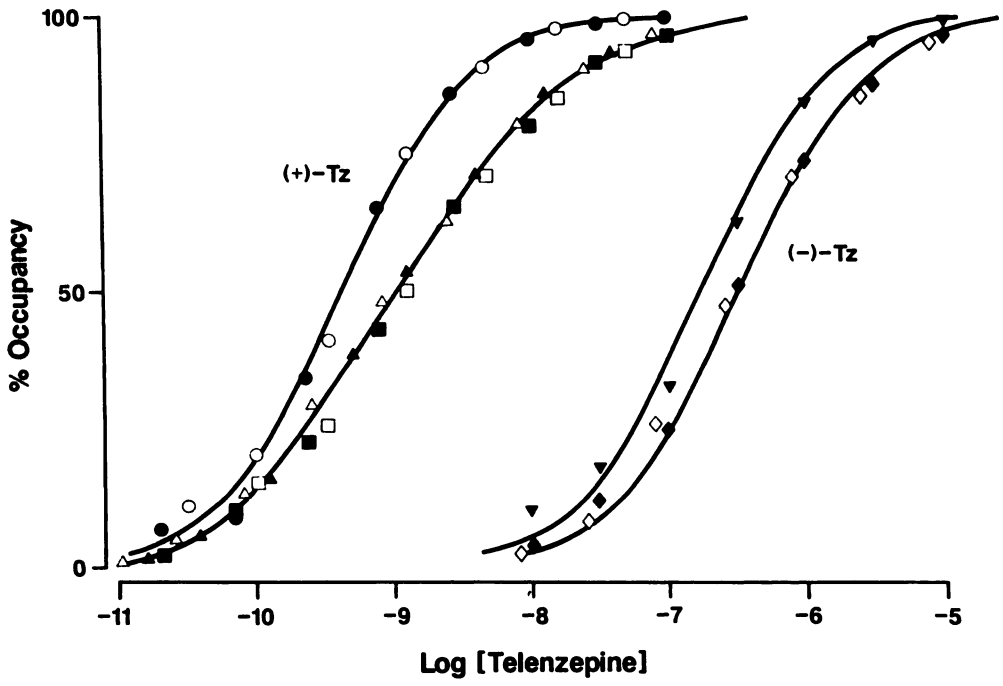

Fig. 5. Occupancy/concentration curves for the binding of (+)- and (−)-Tz to muscarinic receptors in rat cerebral cortex. The binding of (+)-Tz (●, ■, ▲) and (−)-Tz (▼, ♦, ◇) were assessed by competition experiments using [^{3}H]-Pz (1 nM; ●, ▼) to label M_1 sites and [^{3}H]PrBCh (1 nM; ▲, ♦) or [^{3}H]NMS (1.3 nM; ■, ◇) to label all the sites. The [^{3}H]NMS data have been corrected by 1.1 log unit of concentration to account for the high receptor occupancy by the [^{3}H]NMS (K_A = 9 × 10^9 M^{-1}). Also included in this figure are data for (+)-Tz calculated from competition experiments using (+)-Tz, assuming that only the (+)-isomer of the racemate binds with high affinity. These data are represented by the *open symbols* (○, △, □), equivalent to the *closed symbols* used to depict the binding of the pure (+)-enantiomer. The data for (+)-Tz were corrected for depletion. The total concentration of muscarinic receptor sites in this set of experiments, determined using 10 nM [^{3}H]NMS, was 0.52 nM. The data show the superimposability of the occupancy curves estimated using [^{3}H]PrBCh or [^{3}H]NMS as radioligand and the (+)-Tz occupancy curves using (+)-Tz or (±)-Tz as the competing ligand. All the curves are the best fit simple mass action binding curves except for the (+)-Tz/[^{3}H]PrBCh ([^{3}H]-NMS) occupancy curve, which is the best fit of the data to a two-site model with 49 ± 9% high affinity sites (log K_A, 9.55 ± 0.12) and 51 ± 9% low affinity sites (log K_A, 8.35 ± 0.15).

TABLE 1

Affinity constants for the binding of (+)- and (−)-Tz to muscarinic receptors on membranes from different tissues

Values stated are mean log K_A values of 2–5 independent experiments ± standard error (range for n = 2). Values for the high affinity M_1 sites in the cortex were obtained from competition experiments against [^{3}H]Pz (1 nM) and from two-site analysis of data from competition experiments against [^{3}H]PrBCh (1 nM) or [^{3}H]NMS (1–2 nM). Affinity constants for the low affinity sites (40 ± 7% of the total sites) were determined by the two-site analysis of the competition curves against the latter two radioligands. The values for (+)-Tz were combined from experiments using resolved (+)-Tz and (±)-Tz.

	(+)-Tz	(−)-Tz	Stereoselectivity
Cerebral Cortex			
High affinity sites	9.48 ± 0.07 (5)	6.79 ± 0.05 (2)	510 ± 80
Low affinity sites	8.23 ± 0.09 (3)	6.03 ± 0.05 (2)[a]	160 ± 25
Heart	7.77 ± 0.02 (5)	5.89 ± 0.03 (2)	75 ± 7
Lacrimal Gland	8.39 ± 0.03 (5)	6.20 ± 0.03 (2)	160 ± 25

[a] Obtained by two-site analysis of the competition curve with the model restrained by having 60% of the high affinity site, log K_A, 6.79.

Fig. 6 shows the occupancy concentration curves for (+)- and (−)-Tz binding to the cardiac and glandular muscarinic receptor subtypes. As found in the cortical studies, (+)-Tz is very much more potent than (−)-Tz. It also exhibits a slightly greater selectivity for the glandular receptors. All the occupancy curves approximate to simple mass action curves, and the affinity constants for a given isomer are lower than those found for the sites labeled by [^{3}H]Pz in the cerebral cortex (Table 1). The affinity constant for the lacrimal receptor, under these experimental conditions, is similar to that for the cortical non-M_1 receptor, inferred from the two-site fit of the competition curves using the nonselective tritiated antagonists or from the [^{3}H]Tz saturation curve.

An interesting feature of these data is that the stereoselectivity of the isomers varied for the different subtypes (Table 1). The value was approximately 500 for M_1 receptors in the cortex, approximately 160 for the glandular receptors and non-M_1 cortical receptors, and approximately 80 for the cardiac receptors. These data reinforce the fact that the levels of (+)-Tz in the sample of (−)-Tz, if present, are very low.

Discussion

The important finding of this paper is that Tz exists as a mixture of enantiomers, which are resolvable. Although it is not obvious from the chemical structure that Tz is chiral, a three-dimensional structure reveals the asymmetry resulting from the nonplanarity of the tricyclic ring structure. This is illustrated in Fig. 7. Resolution of Tz was initially accomplished on a picomole scale by using muscarinic receptors to selectively bind the active enantiomer of [^{3}H]Tz. Subsequently, it became possible to obtain the enantiomers on a larger scale.

The energy barrier for racemization is very high (35 kcal/mol) and was necessarily determined at high temperatures (90–120°) at which, fortunately, [^{3}H]Tz is stable. This energy barrier has recently been determined over the same temperature range using optical rotation methods on the pure enantiomers.[1] The value obtained, 35.5 kcal/mol, is in excellent agreement with our estimate. The extrapolation of the Arrhenius plot (Fig. 3B) to room temperature (20°) suggests a half-time for racemization in aqueous solutions at neutral pH of over 1000 years! A more relevant figure is the time for 0.1% racemization at 37°, because this level of racemization would begin to produce an increase in the apparent affinity of a sample of the inactive isomer in a binding or a functional experiment. This estimate is 400 hr and, therefore, even accounting for the uncertainties associated with the very considerable extrapolation of the Arrhenius plot, it is unlikely that racemization will be a significant problem in experiments using the inactive isomer.

In contrast, the energy barrier for interconversion of the enantiomers of Pz has been calculated to be 18–20 kcal/mol, too low a level to permit resolution at room temperatures (10). The equivalent calculations for another tricyclic muscarinic antagonist, UH-AH 37, indicate an energy barrier of 40 kcal/mol and enantiomers of this compound have been resolved.[2] [UH-AH 37 has a structure similar to that of Pz but the pyridine

[1] G. Grundler, personal communication.
[2] G. Trummlitz, personal communication.

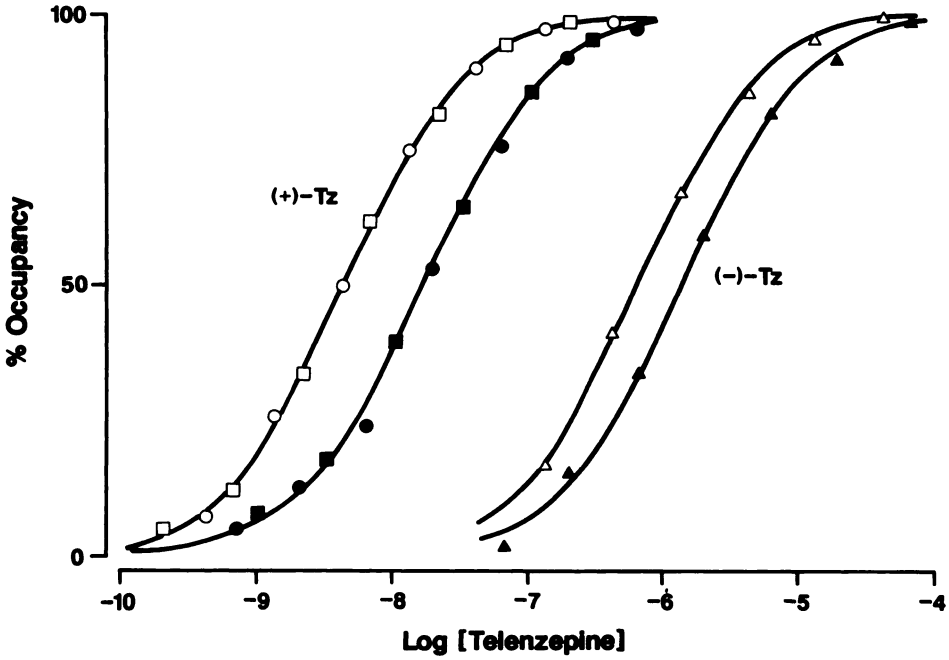

Fig. 6. Occupancy concentration curves for the binding of (+)- and (−)-Tz to muscarinic receptors from rat myocardium and lacrimal glands. The binding of (+)-Tz (*circles*) and (−)-Tz (*triangles*) were assessed by competition experiments using [^{3}H]NMS (0.27 nM; *solid symbols*, myocardium; 0.23 nM, *open symbols*, lacrimal gland). The data are corrected for the shift produced by receptor occupancy by [^{3}H]NMS (log K_A, 9.30 and 9.76 for myocardial and lacrimal receptors, respectively). Included in this figure are receptor occupancy data obtained using (±)-Tz (*squares*) assuming that only the (+)-isomer of the racemate binds with high affinity.

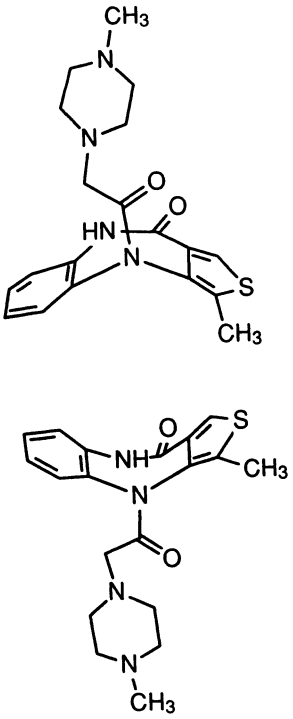

Fig. 7. The enantiomers of Tz. Illustration of the chirality resulting from the nonplanarity of the tricyclic ring.

ring has been replaced by chlorobenzene and the piperazine ring by piperidine.] It appears that bulky substituents (Cl— or CH_3—) in positions on the rings *ortho* to the nitrogen atom bearing the side chain hinder the interconversion of the boat conformers of the seven-membered ring. Another way of viewing the chirality of isomerization is by considering these to be due to slow inversion of the chiral N atom joining the two aromatic rings and concomitant inversion of the endocyclic amide linkage.

[^{3}H]Tz appears to be a good radioligand for binding studies on muscarinic receptors. The ratio of specific to nonspecific binding is excellent in both centrifugation assays described here and in filtration assays described elsewhere (6). Typical values for calf and rat forebrain receptors are 15–30:1 at subnanomolar concentrations of [^{3}H]Tz. These values are comparable to those found for [^{3}H]NMS. However, precautions should be taken in binding studies using [^{3}H]Tz to avoid the insidious artifacts caused both by the depletion of (+)-[^{3}H]Tz caused by its binding to receptors and by its slow kinetics of binding. Problems caused by depletion start to become significant when over 20% of a radioligand binds to receptors. Because only 50% of [^{3}H]Tz binds to muscarinic receptors, attempts should, therefore, be made to keep depletion below 10% of the added radioactivity. The effects of a 60% depletion of (+)-[^{3}H]Tz are manifest in the on-rate measurement of [^{3}H]Tz (Fig. 2A). A simple calculation, assuming a K_A for (+)-[^{3}H]Tz of 3×10^9 M^{-1} and the parameters given in the legend to Fig. 1A, shows that this depletion speeds up the measured on-rate from a $t_{1/4}$ of 43 min (in the absence of depletion) to one of 27 min, which is very close to the observed value. Depletion of this magnitude in the binding of [^{3}H]Tz also has the effect of increasing the IC_{50} value of a competing ligand up to 2-fold above the value calculated with depletion, without changing the shape of the curve (11).

One advantage of [^{3}H]Tz is that its affinity and selectivity are such that it is possible by the choice of concentrations to selectively label either M_1 sites or all the sites and, hence, to be able to monitor directly the binding and kinetics of a selective muscarinic drug at sites for which it does not have its highest affinity. The very slow off-rate of Tz also means that it is possible to use it as a pseudoirreversible ligand to selectively block M_1 sites and to monitor the binding of rapidly equilibrating ligands to the non-M_1 sites.

The preparation and examination of the binding properties of Tz have revealed a number of points. Firstly, the affinities measured for the inactive (−)-isomer reflect the binding properties of the isomer itself and not a residual minor contaminant of the (+)-isomer. This conclusion is drawn from the fact that the selectivity of the (−)-isomer between receptor subtypes is different from that of the (+)-isomer. From the highest stereoselectivity observed, approximately 500-fold at M_1 sites in the cortex, the maximum level of (+)-enantiomer in the (−)-Tz preparation is 0.2%. The second conclusion is that the selectivity of the (−)-enantiomer is much lower than that of the (+)-isomer. In other words, muscarinic antagonist selectivity depends on the chirality of the ligand. The selectivity of the enantiomers in functional studies will be reported elsewhere.

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References

1. Eltze, M., S. Gonne, R. Riedel, B. Schlotke, C. Schudt, and W. A. Simon. Pharmacological evidence for selective inhibition of gastric acid secretion by telenzepine, a new antimuscarinic drug. *Eur. J. Pharmacol.* **112**:211–224 (1985).
2. Eltze, M. Muscarinic M_1 and M_2 receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol.*, 151:205–221 (1988).
3. Watson, M., W. R. Roeske, and H. I. Yamamura. [^{3}H]-Pirenzepine selectively identifies a high affinity population of muscarinic cholinergic receptors in the rat cerebral cortex. *Life Sci.* **31**:2019–2923 (1982).
4. Watson, M., W. R. Roeske, and H. I. Yamamura. [^{3}H]-Pirenzepine and (−)-[^{3}H]quinuclidinylbenzilate binding to rat cerebral cortical and cardiac muscarinic cholinergic sites. II. Characterization and regulation of antagonist binding to putative muscarinic subtypes. *J. Pharmacol. Exp. Ther.* **237**:419–427 (1986).
5. Luthin, G. R., and B. B. Wolfe. [^{3}H]Pirenzepine and [^{3}H]quinuclidinylbenzilate binding to brain muscarinic cholinergic receptors: differences in measured receptor density are not explained by differences in receptor isomerization. *Mol. Pharmacol.* **26**:164–169 (1984).
6. Schudt, C., C. Auriga, B. Kinder, and N. J. M. Birdsall. The binding of [^{3}H] telenzepine to muscarinic acetylcholine receptors in calf forebrain. *Eur. J. Pharmacol.* **145**:87–90 (1988).
7. Schudt, C., C. Auriga, P. Eveleigh, E. C. Hulme, and N. J. M. Birdsall. [^{3}H]-Telenzepine: a new M_1-selective muscarinic antagonist. *Trends Pharmacol. Sci.* (Suppl. 9) 81 (1988).
8. Hammer, R., C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Pirenzepine distinguishes between subclasses of muscarinic receptor. *Nature (Lond.)* **283**:90–92 (1980).
9. Hulme, E. C., N. J. M. Birdsall, A. S. V. Burgen, and P. Mehta. The binding of antagonists to brain muscarinic receptors. *Mol. Pharmacol.* **14**:737–750 (1978).
10. Trummlitz, G., G. Schmidt, H.-V. Wagner, and P. Luger. Conformational analysis of the antiulcer drug pirenzepine. *Arzneim.-Forsch/Drug Res.* **34**:849–859 (1984).
11. Goldstein, A., and R. W. Barrett. Ligand dissociation constants from competition binding assays: errors associated with ligand depletion. *Mol. Pharmacol.* **31**:603–609 (1987).

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