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Cell Biology

Benzodiazepines that bind at peripheral sites inhibit cell proliferation

(binding site/correlated response/Scatchard analysis)

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ABSTRACT **[^{3}H]Ro5-4864 binds to mouse thymoma cells in a specific, saturable, and reversible manner. Scatchard analysis shows a single class of binding sites with a K_d of 4.4 nM and a B_{max} of 477 fmol per 10^6 cells. This benzodiazepine binding site is of the peripheral type, based on the relative potencies of Ro5-4864 and clonazepam in competing for [^{3}H]diazepam binding. Fifteen benzodiazepines that bind to this site with affinities ranging from 6 nM to 1 μM also reversibly inhibit the proliferation of thymoma cells in culture at the micromolar dose range. There is a strong positive correlation ($r = 0.85$) between the binding constants of these compounds for the peripheral-type sites and their ED_{50} in inhibiting the uptake of [^{3}H]thymidine into the cells. These sites may be involved in the regulation of thymoma cell proliferation.**

The specific binding of benzodiazepines (BZDs) has been observed in many tissues and cell types and can be pharmacologically separated into two classes. The first and most studied are the central-type receptors in brain that are thought to mediate the known clinical effects of the BZDs (1–3). The other class is the peripheral-type binding site found in a variety of tissues and cells, including kidney (4, 6), heart (5, 6), platelets (7), mast cells (8), lymphocytes (9), many cell lines (10), and brain (11, 12). BZD binding to this site has no known physiological consequences. Several pharmacological effects of the BZDs have been reported (13–15); however, none has been supported by a positive correlation between the peripheral-type binding and the biological response. We now report the presence of specific, saturable, and peripheral-type BZD binding sites on an AKR mouse thymoma line, BW 5147.G.1.4.Oua^r.1. (Cell Distribution Center, Salk Institute, San Diego, CA), and that the BZDs that bind to these sites inhibit the proliferation of the thymoma cells. The relative potencies of 15 BZDs as antiproliferative agents correlate significantly and positively with their binding constants, suggesting a possible relationship between the growth-inhibitory effect of the BZDs and their binding to the peripheral-type sites.

MATERIALS AND METHODS

Cell Culture and [^{3}H]Thymidine Uptake Assay. Thymoma cells were grown at 37°C in Iscove's modified Dulbecco's medium (GIBCO) containing 5% fetal calf serum in a humidified incubator with 5% CO_2/95% air. For the [^{3}H]thymidine assay, cells were seeded in 96-well tissue culture clusters (Costar, Cambridge, MA) at 1×10^5 cells per well for the 22-hr assay or 1×10^4 cells per well for the 44-hr assay. BZDs were diluted from ethanol stock solutions into Iscove's medium and then were added directly to the wells to yield the appropriate final concentration in a total volume of 200 μl. The ethanol concentration was never >0.4% and controls were included in the experiments to show that this had no effect on the proliferation of thymoma cells. After either 22 hr or 44 hr of incubation at 37°C, each well received 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (New England Nuclear, 101.0 Ci/mmol) and unlabeled 0.5 μM thymidine. After 2 hr, the cells were harvested and washed over glass fiber filters by a Microharvester (Bellco Glass). The filters were dried and placed in 4 ml of Aquasol (New England Nuclear) in minivials for scintillation spectroscopy.

Binding Studies. The thymoma cells were centrifuged at $100 \times g$ and washed once in balanced glucose salt buffer (5.0 mM KCl/120.0 mM NaCl/5.0 mM Na_2HPO_4/5.0 mM Tris/0.6 mM $CaCl_2$/1.0 mM $MgSO_4$/5.5 mM glucose, final pH = 7.4). Binding of [^{3}H]Ro5-4864 (New England Nuclear, 73.8 Ci/mmol) to the cells was carried out in this buffer at 0°C for 40 min with the indicated concentrations of labeled ligand in a total volume of 250 μl. [^{3}H]Diazepam (New England Nuclear, 87.6 Ci/mmol) binding was also carried out at 0°C but the incubation time was 15 min. Binding reaction was terminated by the addition of 3 ml of ice-cold Dulbecco's phosphate-buffered saline and rapid filtration over Whatman GF/B glass fiber filters, followed by two washes of 3 ml of ice-cold phosphate-buffered saline. The filters were air dried and placed in 4 ml of Aquasol for liquid scintillation spectroscopy. Specific binding was defined as the difference between total binding (in the absence of unlabeled ligand) and nonspecific binding (in the presence of unlabeled 10 μM diazepam).

The benzodiazepine compounds were obtained from Hoffmann–La Roche, Inc. All reagents were obtained from commercial sources.

RESULTS

The specific binding of [^{3}H]Ro5-4864, a BZD selective for peripheral-type sites (4), was saturable, whereas nonspecific binding increased linearly with increasing concentrations of the labeled ligand (Fig. 1). Scatchard analysis of the specific binding showed a single class of sites with a K_d (mean ± SEM) of 4.4 ± 1.1 nM ($n = 5$) and a B_{max} (maximal amount of ligand bound) of 477 ± 98 fmol per 10^6 cells. The Hill slope coefficient was 1.01 ± 0.03 for the same binding experiments, further indicating the homogeneity and noncooperative nature of these binding sites. [^{3}H]Diazepam, which binds to both central and peripheral-type BZD sites (4), was inhibited from binding to thymoma cells by 200 nM Ro5-4864 but not by 10 μM clonazepam, the central-selective BZD (data not shown), indicating that all of the BZD sites on the cells are of the peripheral type.

The specific binding of [^{3}H]Ro5-4864 to thymoma cells reached a plateau after 30 min of incubation at 0°C and dissociated in a monophasic manner with a $t_{1/2}$ of 14.5 min when an excess (10 μM) of unlabeled diazepam was added to the incubation (data not shown). The rate constant of association was 6.8×10^7 $M^{-1} \cdot min^{-1}$ and the rate constant of dissociation was 0.048 min^{-1}. Overall, in terms of kinetics, satur-

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Abbreviation: BZD, benzodiazepine.

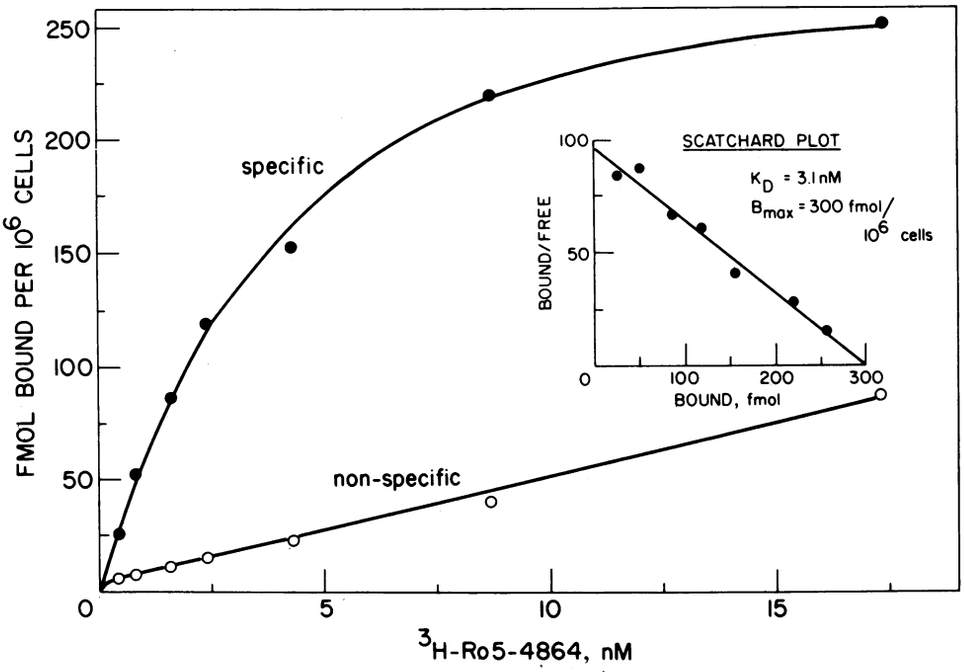


FIG. 1. Saturation and Scatchard plots of [^{3}H]Ro5-4864 binding to thymoma cells. Binding assays were performed as described in *Materials and Methods*. A typical experiment is shown (similar results were obtained in four additional experiments). (*Inset*) Scatchard plot of the binding data using a least-square fit to define the regression line (r = 0.97).

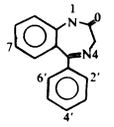
ability and specificity the peripheral-type BZD binding sites on thymoma cells are very similar to those found in other tissues and cell types (5–10).

A series of 15 BZDs were tested for their half-maximal concentrations (IC_{50}) required at 0°C to inhibit [^{3}H]Ro5-4864 binding to thymoma cells. The values obtained spanned about two orders of magnitude and ranged from 6 nM to 1 μM. These values, and the structures of the compounds, are shown in Table 1. Some representative dose–response curves are shown in Fig. 2. When the compounds are included in the thymoma cell culture at micromolar concentrations, they dramatically inhibited the increase in cell number during subsequent days of culture without being cytotoxic (as determined by trypan blue staining). Preliminary experiments indicated that the inhibition of cell proliferation was paralleled by an inhibition of incorporation of [^{3}H]thymidine into the DNA of the cells. Because direct cell count was impractical due to the availability of only small amounts of some BZDs and the large number of assays required, the thymidine assay was employed as a valid and convenient index of the antiproliferative potencies of the BZDs. The inhibitory effect appeared after 8 hr of incubation (roughly the doubling time of the cells) and was marked after 22 hr. The inhibition was reversible by diluting the cells into fresh medium that contained no drug (data not shown). All 15 BZDs inhibited [^{3}H]thymidine incorporation in a concentration-dependent manner (Fig. 2); the half-effective concentrations (ED_{50}) ranged from 6 μM to 180 μM (Table 1). These values were ranked with the IC_{50} of binding and plotted on a log–log scale (Fig. 3), resulting in a positive and highly significant correlation, with a coefficient of correlation r = 0.85 ($P <$ 0.001).

Table 1. Binding constants and antiproliferative potencies of BZDs

BZD	1	7	4′	2′	6′	4	IC_{50}	ED_{50}
Ro5-3448	CH_3	Cl	H	Cl	H	—	35.2	61.8
Ro5-3464	CH_3	H	H	H	H	—	1.0*	174.8
Ro5-4608	CH_3	H	H	Cl	H	—	214.6	176.4
Ro5-4864	CH_3	Cl	Cl	H	H	—	8.5	16.1
Diazepam	CH_3	Cl	H	H	H	—	77.8	26.1
Ro5-5115	CH_3	H	Cl	H	H	—	106.5	37.8
Ro5-5122	CH_3	H	F	H	H	—	441.7	89.1
Ro5-6524	CH_3	F	Cl	H	H	CH_3	340.0	53.3
Ro5-6531	CH_3	F	Cl	H	H	—	33.4	35.7
Ro5-6669	CH_3	Cl	OCH_3	H	H	—	18.7	13.0
Ro5-6900	CH_3	Cl	Cl	Cl	H	—	10.0	17.4
Ro5-6902	CH_3	Cl	Cl	H	Cl	H	23.4	19.9
Ro5-6945	†	Cl	Cl	H	H	—	14.3	5.6
Ro5-6993	‡	Cl	Cl	H	H	—	6.1	12.2
Ro7-5220	CH_3	Cl	H	Cl	Cl	—	93.1	48.8

The positions in the BZDs are shown in the structure



IC_{50} is the binding constant in nM and ED_{50} is the antiproliferative potency in μM as described in the legend to Fig. 2. Each value is the mean of at least four experiments.
*In μM.
†$CH_2CH{=}CH_2$.
‡CH_2CH_3.

Eight of the compounds shown in Fig. 3 also exhibited affinities for the central-type BZD receptor in brain that ranged from 1 nM to 2 μM (Ro5-3448, Ro7-5220, diazepam, Ro5-4608, Ro5-6900, Ro5-3464, Ro5-6902, and Ro5-6524, in decreasing order of binding affinity for the central-type sites). When these central-type binding affinities were ranked with the respective antiproliferative potencies, a log–log plot of no correlation resulted (r = 0.02). The oil–water partition coefficients of seven of these compounds were also ranked with their respective antiproliferative potencies and again no correlation was found (r = 0.03). Because this study specifically addressed the role of peripheral-type BZDs, only data obtained with these compounds were included in the correlation plot in Fig. 3. However, several central-selective BZDs were also examined. At concentrations that were up to the limit of their solubility (keeping below 0.4% ethanol),

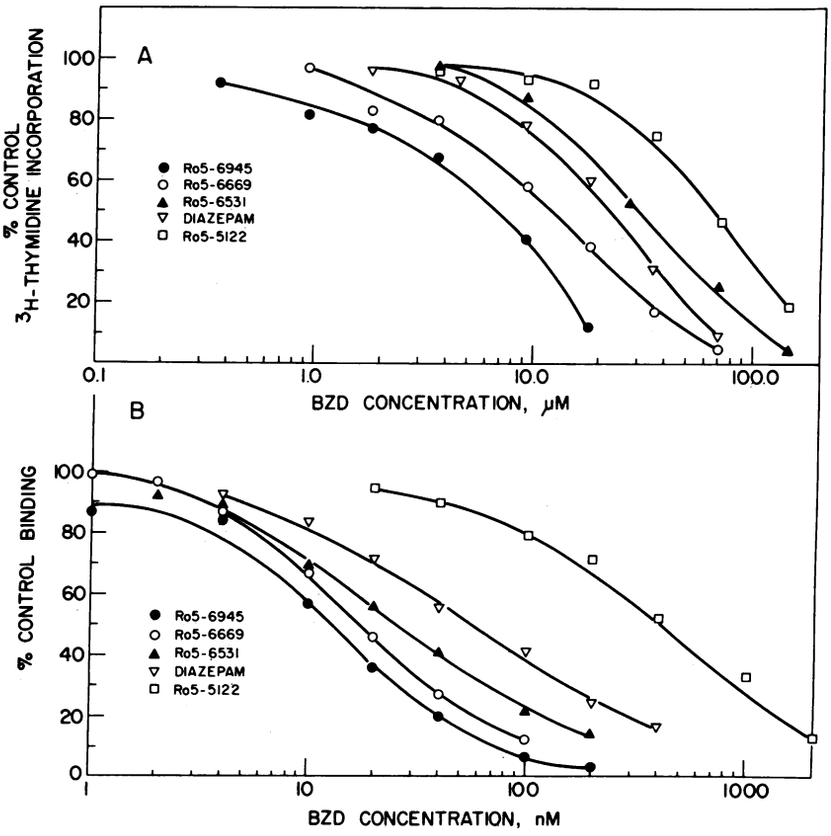


FIG. 2. Dose–response relationships for five representative BZDs inhibiting [^{3}H]thymidine incorporation by thymoma cells (*A*) and inhibiting [^{3}H]Ro5-4864 binding to thymoma cells (*B*). A typical experiment is shown for each compound (at least three additional experiments were performed with similar results). [^{3}H]Thymidine uptake was performed as described in *Materials and Methods*. Control radioactivity was expressed as 100% and all other radioactivity was expressed as % of control. ED_{50} was calculated as the dose required to reduce the [^{3}H]thymidine uptake to 50% of control. For binding assays, cells were incubated with [^{3}H]Ro5-4864 (0.8 nM) and various concentrations of BZDs at 0°C for 40 min. Control binding was defined as specific binding in the absence of any unlabeled BZDs and was expressed as 100%. IC_{50} was calculated as the concentration of BZD that inhibited [^{3}H]Ro5-4864 binding by half.

clonazepam (100 μM) and Ro5-4022 (100 μM) did not produce any significant inhibition of [^{3}H]thymidine incorporation. In addition, direct cell count showed that the central-selective BZD compounds Ro14-7434, Ro15-1788, Ro5-4022, and clonazepam produced no significant decrease in overall cell proliferation. Ro5-3027 and Ro5-2180, which did produce significant inhibition of [^{3}H]thymidine incorporation, also exhibited very weak affinities for the peripheral-type sites in addition to much higher affinities for the central-type sites. Nevertheless, these central-type BZDs are unequivocally less potent inhibitors of proliferation than are the peripheral-type BZDs.

DISCUSSION

The strong correlation between the antiproliferative potencies of a series of BZDs and their binding constants for the peripheral-type BZD sites suggests that these sites may be involved in the control of cell proliferation. The antiproliferative effect of the BZDs is not limited to the thymoma cells and has been observed in a number of cultured cell lines, including Swiss 3T3 cells (14), B103 and B104 neuroblastoma cells, and Friend erythroleukemia cells (unpublished observations). Although detailed correlation studies on these cell lines have not yet been carried out, these observations do indicate that the antiproliferative effect of the BZDs is a generalized phenomenon. The total lack of correlation between the central-type binding affinities of some of the compounds and their potencies in inhibiting cell proliferation rules out any direct involvement of the central-type BZD sites in this effect.

The discrepancy between the doses of BZDs needed to inhibit [^{3}H]thymidine uptake at 37°C and their binding constants at 4°C may be due to a number of factors: (*i*) binding of BZDs to the peripheral-type sites is inversely related to temperature, as is the case with the central-type sites (4, 7, 8); (*ii*) diazepam binds to plasma proteins (16) and this property is probably shared by all BZDs; and (*iii*) the metabolic fate of these compounds in culture for extended periods is unknown.

Clarke and Ryan (14) have reported that some BZDs inhibit the proliferation of Swiss 3T3 cells in culture. However, most of the compounds in their study were selective for central-type sites and displayed ED_{50} values substantially greater than 100 μM, in contrast to the ED_{50} of 10 μM that we obtained for the potent peripheral site-selective BZDs. The two most active compounds reported by Clarke and Ryan, diazepam and temazepam, bind to both peripheral and central-type sites, and the ED_{50} of 30 μM for diazepam is in good agreement with data reported here. Because the above authors did not determine BZD binding constants for the 3T3 cells, no further correlation with the antiproliferative effects could be made.

BZDs are also known to inhibit both the contractions of longitudinal muscle strips from guinea pig ileum (15) and the uptake of serotonin by platelets (unpublished observation). However, neither effect was correlated with binding to the peripheral-type BZD sites. BZDs also induce melanoma cell (13) and Friend erythroleukemia cell (14) differentiation. However, neither study linked these effects to either central or peripheral-type BZDs. In fact, in the study using erythro-

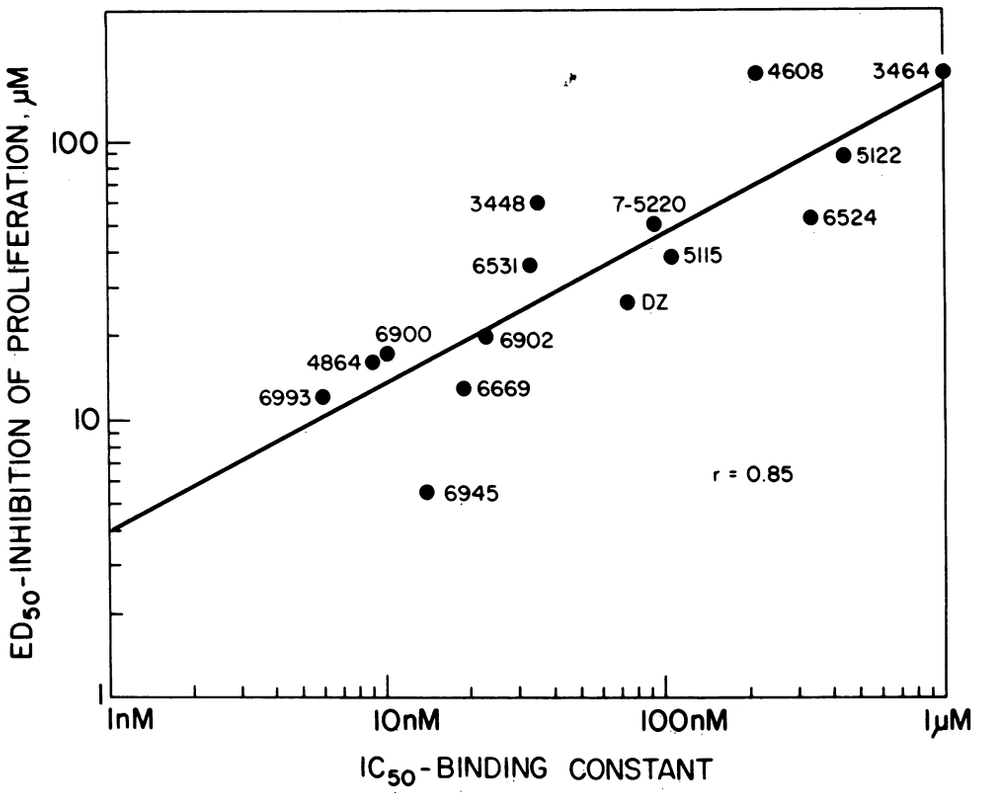


FIG. 3. Correlation between binding constants of 15 BZDs and their ED_{50} values in inhibiting [^{3}H]thymidine uptake by thymoma cells. The binding constants (IC_{50}) and ED_{50} values in inhibiting [^{3}H]thymidine uptake were determined as described in the legend to Fig. 2. Each value is the mean of at least four experiments. All compounds except Ro7-5220 were of the Ro5 series. DZ, diazepam.

leukemia cells (14) it was concluded that differentiation was induced by simple lipophilic properties of certain BZDs. Recent results from this laboratory sharply contradict this conclusion, inasmuch as peripheral but not central BZDs induce differentiation of Friend erythroleukemia cells in culture (unpublished observation). The highly significant correlation between the ED_{50} of inhibition of [^{3}H]thymidine incorporation into thymoma cells by BZDs and the binding constants of these compounds presents evidence that the peripheral-type BZD site may indeed mediate a physiological function.

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