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Antiproliferative and Differentiating Effects of Benzodiazepine Receptor Ligands on B16 Melanoma Cells

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ABSTRACT. In this study, we evaluated the effect of several ligands active at the central-type and peripheral-type benzodiazepine receptor (BzR) (clonazepam, diazepam, PK11195 and Ro5-4864) on the growth and differentiation of B16 melanoma cells. All tested BzR ligands were able to suppress proliferation of the cells at the micromolar range and in a concentration-dependent manner. However, agents selectively active at the peripheral-type BzR (PK11195 and Ro5-4864) exhibited more potent antiproliferative activity. In addition, the BzR ligands were demonstrated to affect the cell cycle by reducing the percent of cells in the S phase and increasing the percent in the G2/M phase. BzR ligands induced cellular phenotypic alterations, which have been previously shown to be associated with melanoma cell differentiation. These alterations included: marked morphological changes, enhancement of melanogenesis, lipid accumulation and increase in the activity of γ glutamyl transpeptidase. All BzR ligands induced a marked reduction in the concentration of UTP and most of them did the same in GTP and CTP, while ATP levels were not significantly altered. In summary, BzR ligands (clonazepam, diazepam, PK11195 and Ro5-4864) were found to exert antitumor effects in B16 melanoma cells. These findings encourage further studies of a possible therapeutic potential of BzR ligands in treatment of melanoma. BIOCHEM PHARMACOL **56**;8:1029–1034, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. melanoma; benzodiazepine receptor; cell proliferation; cell cycle; γ glutamyl transpeptidase

BZDs** are well known as anxiolytic, hypnotic and anticonvulsant drugs, active via the central-type BzR [1]. Peripheral-type mitochondrial (as well as membranal) BzRs were found in a variety of cells. Several reports have demonstrated the antiproliferative effect of BZD ligands in normal and transformed cells, including lymphocytes [2], thymoma [3], glioma [4] and human melanoma cells [5]. It is unclear whether this antiproliferative effect of BzR ligands is mediated via the peripheral-type (mitochondrial) BzRs [6]. Furthermore, nanomolar concentrations of BZDs induced proliferation of murine glioma [7] and tymoma cells [8]. Diazepam and temazepam induced cell differentiation in Friend erythroleukemia cells [9]. The peripheral-type BZD ligand, Ro5-4864, increased melanogenesis in melanoma cells [5]. BZDs enhanced nerve growth factor-induced c-fos expression [10]. Only a few inducers of differentiation have been introduced thus far into anticancer clinical use. Thus, there is a special interest in evaluating the activity of BzR ligands as antitumor drugs. The aim of the present study was to compare the activity of several BzR ligands on proliferation and differentiation of B16 mouse melanoma cells.

MATERIALS AND METHODS

Clonazepam, diazepam, PK11195 and Ro5-4864 were purchased from Sigma Chemical Co. The drugs were dissolved in saline with up to 0.1% of DMSO. DMSO (at 0.1%) was added to untreated B16 cells in all control incubations.

Cell Cultures

B16 melanoma cells, grown in our laboratory for several years and originally obtained from A. Raz (The Weizmann Inst., Rehovot, Israel), were incubated in RPMI 1640, supplemented with 10% fetal calf serum (FCS), antibiotics and antimycoplasma agent at 37°, in an atmosphere of 95%

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** *Abbrevations:* BZD, benzodiazepine; and BzR, benzodiazepine receptor.

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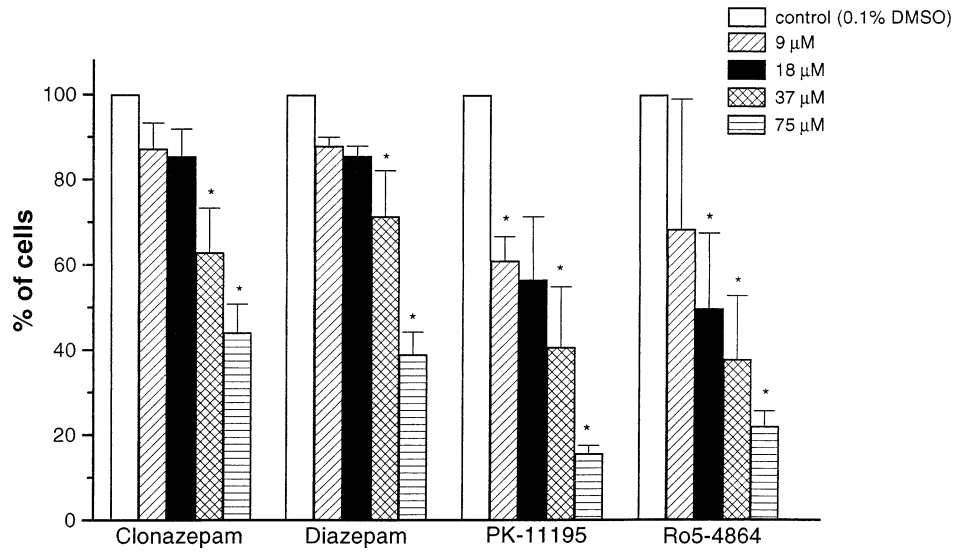

FIG. 1. The effect of BzR ligands on the proliferation of B16 melanoma cells. B16 cells were incubated in the presence of DMSO 0.1% (control) or increasing concentrations of BzR ligands for 72 hr. After detachment with EDTA (1 mM) in PBS, the cells were counted in a Coulter counter. Values are means of 5–8 independent experiments ± SE. *Treatment versus control: $P < 0.01$.

air and 5% CO_2. Cells were found to be free of mycoplasma. Cells were transferred 2–3 times weekly.

Evaluation of Cell Proliferation

B16 melanoma cells (2×10^4/0.5 mL of culture medium) were incubated in 24-well plates in the presence or absence of increasing concentrations of clonazepam, diazepam, PK11195 or Ro5-4864 for 72 hr. Cells were detached with EDTA (1 mM) in PBS and counted in a Coulter counter [11].

Cell-Cycle Analysis

Cells (5×10^5 cells/10 mL of medium) were incubated in the presence or absence of the central-type BzR ligand, clonazepam (75 μM), the mixed-type BzR ligand, diazepam (75 μM), and the peripheral-type BzR ligands, PK11195 (37 μM) and Ro5-4864 (37 μM) for 72 hr. Cells were detached with trypsin and washed with PBS. Nuclei obtained from 10^6 cells were prepared for flow-cytometric DNA analysis by a detergent- trypsin method [12] and stained with propidium iodide. Flow cytometry was performed on a Becton Dickinson FACScan at ex. 488 and em. 580. The proportion of cells in the different phases of the cell cycle was determined from the DNA histogram, using the Cell FIT program (Becton Dickinson).

Assessment of Cell Morphology

Cells (4×10^4 cells/1 mL of medium) were incubated in the presence or absence of diazepam, clonazepam, PK11195 or Ro5-4864 for 72 hr. Cell morphology was assessed by light microscopy following hematoxylin eosin staining, as previously described [13]. Lipid staining was performed by the oil red O method, as previously described [14].

γ Glutamyl Transpeptidase Activity

Cells (4×10^5 cells/10 mL of medium) were incubated in tissue culture plates in the presence or absence of diazepam, clonazepam, PK11195 or Ro5-4864 (18 μM and 75 μM) for 72 hr. γ Glutamyl transpeptidase activity was determined spectrophotometrically as previously described [15] and results were expressed in μmol/mg of DNA/hr. DNA was determined by the method of Labarca *et al.* [16].

Intracellular Nucleotide Levels

Cells (6×10^5 cells/10 mL of medium) were incubated in the presence or absence of diazepam (75 μM), clonazepam (75 μM), PK11195 (37 μM) or Ro5-4864 (37 μM) for 72 hr. Nucleotides were extracted with perchloric acid (1 M) and levels were determined by using anion-exchange column (Sax-10 Whatman) on HPLC (Varian 9050), as previously described [17].

Statistical Analysis

Statistical significance of the data were evaluated using paired Student's *t*-test.

RESULTS

Effect of BzR Ligands on B16 Melanoma Cell Growth

The effects of micromolar concentrations of clonazepam, diazepam, PK11195 and Ro5-4864 on the proliferation of B16 melanoma cells were assessed (Fig. 1). The prolifera-

TABLE 1. The effect of BzR ligands on the cell cycle of the B16 melanoma cell line

Treatment	Percent of cells in each phase of the cell cycle		
	G1	S	G2/M
Control (DMSO 0.1%)	46.7 ± 2.8	49.9 ± 2.6	3.4 ± 0.8
Diazepam (75 μM)	49.6 ± 1.5	36.3 ± 1.3‡	14.1 ± 0.9‡
Clonazepam (75 μM)	48.3 ± 1.5	40.6 ± 2.2*	11 ± 1.5‡
PK11195 (18 μM)	56.7 ± 1.5†	35.1 ± 2.1‡	8.3 ± 1.3‡
Ro5-4864 (18 μM)	56.4 ± 0.6†	33.5 ± 1.9‡	10.1 ± 0.9‡

B16 cells were incubated in the presence of the BzR ligands or DMSO 0.1% (control) for 72 hr and cell cycle kinetics were determined as described in Methods. Values are means ± SE for 5–7 replicates carried out with three different cell preparations.
*Treatment vs. control: $P < 0.03$.
†Treatment vs. control: $P < 0.01$.
‡Treatment vs. control: $P < 0.003$.

tion of B16 melanoma cells was inhibited by all four compounds. However, the peripheral-type BzR ligands, PK11195 and Ro5-4864, were more potent than clonazepam and diazepam in inhibiting cell growth. Fifty percent inhibition of cell growth was achieved by 75 μM clonazepam or diazepam, and 37 μM of PK11195 or Ro5-4864. Previous reports suggested a growth-stimulatory effect for BzR ligands at nanomolar concentrations [7, 8]. Incubation of B16 melanoma cells in the presence of 1–100 nM of each of the compounds did not induce any significant change in cell proliferation (data not shown). The growth-inhibitory effect of the BzR ligands seems to be due to a cytostatic rather than a cytotoxic effect, because cell viability, as assessed by the trypan blue dye exclusion test, was not reduced significantly by these agents. However, PK11195 and Ro5-4864 at concentrations higher than 37 μM slightly reduced cell viability.

Effect of BzR Ligands on Cell-Cycle Kinetics of B16 Melanoma Cells

Cell-cycle analysis was performed following incubation of B16 melanoma cells with BzR ligands (at concentrations leading to 50% growth inhibition after 72 hr) for 72 hr. All four compounds induced a significant decrease in the percent of cells in the S phase and an increase in cells in the G2/M phase, as compared to untreated cells. PK11195 and Ro5-4864 also increased the percent of cells in the G1 phase, in comparison to untreated cells (Table 1).

Induction of Phenotypic Alterations in B16 Melanoma Cells by BzR Ligands

BzR ligands induced marked morphological changes in B16 melanoma cells, which included production of dendrite-like structures, cell flattening and induction of granules. Diazepam, PK11195 and Ro5-4864 induced formation of very long dendrites (Fig. 2A–C, F–I). Clonazepam, PK11195 and Ro5-4864 led to accumulation of lipid droplets (Fig. 2D–I). Diazepam and Ro5-4864 also induced melanogenesis (Fig. 3), while clonazepam and PK11195 did

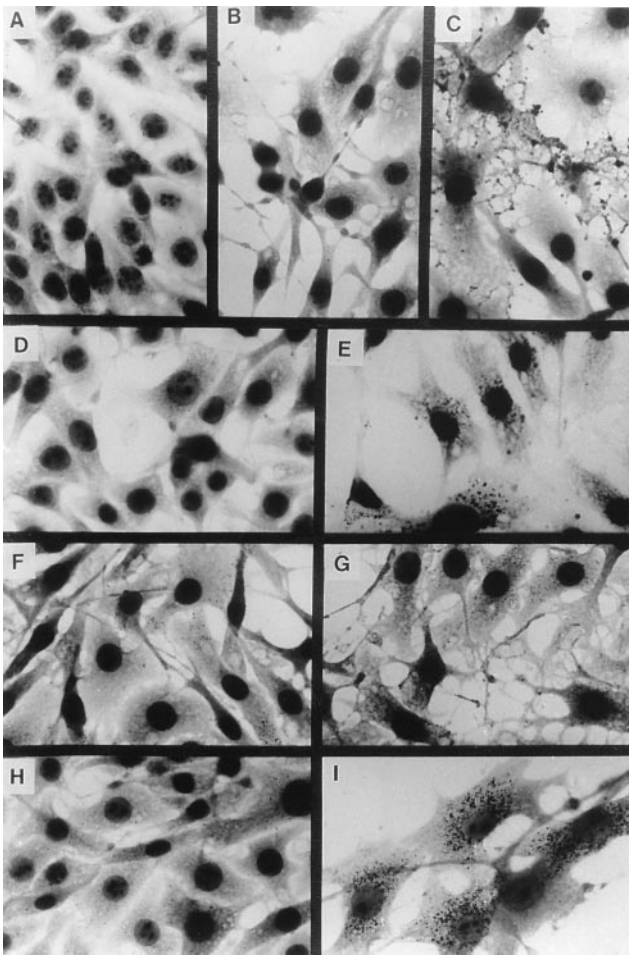

FIG. 2. The effect of BzR ligands on B16 melanoma cell morphology. B16 cells were incubated for 72 hr in the presence of BzR ligands or DMSO 0.1% (control). Cell morphology was assessed by light microscopy following hematoxylin-eosin and oil red O staining, as described in Materials and Methods. (A) Control B16 cells are round amelanotic cells with no dendrite-like projections or lipid granules; following 72-hr incubation with: diazepam B (37 μM) and C (75 μM), long dendrite projections were formed and dark granules appeared; clonazepam D (37 μM) and E (75 μM) lipid droplets were accumulated; Ro5-4864 F (37 μM) and G (75 μM) long dendrite projections were formed and lipid and dark granules were accumulated; PK11195 H (37 μM) and I (75 μM) dendrites were formed and lipid droplets were accumulated (×400).

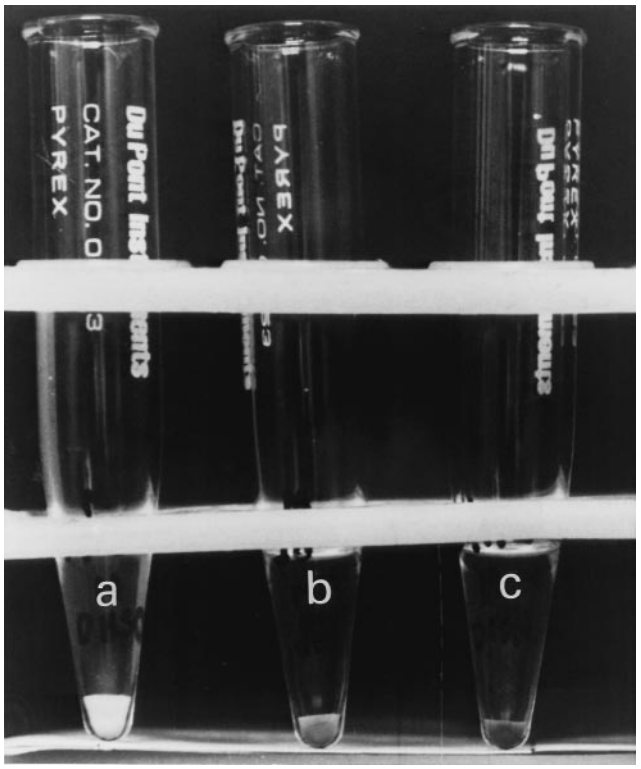

FIG. 3. The effect of BzR ligands on melanogenesis in B16 melanoma cells. The sediment of B16 melanoma cells following incubation in the presence of BzR ligands or DMSO 0.1% (control) for 72 hr. As shown, in comparison with the (A) control, (B) Ro5-4864 (37 μM) and (C) diazepam (75 μM) induced marked melanogenesis.

not affect melanogenesis at concentrations of 37 μM and 75 μM (data not shown).

Effect of BzR Ligands on γ Glutamyl Transpeptidase Activity

The enzyme γ glutamyl transpeptidase participates in melanin biosynthesis [15] and was previously shown to be enhanced by differentiating agents in melanoma cells [18, 19]. The effect of BzR ligands on γ glutamyl transpeptidase activity is shown in Table 2. All BzR ligands markedly increased the activity of γ glutamyl transpeptidase at a concentration (75 μM) which inhibited cell proliferation significantly (Fig. 1). PK11195 and Ro5-4864, which were found to be more potent than diazepam and clonazepam in inhibiting cell growth, also enhanced γ glutamyl transpeptidase activity at 18 μM (Table 2).

TABLE 2. The effect of BzR ligands on γ glutamyl transpeptidase activity in B16 melanoma cells

Treatment	γ glutamyl transpeptidase activity (μmols/mg DNA/hr)
Control (DMSO 0.1%)	8.68 ± 0.51
Diazepam (18 μM)	11.2 ± 1.16
Diazepam (75 μM)	24.11 ± 2.50*
Clonazepam (18 μM)	9.33 ± 0.28
Clonazepam (75 μM)	23.36 ± 2.78*
PK11195 (18 μM)	15.66 ± 12.0*
PK11195 (75 μM)	27.7 ± 1.80*
Ro-4864 (18 μM)	15.43 ± 1.77†
Ro5-4864 (75 μM)	24.7 ± 1.47*

B16 cells were treated with BzR ligands or DMSO 0.1% (control) for 72 hr. Enzyme activity was extracted and determined as described in Methods. Values are means of six experiments ± SE.

*Treatment vs. control: $P < 0.001$.

†Treatment vs. control: $P < 0.05$.

Effect of BzR Ligands on Intracellular Levels of Purine and Pyrimidine Nucleotides

It was suggested that ligand binding to mitochondrial BzR results in inhibition of mitochondrial respiratory control [20]. We assessed changes in intracellular levels of purine and pyrimidine nucleotides following treatment with BzR ligands. No significant effect of BzR ligands on the levels of ADP and ATP was found. However, all four agents induced a marked reduction in the concentration of UTP. Diazepam, clonazepam and PK-11195 also led to a decrease in the concentrations of CTP and GTP (Table 3).

DISCUSSION

In this study, we demonstrated the antiproliferative and differentiating effects of four BzR ligands on B16 melanoma cells. The BzR ligands tested included four compounds: clonazepam, which is active at the central-type BzR; diazepam, active both at the central- and the peripheral-type receptor; Ro5-4864 and the isoquinoline carboxamide, PK11195, both selectively active at the peripheral-type receptor. All BzR ligands inhibited the proliferation of B16 cells at micromolar concentrations. The peripheral-type BzR ligands, Ro5-4864 and PK11195, were shown to be more potent inhibitors of B16 cell growth than the central-type BzR ligands, clonazepam and diazepam (Fig. 1). All ligands induced phenotypic alterations, known to be associated with a more differentiated phenotype of B16 melanoma [20]. However, the pattern of phenotypic alterations induced was not identical. Distinct morphological changes were induced by each individual compound. Only diazepam and Ro5-4864 enhanced melanogenesis and lipid accumulation. B16/C3 melanoma were reported to possess high-affinity binding sites for diazepam, which enhanced melanogenesis in these cells [5]. Solowey *et al.* showed that peripheral-type BzR ligands inhibited the growth of human melanoma cells and potentiated the antiproliferative activity of recombinant human interferons at micromolar concentrations [21]. It should be noted that diazepam and clonazepam, which are widely used clinically, exert their therapeutic effects at lower concentrations. Wang et al. showed antiproliferative effects of peripherally acting BzR ligands at nanomolar concentrations in thymoma cells. These authors suggest a strong positive correlation between the binding characteristics of peripherally acting BzR ligands and their growth-inhibitory potency [3]. It seems

TABLE 3. The effect of BzR ligands on intracellular nucleotide levels in the B16 melanoma cell line

Nucleotide level (nmol/mg protein)	Control	Diazepam	Clonazepam	PK11195	Ro5-4864
ATP	17.14 (±2.06)	14.08 (±1.25)	13.75 (±0.86)	17.6 (±1.13)	19.7 (±1.87)
ADP	5.26 (±1.19)	6.85 (±0.56)	5.61 (±0.63)	7.17 (±0.47)	8.85 (±1.31)
GTP	4.25 (±0.32)	2.54* (±0.23)	2.30* (±0.20)	2.89* (±0.08)	3.4 (±0.33)
UTP	10.75 (±0.99)	3.60* (±0.47)	2.30* (±0.36)	2.87* (±0.35)	6.33* (±0.77)
CTP	2.81 (±0.37)	1.30* (±0.14)	1.24* (±0.17)	1.35* (±0.07)	2.19 (±0.34)

B16 cells were treated with BzR ligands (diazepam 75 μM, clonazepam 75 μM, PK11195 37 μM or Ro5-4864 37 μM), or DMSO 0.1% (control) fcor 72 hr. Nucleotides were extracted as described in Methods. Values are means ± SE for 4–9 replicates carried out with different cell preparations.

*Treatment vs control: $P < 0.05$.

unlikely that the cellular responses induced by BzR ligands were mediated by peripheral BzRs, because in our study both peripheral- and central-type BzR ligands affected the cells at the micromolar (rather than nanomolar) concentration range. Furthermore, equiactive concentrations of clonazepam and diazepam were four times higher than those of PK11195 and Ro5-4864 (rather than 1000×).

In the present study, we demonstrated for the first time that BzR ligands induced alterations in the cell cycle kinetics. All BzR ligands increased the percent of cells in the G2/M phase of the cell cycle and reduced the percent of cells in the S phase. It should be noted that G2/M arrest is known to be induced in tumor cells by irradiation and various chemotherapeutic agents, such as taxol, etoposide and vincristine [22–24]. PK11195 and Ro5-4864 also led to an increase in the percent of cells in the G1 phase, similar to established differentiating agents such as butyrate [25].

In this study, all BzR ligands were found to increase γ glutamyl transpeptidase activity significantly. These results resemble the effect of known differentiating agents such as butyrate [26], histidinol [27], dimethylthiourea [28], 8-hydroxyquinoline [29] and novobiocin [11] on this enzyme.

γ Glutamyl transpeptidase was suggested to play a role in melanin biosynthesis [15]. In this study, the BzR ligands diazepam and Ro5-4864 increased both melanogenesis and γ glutamyl transpeptidase activity, while clonazepam and PK11195 enhanced γ glutamyl transpeptidase activity, but did not enhance melanogenesis. These results suggest that B16 melanoma cells possess bypass pathways for melanogenesis not involving γ glutamyl transpeptidase.

The present results again show that the phenotypic alterations induced by various maturating agents are not identical. For example, the known differentiating agents sodium butyrate and DMSO differ in their ability to induce melanogenesis and γ glutamyl transpeptidase activity [26].

In our study, BZDs reduced UTP, CTP and GTP levels. We have previously described that inhibition of melanoma cell proliferation and induction of differentiation by dimethylthiourea derivatives was associated with reduction of ATP and mitochondrial respiration [28]. Because ATP and ADP levels were not markedly affected by BzR ligands, it is unlikely that their growth-inhibitory and differentiating activity is mediated by altering mitochondrial respiration. The fact that all the compounds led to a reduction in the levels of at least one nucleotide (UTP) is compatible with interference of either synthesis or degradation pathways of nucleotides by BzR ligands. It was reported that dipyridamole, which inhibited hepatoma cell proliferation via inhibition of the salvage pathway, led to a decrease in GTP, UTP and CTP levels [30]. The decrease in the intracellular level of a single nucleotide may inhibit nucleic acid synthesis and, thus, cell proliferation. It is known that GTP-depleting agents, such as mycophenolic acid or tiazofurin, induce cell differentiation [31, 32]. In our study, diazepam, clonazepam and PK11195 were found to decrease GTP levels. However, our results do not reveal whether the reduction in cellular nucleotide levels induces growth arrest and cell differentiation, or whether this phenomenon is an indicator of development of a more mature cellular phenotype.

In conclusion, it appears that agents active at the BzR, irrespective of the specificity to the central- or peripheral-type receptor, exert antitumor effects in B16 melanoma cells. The possible therapeutic potential of BzR ligands in the *in vivo* treatment of melanoma merits further investigation.

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