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Antiproliferative Action of Pyrrolobenzoxazepine Derivatives in Cultured Cells: Absence of Correlation with Binding to the Peripheral-Type Benzodiazepine Binding Site

Daniela M. Zisterer,‡ Nicole Hance,* Giuseppe Campiani,† Antonio Garofalo,† Vito Nacci† and D. Clive Williams**

*Department of Biochemistry, Trinity College, Dublin 2, Ireland and †Dipartimento Farmaco Chimico Technologico, Universita 'Di Siena, Bancha Di Sotto 55, 53100 Siena, Italy

ABSTRACT. Three novel peripheral-type benzodiazepine binding site (PBBS) ligands, NF 182, 213 and 262, along with the classically used PBBS ligands, PK 11195 and Ro5-4864, were found to inhibit, at micromolar concentrations and in dose-dependent manner, the proliferation of rat C6 glioma and human 1321N1 astrocytoma, without being cytotoxic. This antiproliferative effect is mediated by arrest in the G1 phase of the cell cycle and does not appear to be mediated by a specific interaction of these ligands with the peripheral-type benzodiazepine binding site. BIOCHEM PHARMACOL 55;4:397–403, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. peripheral-type benzodiazepine binding site; cell proliferation; cell cycle; flow cytometry

Benzodiazepines, such as valium, are among the most highly prescribed drugs due to their anxiolytic, relaxant and sedative effects. These pharmacological effects of benzodiazepines are mediated through their interaction with the central-type binding sites or $GABA_A$/benzodiazepine receptor [1]. However, benzodiazepines also bind to another distinct recognition site, the "peripheral-type" binding site (PBBS)§, so called because it was discovered initially in peripheral tissues, but subsequently also in brain [2].

The function of the PBBS remains unclear although PBBS ligands such as PK 11195 and Ro5-4864 have been reported to have a wide range of biological effects including alteration in cardiac action potentials [3] and calcium channels [4], alterations of proto-oncogene expression [5], alteration of immune function [6] and stimulation of steroidogenesis [7].

PBBS ligands such as diazepam, PK 11195 and Ro5-4864, have also been shown to inhibit cell growth in a variety of cell lines. Wang *et al.*, [8], tested the effect of a range of benzodiazepines on the growth of mouse thymoma cells and demonstrated a strong positive correlation between the binding constants for these benzodiazepines at the peripheral-type site and their ability to inhibit the proliferation of thymoma cells in culture at the micromolar range. Gorman *et al.*, [9] found that the benzodiazepines Ro5-4864, diazepam and clonazepam along with PK 11195 inhibited the proliferation of rat C6 glioma and mouse neuro-2A neuroblastoma cells again in the micromolar range. However the different potencies and specificities of these compounds for the antiproliferative actions and binding affinities for the binding site suggested that these antiproliferative actions were not mediated through the PBBS. More recently Camins *et al.*, [10], demonstrated that Ro5-4864, PK 11195 and diazepam inhibited in a concentration-dependent manner the proliferation of V79 Chinese hamster lung cells. However it was again concluded that it was unlikely that these antiproliferative effects were mediated through an interaction with the PBBS as far higher concentrations (approximately 1,000-fold) than are necessary to saturate the PBBS were required to elicit such effects.

Recently a novel series of high affinity PBBS ligands based on a pyrrolobenzoxazepine skeleton, classed here as NF compounds, have been synthesised [11]. One of the proposed roles of the PBBS is in the regulation of steroid production [7], and some of these ligands have been shown to stimulate steroidogenesis in a mouse Y-1 adrenocortical cell line model with similar potencies and efficacies to PK 11195 and Ro5-4864, thus demonstrating their biological activity [11]. These NF compounds therefore represent novel probes for the PBBS and it would be of interest to examine their effect on cell growth and regulation to further determine any involvement of the PBBS in the process.

The aim of this study was to examine the action of three novel PBBS ligands on the growth of glial cell lines. To determine involvement of the PBBS in any antiprolifera-

‡ Corresponding author: TEL. (353) 1-608-1802; FAX (353) 1-677-2400.

§ *Abbreviations:* FACS, fluoresence activated cell sorting; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide); PBBS, peripheral-type benzodiazepine binding site; PI, propidium iodide; phosphate buffered saline.

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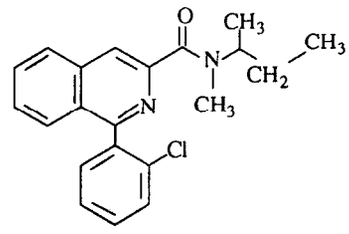
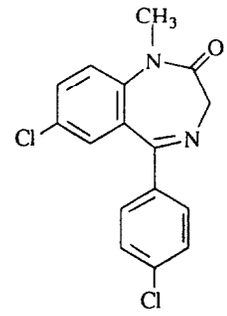


PK 11195



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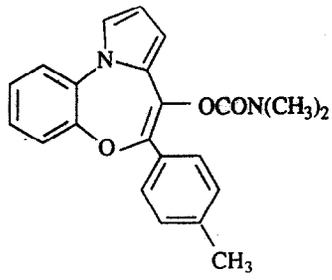
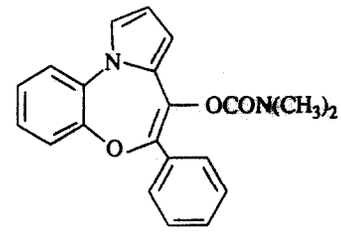
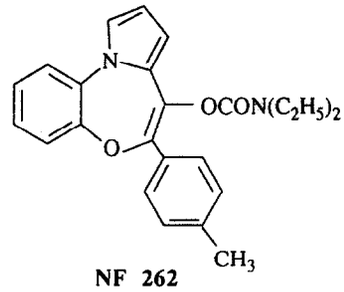


NF 213



NF 182



NF 262

FIG. 1. Structures of PBBS ligands, PK 11195, Ro5-4864 and NF drugs

tive effects, cell lines of both rodent and human origin were examined. The binding of PBBS ligands to the PBBS from rodent tissue is of high affinity whereas some ligands (e.g. Ro5-4864) bind with lower affinity to the PBBS in human tissue [12]. A comparison of the affinity of ligands for the PBBS with the potency of their antiproliferative action would help to determine any involvement of the PBBS in this process. The first cell line chosen was the rat C6 glioma cell line. C6 cells have been extensively studied in determining the antiproliferative actions of benzodiazepines, hence it was also thought to be a suitable cell line to use for comparative purposes. The second cell line examined in this study was the human astrocytoma 1321N1 cell line. Also in view of the wide use of benzodiazepines as psychotropic drugs, it was interesting to examine if these compounds inhibit proliferation of human brain cells. For comparative purposes the effect of the classically used PBBS ligands, PK 11195 and Ro5-4864, on the proliferation of these two cell lines was also examined. Any cell cycle arrest was further investigated using the sensitive analytical technique of flow cytometry. Identifying the point at which the cell cycle is arrested would provide further information on the mechanism of these antiproliferative actions. In conclusion it is hoped that this study will further our understanding of cell growth and its regulation and any involvement of the PBBS in this process.

MATERIALS AND METHODS

Chemicals

PK 11195 was a gift from Dr. A. Doble (Rhone-Poulenc Rorer). Ro5-4864 was obtained from Fluka Chemical Company. NF 182, 213 and 262 were synthesised [11] by Prof. Giuseppe Campiani (University of Siena, Italy) (see Fig. 1).

DMEM, foetal calf serum, gentamycin, trypsin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and propidium iodide were obtained from Sigma Chemical Company (Poole). [^{3}H]PK 11195 (specific activity 85.8 Ci/mmol) was obtained from NEN Research Products. CytoTox 96 nonradioactive cytotoxicity assay kit was obtained from Promega.

Cells

The cell lines used were obtained from the European Collection of Animal Cell Cultures (E.C.A.C.C.). The rat C6 glioma and human 1321N1 cells were cultured in DMEM. All media were supplemented with foetal calf serum (FCS, 10% v/v), gentamycin (100 mg/mL) and L-glutamine (2 mM). Stock cultures were maintained at 37° in a humidified atmosphere with 5% CO_2 in air. 1321N1 and C6 cells were passaged using 0.25% (w/v) of trypsin in DMEM.

Preparation of Cell Homogenates

Near-confluent cells were washed twice with 5 mL of ice-cold phosphate-buffered saline (PBS) and harvested by scraping into this buffer (approximately 5 mL). The cell suspension was centrifuged at 600 × *g* for 5 min and the pellet homogenised in 50 mM Tris-HCl buffer, pH 7.4 (2 mL), using an Ultraturrax homogenizer (10 sec).

Cytotoxicity Assay

The effect of the PBBS ligands on 1321N1 and C6 cell viability was assessed by the use of the CytoTox 96 nonradioactive cytotoxicity assay. Cells were plated at a density of 0.5 × 10^4 per well in a 96-well plate. After incubation at 37°, 5% CO_2 for 24 hr the medium was removed and 100 μL fresh medium containing 100 μM of the various compounds was added. Following a further incubation for 48 hr, 50 μL aliquots of media were removed to a fresh 96-well plate. Lactate dehydrogenase present in the media was then measured using the kit obtained from Promega under conditions described by the manufacturer.

Cell Proliferation Assay (MTT)

The cells were seeded at a density of 0.5 × 10^4 cells/well into a 96-well plate and incubated at 37°, 5% CO_2 for 24 hr. They were then treated with various concentrations of the compounds (0–100 μM) in 100 μL DMEM/10% FCS, for a further 48 hr. Higher concentrations of the various compounds were not used due to limits of solubility. The final concentration of ethanol was constant for all the wells within each experiment and did not exceed 1% (v/v), a concentration which, on its own had no effect on cell growth. The medium was removed and the cells washed with 125 μL of phosphate buffered saline (PBS). Following incubatation with 50 μL of MTT in PBS (final concentration of 0.2 mg/mL) for 1 hr at 37° in the dark, 125 μL of isopropanol containing 0.04 M HCl and 10% Triton X-100 was added and left overnight in the dark at 4°. The absorbance of the plate was then measured at 570 nm.

Radioligand Binding Assays

Cell homogenates (15–35 μg protein) were incubated with 0.5–50 nM [^{3}H]PK 11195 (85.8 Ci/mmol) in 50 mM Tris/HCl buffer, pH 7.4, (incubation buffer), in a total volume of 0.5 mL on ice. Total and non-specific/non-saturable binding in each case was determined in the absence and presence of 10 μM unlabelled PK 11195 respectively. All samples were incubated in triplicate for 60 min. The incubation mixtures were then filtered and counted as previously described [13]. When testing the potency of a compound to inhibit [^{3}H]PK 11195 binding, samples were incubated with 5 nM [^{3}H]PK 11195 and various concentrations (1 nM to 1 μM) of compound and subsequently treated exactly as above. The resulting K_i values were then generated by the use of the computer programs EBDA and LIGAND [14].

Fluorescence Activated Cell Sorting

Cells were seeded at the appropriate density (in the case of C6 glioma cells at a density of 5 × 10^4 cells/5 mL for control samples and at a density of 25 × 10^4 cells/5 mL for drug treated samples and in the case of 1321N1 cells at a density of 15 × 10^4 cells/5 mL for control samples and at a density of 30 × 10^4 cells/5 mL for drug treated samples) into 25 cm^2 flasks and incubated at 37°, 5% CO_2 for 24 hr. They were treated with the various compounds (100 μM) for a further 48 hr. The cells were seeded at these various densities to ensure they had not reached confluency and therefore out of the log phase of growth prior to their analysis by FACS. The cells were then washed three times with PBS before being trypsinized and centrifuged at 300 × *g* for 5 min. They were resuspended in 200 μL PBS, made up to 2 mL with ice-cold ethanol (70%; v/v) and left to sit on ice for at least 1 hr to fix them. Approximately 1 hr prior to use they were centrifuged at 300 × *g* for 3 min and the supernatant carefully pipetted off. The pellet was resuspended in 800 μL PBS. RNAase (100 μL; 1 mg/mL) and 100 μL of the fluorescent dye propidium iodide (PI, 400 μg/mL) which binds DNA were added. The tubes were vortexed and incubated at 37° for 30 min. As the PI is light sensitive the tubes were covered with foil at all times. The cells were then passed single file through a laser beam, and fluorescent light emitted by each cell was measured; from this the size and shape of the cell and its DNA content could be determined. Flow cytometry was performed with a FACStar-PLUS from Becton Dickinson. FACS data was analysed using the program WinMDI.

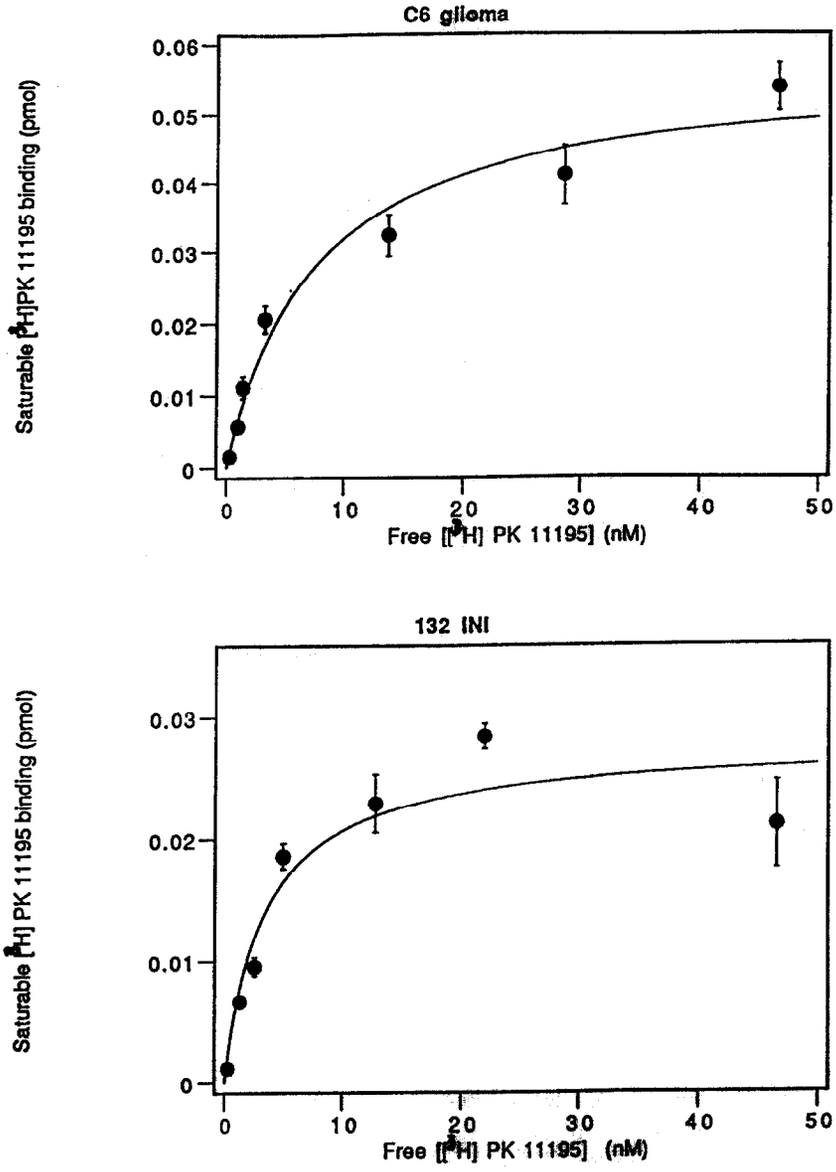


FIG. 2. Saturable binding of [^{3}H]PK 11195 to homogenates of C6 glioma and 1321N1 cells by NF 182. Homogenates of C6 glioma and 1321N1 cells (35μg of protein) were assayed for [^{3}H]PK 11195 binding over a range of concentrations (0–50nM) as described in "Materials and Methods." Each point represents the mean of triplicate determinations and the error bars represent the SEM. Absence of error bars indicate the error was smaller than the size of the symbol. The data were fitted with the use of the computer program MacCurve Fit to the equation describing a rectangular hyperbole (of "bound" versus "free ligand") yielding K_d and B_{max} values of 7.8 ± 2.5 nM and 16.3 ± 1.5 pmols/mg protein respectively, for C6 glioma cells and 2.5 ± 1 nM and 8.5 ± 1 pmol/mg protein, respectively for 132 INI cells.

Protein Determination

Protein was quantitated by the method of Markwell *et al.*, [15], using bovine serum albumin as a standard.

Statistics

Statistical analysis of differences between control values and various drug treatment values for cell proliferation and FACS analysis were performed with a Student's *t*-test and analysis of variance (ANOVA test) respectively using the Apple Macintosh program INSTAT.

RESULTS

Rodent C6 glioma homogenates displayed saturable, high affinity binding of [^{3}H]PK 11195, a selective ligand for the PBBS, yielding K_d and B_{max} values of 7.8 ± 2.5 nM and 16.3 ± 1.5 pmol/mg of protein, respectively (Fig. 2). Human 1321N1 astrocytoma homogenates also displayed saturable high affinity binding of [^{3}H]PK 11195, yielding K_d and B_{max} values of 2.5 ± 1.0 nM and 8.5 ± 1.0 pmol/mg protein, respectively (Fig. 2).

NF 182, 213 and 262 were shown to inhibit ^{3}[H]PK 11195 binding to homogenates of both C6 glioma and

TABLE 1. High affinity binding NF 182, 213 and 262 to homogenates of C6 and 1321N1 cells

	Inhibition of [^{3}H]PK 11195 binding to the PBR K_i value (nM)			
	NF 182	NF 213	NF 262	Ro5-4864
C6 glioma	2.8 ± 0.7	2.1 ± 1.0	18.7 ± 4.5	15.3 ± 1.4
1321N1 astrocytoma	2.7 ± 1.6	2.7 ± 1.6	2.5 ± 0.8	113 ± 29

Homogenates (35 μg of protein) of C6 and 1321N1 cells were assayed for specific binding of [^{3}H]PK 11195 (5 nM) in the presence or absence of the indicated unlabelled compounds (0–1 μM). Control binding is defined as specific binding in the absence of unlabelled displacing compound. The amount of radioactivity bound in the presence of displacer was expressed as a percentage of control binding and the points represent mean ± SEM of triplicate determinations. The K_i values were obtained using the computer program EBDA and LIGAND as described in "Materials and Methods."

1321N1 cells with K_i values between 1–20 nM (Table 1). A representative graph demonstrating displacement of [^{3}H]PK 11195 binding by NF 182 to homogenates of C6 glioma cells is shown in Fig. 3. Ro5-4864 was also shown to inhibit [^{3}H]PK 11195 binding to homogenates of C6 glioma and 1321N1 cells with K_i values as expected for rodent and human PBBS (Table 1).

Possible cytotoxic effects of each of the PBBS ligands were determined on each cell line at a concentration of 100 μM (the highest concentration used in proliferation studies). None of the compounds were cytotoxic as determined by their failure to cause significant (>5%) release of lactate dehydrogenase (results not shown).

In C6 glioma (Table 2a) and 1321N1 astrocytoma (Table 2b), NF 182, 213 and 262, induced a dose-dependent inhibition of cell proliferation in the micromolar range. Similar antiproliferative effects were demonstrated with PK 11195 and Ro5-4864 in C6 glioma (Table 2a) and 1321N1 cells (Table 2b). In each of the cell types no antiproliferative effects were observed at the lowest concentration of drug tested (1 nM).

Table 3 demonstrates the lack of correlation between the

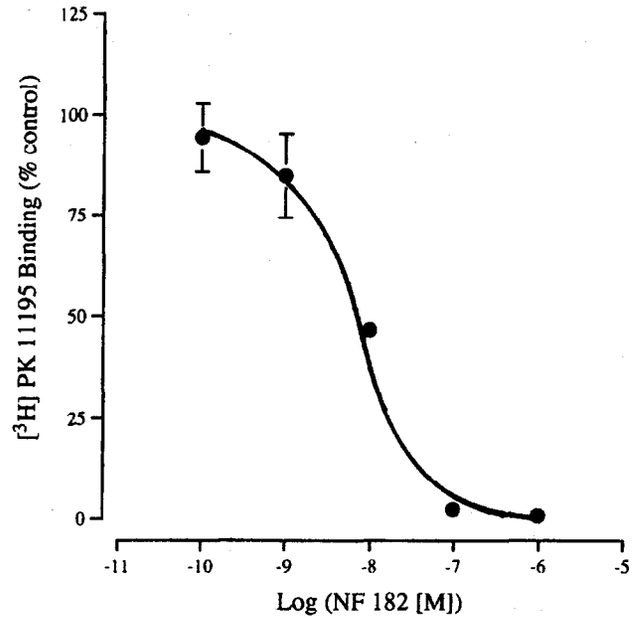


FIG. 3. Displacement of [^{3}H]PK 11195 binding to homogenates of C6 cells by NF 182. Homogenates (35μg of protein) of C6 cells were assayed for specific binding of [^{3}H]PK 11195 (5nM) in the presence or absence of NF 182 (0-1μM). Control binding is defined as specific binding in the absence of NF 182. The amount of radioactivity bound in the presence of NF 182 was expressed as a percentage of control binding and the points represent mean ± SEM of triplicate determinations. Absence of error bars indicate the error is smaller than the size of the symbol. The data was fit using the computer programs EBDA and LIGAND as described in "Materials and Methods" yielding a K_i value of 2.8 ± 0.7 nM.

ED_{50} values obtained for inhibition of either C6 or 132 INI cell proliferation by the PBBS ligands and their affinity (K_i values) for the PBBS in the respective cell lines.

Flow cytometry was employed to determine whether these compounds caused a stage-specific inhibition of the cell cycle. All the PBBS ligands tested were found to induce a specific accumulation of C6 and 1321N1 cells in the G1

TABLE 2. Antiproliferative activity of PBBS ligands on C6 glioma and 1321N1 astrocytoma

	Cell proliferation (%)				
Drug	PK 11195	Ro5-4864	NF 182	NF 213	NF 262
(a) C6 glioma					
1 nM	98.0 ± 2.8	95.9 ± 2.9	97.9 ± 0.2	94.7 ± 12.9	111 ± 7
10 μM	84.7 ± 3.7*	82.1 ± 2.4**	77.8 ± 0.1**	85.5 ± 3.7*	93.9 ± 7.2
50 μM	68.7 ± 5.1**	74.7 ± 2.1**	37.6 ± 4.2**	55.3 ± 2.3**	80.4 ± 3.1**
100 μM	27.9 ± 3.1**	47.3 ± 0.8**	32.3 ± 0.8**	46.8 ± 4.4**	65.9 ± 4.1**
(b) 1321N1					
1 nM	97.1 ± 2.9	94.3 ± 5.7	100.5 ± 5.5	84.5 ± 9.5	93.8 ± 5.3
10 μM	91.4 ± 8.6	91.4 ± 5.7	77.4 ± 7.4*	86.9 ± 6.9	103.4 ± 7.5
50 μM	57.1 ± 2.9**	62.9 ± 5.9**	33.7 ± 6.4**	60.3 ± 14.8**	64.8 ± 8.5**
100 μM	18.4 ± 8.2**	28.6 ± 6.9**	41.2 ± 13.8**	62.2 ± 7.8**	46.8 ± 11.6**

C6 glioma (a) and 1321N1 astrocytoma (b), grown in 96-well plates to a density of 0.5 × 10^4 cells per well were incubated either in absence or presence of increasing concentrations of either PK 11195, Ro5-4864, NF 182, NF 213 or NF 262. The volume of ethanol in each well remained constant and did not exceed 1% (v/v). After 48 hr, proliferation of cells was measured by the MTT assay as described in the "Materials and Methods" section. Inhibition of cell proliferation is expressed as a percentage of the value obtained in the absence of drug but presence of 1% ethanol (v/v). The values represent the mean ± SEM of three experiments each performed in triplicate. *$P < 0.05$, **$P < 0.01$ (vs. control value; Student's t-test).

TABLE 3. Lack of correlation between antiproliferative potency of PBBS ligands and their affinity for the PBBS

PBBS ligand	C6 glioma		1321N1	
	ED_{50} (μM)	K_i (nM)	ED_{50} (μM)	K_i (nM)
PK 11195	73.0 ± 5.0	7.8 ± 2.5	50.3 ± 8.3	2.5 ± 1.0
Ro5-4864	95.0 ± 2.0	15.3 ± 1.4	55.5 ± 13.5	113 ± 29
NF 182	37.5 ± 2.5	2.8 ± 0.7	35.8 ± 1.3	2.7 ± 1.6
NF 213	82.5 ± 17.5	2.1 ± 1.0	>100	2.7 ± 1.6
NF 262	>100	18.7 ± 4.5	74.0 ± 16.0	2.5 ± 0.8

The ED_{50} values represent the concentrations of PBBS ligands which resulted in 50% inhibition of cell proliferation and are the mean ± SEM of triplicate experiments. The K_i values were obtained as previously described (in the case of PK 11195 the K_D value is given).

phase of the cell cycle (Table 4). In both cell types there was a significant decrease in the proportion of cells in the S and G2 + M phases of the cell cycle with a concomitant increase of cells in the G0–G1 phase. It should also be noted that FACS analysis of C6 cells demonstrated a proportion of cells in a pre-G1 peak. This pre-G1 peak may represent cell debris. However incubation of C6 cells for 48 hr in the presence of the PBBS ligands had no effect on the percentage of cells distributed in this pre-G1 peak (Table 4).

DISCUSSION

It has been demonstrated in this study that the rat C6 glioma and human 1321N1 astrocytoma cell lines contain significant levels of the peripheral-type benzodiazepine binding site as determined by high affinity, saturable binding of [^{3}H]PK 11195. This is the first report of the presence of the PBBS in 1321N1 cells. The binding parameters determined for C6 glioma agree with values reported previously [9, 16]. While the affinity of [^{3}H]PK 11195 for the PBBS in the two cell lines is similar, there is approximately twice the amount of peripheral-type binding sites in C6 glioma when compared to 1321N1 cells.

The benzodiazepine-like compounds NF 182, 213 and 262 were shown to specifically bind to the PBBS with K_D values in the nanomolar range in C6 and 1321N1 cells as judged by their ability to displace [^{3}H]PK 11195 binding to homogenates of these cells. Ro5-4864 was also found to displace [^{3}H]PK 11195 binding to 1321N1 cells with a K_i value approximately eight times of that for C6 glioma. This result is in agreement with previous reports that Ro5-4864 binding is species dependent, binding with higher affinity to rodent tissues and with lower affinity to human tissues [12]. It is interesting that although the structures of the NF compounds were designed to be benzodiazepine analogues [11], the binding of NF 182, 213 and 262 to the PBBS is not species dependent, as exhibited by their similar binding affinity to both rodent C6 glioma cells and human 1321N1 cells. In this manner the NF compounds behave more like the isoquinoline carboxamide PK 11195.

In the present study, NF 182, 213 and 262 at micromolar concentrations exhibited antiproliferative effects in the two cell types tested. The compounds were not cytotoxic, because even after 48 hr in the presence of the drugs, greater than 95% of the cells were intact as judged by their lack of release of LDH into the culture medium. The concentration range (10–100 μM), with which these NF compounds exhibited their antiproliferative effects was similar to that demonstrated by PK 11195 and Ro5-4864. These novel PBBS ligands have previously been shown to exhibit stimulatory effects on steroidogenesis in Y1 adreno-

TABLE 4. Effect of NF drugs on the distribution of C6 and 1321N1 cells within each phase of the cell cycle

Drug	Cell phase (%)			
	Pre G1	G0–G1	G2 + M	S
(a) C6 glioma				
Control	8.2 ± 0.3	62.3 ± 1.0	17.0 ± 1.7	13.1 ± 0.8
NF 182	9.9 ± 0.5	72.6 ± 2.1*	10.9 ± 1.1†	7.0 ± 1.4‡
NF 213	11.0 ± 0.9	69.7 ± 1.0*	12.9 ± 1.0†	6.3 ± 0.8‡
PK 11195	14.3 ± 5.2	70.9 ± 2.3*	10.6 ± 1.3†	4.0 ± 0.6‡
Ro5-4864	10.7 ± 0.8	67.9 ± 1.3*	14.4 ± 1.8†	7.5 ± 1.2‡
(b) 1321N1				
Control		61.8 ± 2.9	23.3 ± 1.9	14.7 ± 1.3
NF 182		91.5 ± 1.8§	5.7 ± 1.2¶	2.7 ± 1.2#
NF 213		90.5 ± 1.4§	5.6 ± 0.5¶	3.7 ± 1.3#
PK 11195		89.6 ± 6.0§	3.8 ± 0.2¶	6.6 ± 2.0#
Ro5-4964		91.3 ± 1.7§	6.4 ± 1.6¶	2.1 ± 0.7#

C6 glioma (a) and 1321N1 cells (b) were seeded at the appropriate density into 25-cm^2 flasks as described in "Materials and Methods." After 24 hr they were incubated either in absence or presence of 100 μM NF 182, NF 213, PK11195 or Ro54864. The volume of ethanol in each well remained constant and did not exceed 1% (v/v). After 48 hr, the percentage of cells in each phase of the cell cycle was determined by flow cytometry as described in the "Materials and Methods" section. The values represent the mean ± SEM of three experiments. For experiment (a) *$P < 0.01$ (vs control, G0–G1 value) one-way ANOVA; †$P < 0.05$ (vs control, G2 + M value) one-way ANOVA; ‡$P < 0.001$ (vs control, S value) one-way ANOVA. For experiment (b) §$P < 0.001$ (vs control, G0–G1 value) one-way ANOVA; ¶$P < 0.005$ (vs control, G2 + M value) one-way ANOVA; #$P < 0.001$ (vs control, S value) one-way ANOVA.

cortical cell line with similar potency to the classically used PBBS ligands PK 11195 and Ro5-4864 [11]. This study demonstrates another biological effect of these NF compounds that is similar to that demonstrated by classic PBBS ligands.

No difference could be found between the antiproliferative potencies of Ro5-4864 and PK 11195 in 1321N1 cells although the latter was found to bind with higher affinity to the PBBS in this cell line. This finding, together with the quantitative discrepancy of approximately 1000-fold in the concentrations of drugs necessary to produce the antiproliferative effects and saturate the PBBS, argues against these effects being mediated through this binding site. This conclusion is in agreement with previous reports of a study of C6 glioma [9] and a Chinese hamster lung cell line V79 [10]. The present findings that PBBS are not involved in inhibition of glioma cell proliferation may be relevant to the suggestion of Miettinen *et al.*, [17], on the basis of high expression of PBBS in astrocytic tumours, that the PBBS play a role in astrocytic tumour cell proliferation.

The results of the flow cytometric studies on the cell cycle demonstrate that the antiproliferative action of NF compounds and of PK 11195 and Ro5-4864 on C6 glioma and 1321N1 cells is mediated by arrest in the G1 phase. Thus, it seems that in the micromolar range, these peripheral-type ligands have an antiproliferative effect without affecting DNA synthesis. This demonstration of the point at which the cell cycle is arrested helps to elucidate possible targets for these antiproliferative affects. A previous study [10] reported that the antiproliferative action of PK 11195, Ro5-4864 and diazepam on Chinese hamster lung cells was mediated by mitosis arrest in the G2 + M phase. The nature of cell cycle arrest by micromolar concentrations of PBBS ligands seems to be dependent on the cell type under examination although the flow cytometry results from this study are similar in glial cells from different species.

In conclusion the novel PBBS ligands in this NF series along with the classically used PBBS ligands Ro5-4864 and PK 11195 were found to exhibit antiproliferative effects on the two cell lines under examination. These effects seem unrelated to a specific interaction of these drugs with peripheral-type binding sites as there was no correlation between the affinity of the ligands for the PBBS and the potency of their antiproliferative action. This inhibition of cell growth was mediated by arrest in the G1 phase of the cell cycle. This study demonstrates the use of these NF drugs as novel probes in determining the physiological function of the PBBS and might suggest the potential of these compounds as anti-tumour drugs.

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