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A new aspect of the antiproliferative action of peripheral-type benzodiazepine receptor ligands

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Abstract

Ro 5-4864 (4-chlorodiazepam), PK 11195 (1-(2-chlorophenyl)-1,3-dihydro-1-methyl-propyl)isoquinoline carboxamide) and diazepam inhibit, in a concentration-dependent way, the proliferation of V79 Chinese hamster lung cells (IC_{50} values were: 65.0 ± 3.73 μM, 57.70 ± 4.75 μM and 106.80 ± 8.89 μM, respectively) without being cytotoxic. This antiproliferative effect is mediated by mitosis arrest in the $G_2 + M$ stage without affecting DNA synthesis and seems unrelated to a specific interaction of these drugs with the peripheral-type benzodiazepine receptor.

Keywords: Benzodiazepine; Cell proliferation; Lung; (Chinese hamster)

1. Introduction

Benzodiazepines are clinically effective drugs used as hypnotics and anxiolytics. It is known that these compounds bind to at least two different classes of binding sites termed central and peripheral-type benzodiazepine receptors. Whereas the central benzodiazepine binding sites are coupled to the γ-aminobutyric acid anion channel receptor, the peripheral-type benzodiazepine receptor is a non-ligand-gated channel receptor located on the outer mitochondrial membrane (Krueger and Papadopoulos, 1992), and different ligands such as Ro 5-4864 (4-chlorodiazepam, a specific agonist) and PK 11195 (1-(2-chlorophenyl)-1,3-dihydro-1-methyl-propyl)isoquinoline carboxamide, a specific putative antagonist) show high affinity for this receptor type.

Previous studies have characterized the peripheral-type benzodiazepine receptor in different species and tissues (Camins et al., 1994). Although the physiological function of the peripheral-type benzodiazepine receptor remains unclear, some actions of peripheral-type benzodiazepine receptor ligands, at nanomolar concentrations, have been described as stimulating steroid synthesis (Krueger and Papadopoulos, 1992) and monocyte chemotaxis (Ruff et al., 1985). In the micromolar range, peripheral-type benzodiazepine receptor ligands inhibit cell growth (Wang et al., 1984) and insulin secretion (Petit et al., 1992).

Ro 5-4864 is also able to bind to calmodulin with an associated equilibrium dissociation rate constant of about 600 nM (Morgan et al., 1987) and such an interaction may underlie some of the pharmacological actions of benzodiazepine compounds at micromolar concentrations.

Since the role that peripheral-type benzodiazepine receptor play in cell growth and differentiation has been described, the purpose of the present study was to investigate the influence of Ro 5-4864 and PK 11195 (specific peripheral-type benzodiazepine receptor ligands) and diazepam (which binds to central and peripheral-type receptors) on cell growth and survival, using the Chinese hamster lung cell line V79, and to study the effect of these compounds on the different

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phases of the cell cycle. This was done by using a sensitive analytical technique, namely flow cytometry.

2. Materials and methods

2.1. Materials

The pharmacological agents used were: Ro 5-4864 from Fluka, AG (Basel, Switzerland). Diazepam was a gift of Hoffmann-La Roche (Basel, Switzerland). PK 11195 was a gift of Pharmuka Laboratories (Asnières, France). Minimum essential medium-Eagle, foetal calf serum, L-glutamine, penicillin, streptomycin and trypsin were purchased from GIBCO (Uxbridge, England). Propidium iodide was obtained from Sigma Chemical Co. (St. Louis, MO). V79 Chinese hamster lung cells were obtained from M. Fox, Paterson Laboratories, Christie Hospital and Holt Radium Institute (Manchester, UK). Cycle-Test DNA reagent (containing the nonionic detergent Nonidet P-40, trypsin, spermine, RNase and propidium iodide) was from Beckton Dickinson (San Jose, CA). All other reagents were of analytical grade.

2.2. Cell culture

V79 Chinese hamster lung cells were routinely cultured (37°C gassed with carbogen) as monolayers in Eagle's minimal essential medium, supplemented with 10% foetal calf serum, penicillin (10000 IU/ml and streptomycin (10000 μg/ml). Petri dishes (9-cm diameter) were seeded with 300000 to 500000 cells in 10 ml of culture medium and the generation time was about 12 h. After 24 h, the cells were exposed to different drug concentrations dissolved in dimethylsulfoxide for a further 24 h. After being washed with 5 ml of phosphate buffer solution 0.01 M (pH 7.4), the cells were collected by trypsinization (2 ml trypsin 0.25%/EDTA 1 mM for 10 min) and resuspended in 8 ml of Eagle's minimal essential medium in order to be quantified with a Sysmex F300 M counter.

2.3. Flow cytometric assays

2.3.1. Cytotoxicity studies

Cells were exposed for 24 h to different drug concentrations ranging from 10^{-7} M to 2.5×10^{-4} M. Propidium iodide was added at a final concentration of 0.1 mg/ml for 10 min. Samples were analyzed by flow cytometry in a FACScan (Beckton Dickinson), the percentage of cells incorporating the fluorochrome and the percentage of those not being quantified.

2.3.2. DNA measurements

Viable cells were stained with propidium iodide according to the method of Vindelov et al. (1983) using the cell cycle test. The emitted fluorescence of the DNA-propidium iodide complex was also measured in the FACScan. As emitted fluorescence is proportional to DNA cell content, it is possible to measure and differentiate diploid from tetraploid fractions that correlate well with the G_1 and $G_2 + M$ cell phases. A double discriminator module was used to distinguish between signals coming from a single nucleus and those produced by nuclear aggregation.

IC_{50} values (a concentration which inhibits cell proliferation by 50%) were calculated by non linear analysis from the complete concentration-inhibition curves. Results are reported as means ± S.E.M. from six to seven different experiments.

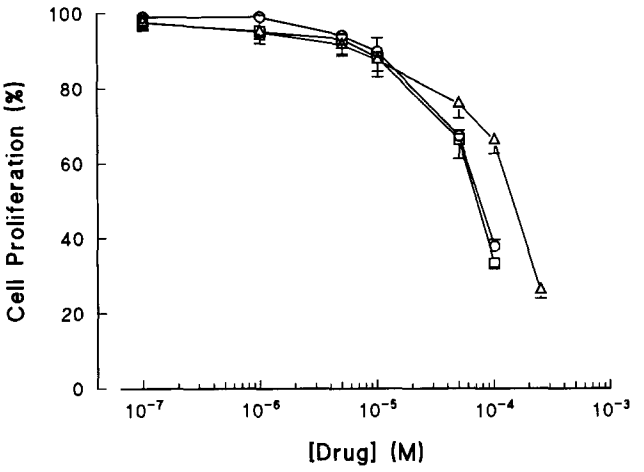

Fig. 1. Inhibition of proliferation of V79 Chinese hamster lung cells by Ro 5-4864 (○), PK 11195 (□) and diazepam (△). Data are the mean ± S.E.M. from six to seven different experiments.

3. Results

In V79 Chinese hamster lung cells, Ro 5-4864 and PK 11195 (10^{-7} to 10^{-4} M) and diazepam (10^{-7} to 2.5×10^{-4} M) induced, in a concentration-dependent manner, an inhibition of cell proliferation (see Fig. 1). This inhibitory effect was not due to cell toxicity because in all samples cell viability was greater than 95%, as assessed by the propidium iodide assay. IC_{50} values of Ro 5-4864, PK 11195 and diazepam were: $65.0 \pm 3.73 \times 10^{-6}$ M, $57.70 \pm 4.75 \times 10^{-6}$ M and $106.80 \pm 8.84 \times 10^{-6}$ M, respectively.

In order to determine the cellular DNA content, a flow cytometric analysis was carried out. Each result is the mean of at least three separate experiments. The distribution of populations, according to DNA content, is summarized in Table 1. Our results showed that all compounds induced a specific accumulation of V79 Chinese hamster lung cells in the $G_2 + M$ phase (see Fig. 2).

Table 1
Effect of peripheral-type benzodiazepine receptor ligands on the distribution of V79 chinese hamster lung cells according to DNA content

Drug	Conc. (μM)	Cell phase (%)		
		$G_0 - G_1$	$G_2 + M$	S
–	0	57.1	12.8	30.0
Ro 5-4864	1	58.6	16.0	25.4
	50	55.8	19.4	25.1
	100	49.1	26.4	24.5
PK 11195	1	57.1	13.3	29.6
	50	58.6	14.4	26.9
	100	55.3	25.7	19.0
Diazepam	1	57.2	13.3	29.5
	50	55.1	18.1	26.7
	100	51.3	22.4	26.2

4. Discussion

The results of the present experiments clearly indicate that Ro 5-4864, PK 11195 and diazepam inhibit, in the micromolar range, the proliferation of V79 Chinese hamster lung cells. The rank order of potency was: PK 11195 > Ro 5-4864 ≫ diazepam. At the concentrations assayed (up to 2.5×10^{-4} M), none of the drugs tested were cytotoxic. Even after 24 h of incubation with these compounds, more than 95% of V79 cells remained intact, confirming that a toxic effect of these drugs on cell growth can be discarded.

The antiproliferative effect of Ro 5-4864, PK 11195 and diazepam described in this paper appeared at micromolar concentrations, thus excluding a specific interaction with peripheral-type benzodiazepine recep-

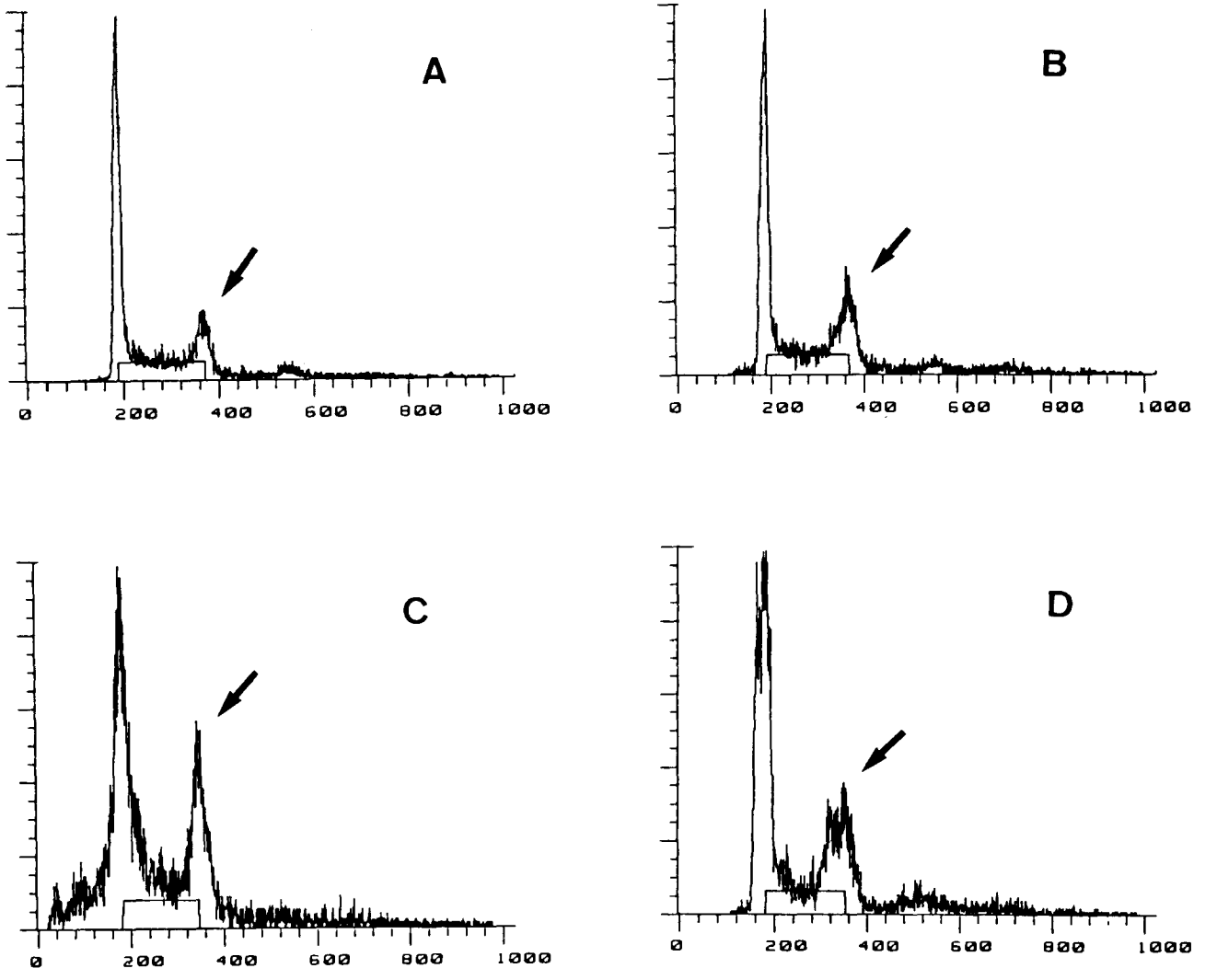

Fig. 2. Flow cytometric determination of changes in cell cycle distribution in V79 Chinese hamster lung cells, following 24 h exposure to (A) Control, (B) Diazepam, (C) Ro 5-4864 and (D) PK 11195 at a concentration of 10^{-4} M. Ordinates: cell number (counts full scale). Abscissae: DNA content based on propidium iodide fluorescence intensity. Arrows indicate the increased fluorescence corresponding to cells in the $G_2 + M$ stage.

tors. Previous studies on rat duodenum and vas deferens (Escubedo et al., 1992) demonstrated an inhibitory effect of these compounds, also at micromolar concentrations, which was related to cell Ca^{2+} bioavailability. Because a new binding site for Ro 5-4864 on calmodulin, with micromolar affinity, was described by Morgan et al. (1987), we can hypothesize that the antiproliferative effect shown by these drugs is probably related to calmodulin-dependent Ca^{2+} events.

From our results of flow cytometric studies on the cell cycle, it can be concluded that this antiproliferative effect of benzodiazepine compounds is mediated by mitosis arrest in the $G_2 + M$ stage. Thus, it seems that, in the micromolar range, peripheral-type benzodiazepines have an antiproliferative effect without affecting DNA synthesis. Previous studies (Clarke and Ryan, 1980) reported that diazepam inhibits the proliferation of 3T3 cells in pre-S stage, but Andersson et al. (1980) stated that this drug inhibits the fibroblast cell cycle in mitosis, which is arrested at prometaphase, and Miernik et al. (1986) described an antiproliferative effect of central benzodiazepine compounds on green alga, which inhibit the duplication and separation of centrioles. The results obtained in this study suggest that, in V79 Chinese hamster lung cells, Ro 5-4864, PK 11195 and diazepam have an antimitotic effect, inhibiting any phase of mitosis, as indicated by the accumulation of cells in the $G_2 + M$ phase, but it remains to be determined if Ro 5-4864 and PK 11195 act, like diazepam, at the prometaphase stage.

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