Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 550 (2006) 8–14
www.elsevier.com/locate/ejphar

Effects of peripheral-type benzodiazepine receptor ligands on Ehrlich tumor cell proliferation

Mônica Sakai [a], Evelise Souza Monteiro Fonseca [a], Silvia Catarina Salgado Oloris [b], Patrícia Matsuzaki [b], Andréia Hanada Otake [c], Kátia Ramos Moura Leite [d], Cristina Oliveira Massoco [d], Maria Lúcia Zaidan Dagli [b], João Palermo-Neto [a,*]

[a] *Laboratory of Applied Pharmacology and Toxicology, School of Veterinary Medicine, University of São Paulo, São Paulo, SP, Brazil*
[b] *Laboratory of Experimental Oncology, School of Veterinary Medicine, University of São Paulo, São Paulo, SP, Brazil*
[c] *Laboratory of Experimental Oncology, Faculty of Medicine, University of São Paulo, São Paulo, SP, Brazil*
[d] *Laboratory of Pathology, Sírio Libanês Hospital, São Paulo, SP, Brazil*

Received 10 November 2005; received in revised form 11 July 2006; accepted 26 July 2006
Available online 23 August 2006

Abstract

Peripheral-type benzodiazepine receptors have been found throughout the body, and particularly, in high numbers, in neoplastic tissues such as the ovary, liver, colon, breast, prostate and brain cancer. Peripheral-type benzodiazepine receptor expression has been associated with tumor malignity, and its subcellular localization is important to define its function in tumor cells. We investigated the presence of peripheral-type benzodiazepine receptors in Ehrlich tumor cells, and the *in vitro* effects of peripheral-type benzodiazepine receptors ligands on tumor cell proliferation. Our results demonstrate the presence of peripheral-type benzodiazepine receptor in the nucleus of Ehrlich tumor cells (85.53 ± 12.60%). They also show that diazepam and Ro5-4864 (peripheral-type benzodiazepine receptor agonists) but not clonazepam (a molecule with low affinity for the peripheral-type benzodiazepine receptor) decreased the percentage of tumor cells in G0–G1 phases and increased that of cells in S-G2-M phases. The effects of those agonists were prevented by PK11195 (a peripheral-type benzodiazepine receptor antagonist) that did not produce effects by itself. Altogether, these data suggest that the presence of peripheral-type benzodiazepine receptor within the nucleus of Ehrlich tumor cells is associated with tumor malignity and proliferation capacity.

Keywords: Peripheral-type benzodiazepine receptor; Cancer; Ehrlich tumor; Diazepam; Ro5-4864; Proliferation

1. Introduction

Benzodiazepines are the most frequently prescribed class of psychotropic drugs in Brazil, in the United States, in Europe, and possibly worldwide (Ruiz et al., 1993; Stiefel et al., 1999). Benzodiazepines, such as diazepam, are commonly used for their anxiolytic and sedative effects, i.e., by their action on high affinity receptor sites coupled to $GABA_A$ complex, present in the central nervous system (CNS) (Costa et al., 1975). Nevertheless, in addition to the central-type of benzodiazepine receptors, peripheral-type binding sites have been identified. Expression of peripheral-type benzodiazepine receptor (PBR) has been found throughout the body, including endocrine steroidogenic tissues (Zilz et al., 1999), immune cells (Zavala, 1997), and also in tumor cells (Han et al., 2003; Hardwick et al., 1999).

Peripheral-type benzodiazepine receptor expression was shown to be increased in some neoplastic tissues and tumor cells, particularly in the ovary (Katz et al., 1990a), liver (Venturini et al., 1999), colon (Katz et al., 1990b), breast (Beinlich et al., 1999; Hardwick et al., 1999) and in the brain (Miettinen et al., 1995). Peripheral-type benzodiazepine receptor expression has also been associated with both tumor progression and aggressiveness, since higher levels of its expression

* Corresponding author. Faculdade de Medicina Veterinária e Zootecnia, Laboratório de Farmacologia Aplicada e Toxicologia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, no. 87, CEP: 05508-900 Cidade Universitária, São Paulo, SP, Brazil. Tel.: +55 11 3091 7957; fax: +55 11 3091 7829.
E-mail address: jpalermo@usp.br (J. Palermo-Neto).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.07.057

were found in tumor cells that display increased rate of proliferation, such as breast cancer cells (Corsi et al., 2005; Hardwick et al., 1999), hepatic cancer cells (Corsi et al., 2005) and glioma cells (Brown et al., 2000). These facts led some authors to suggest that peripheral-type benzodiazepine receptor expression could even be used as a prognostic tool in breast, colorectal and prostate tumors. Indeed, increased levels of peripheral-type benzodiazepine receptor expression were reported in malignant tumors, and in tumors with increased metastatic capability (Galiegue et al., 2004; Han et al., 2003).

Peripheral-type benzodiazepine receptor expression localization within tumor cells has been assessed, aiming at a better understanding of its function on tumor progression. Thus, the presence of peripheral-type benzodiazepine within the nucleus of tumor cells was reported to be positively correlated to an increase on breast cancer cell aggressiveness (Hardwick et al., 1999). Furthermore, those breast tumor cells were shown to proliferate in response to peripheral-type benzodiazepine receptor ligands (Brown and Papadopoulos, 2001; Corsi et al., 2005; Hardwick et al., 1999). Accordingly, *in vitro* treatment with smaller doses of peripheral-type benzodiazepine receptor ligands caused an increase in MDA-231 breast cancer cell proliferation (Beinlich et al., 1999; Hardwick et al., 1999), BT-20 breast cancer cells (Beinlich et al., 1999) and MGM-1 humans glioma cells (Brown and Papadopoulos, 2001). On the other hand, it was reported that *in vitro* treatment with two peripheral-type benzodiazepine receptor ligands decreased melanoma B16 cell (Landau et al., 1998), lymphoma cell (Alexander et al., 1992), thymoma cell (Wang et al., 1984) and glioma C6 cell (Zisterer et al., 1998) proliferation rates.

In a previous study we showed that *in vivo* treatment with diazepam increases Ehrlich tumor growth (Sakai et al., 2006). In the present study, we looked for peripheral-type benzodiazepine receptor expression and localization within Ehrlich tumor cells. The *in vitro* effects of peripheral-type benzodiazepine receptor ligands on Ehrlich tumor cell proliferation were also analyzed.

2. Material and methods

2.1. Ehrlich tumor model and cell culture

Ehrlich tumor cells syngeneic to mice were used in this study. This tumor is maintained in its ascitic form by weekly i.p. transplantations in mice (Matsuzaki et al., 2003; Salgado Oloris et al., 2002). Animals were housed and used in accordance to the Ethical Principles in Animal Research adopted by the Bioethics Commission of the School of Veterinary Medicine, University of São Paulo (protocol 160/2002); these guidelines are similar to those of the National Research Council, USA.

Primary tumor cell culture was carried out as described elsewhere (Obrador et al., 2001). Briefly, the ascitic Ehrlich tumor fluid was collected through an abdominal incision with a sterile syringe. Ehrlich tumor cells were subsequently rinsed twice in Phosphate-Buffered Saline (PBS) for 5 min, centrifuged at 250 ×*g* and then suspended in RPMI medium (10 mM Hepes, 40 mM $NaHCO_3$, 100 units/ml penicillin and 100 μg/ml streptomycin) supplemented with 10% fetal bovine serum (FBS). Tumor cell counts were performed in a Neubauer chamber using the Trypan Blue dye exclusion method.

For *in vitro* studies, 1 ml of Ehrlich tumor cells (5×10^5 cells/ml) was seeded in 24-well plates. The peripheral-type benzodiazepine receptor agonists diazepam (Sigma, St Louis, MO, USA) and Ro5-4864 (4′-chloro-diazepam, Fluka Sigma-Aldrich, St Louis, MO, USA) were used in several concentrations (100 nM, 300 nM, 600 nM, 1 μM) for the *in vitro* treatment. Clonazepam (Renochen) — a molecule with low affinity for the peripheral-type benzodiazepine receptor and high affinity for $GABA_A$ receptors, and the peripheral-type benzodiazepine receptor antagonist PK11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide, Sigma, St Louis, MO, USA) were also used in two different concentrations (100 nM and 1 μM). For the experiments in which tumor cells were treated with two different drugs, the 1st treatment was performed just before incubation, and the 2nd treatment was added 24 h later; evaluations were performed 48 h after the 1st treatment. All drugs were diluted in 0.01% ethanol, and this vehicle was used as control solution. Cells were kept in a humid atmosphere of 5% CO_2 in air, at 37 °C for 48 h.

2.2. Immunohistochemistry

Peripheral-type benzodiazepine receptor immunohistochemistry was performed in order to analyze its expression and localization in tumor cells. Since peripheral-type benzodiazepine receptors are known to be highly expressed in adrenal cells, immunoreactivity of mouse adrenal tissue was used as a positive control for peripheral-type benzodiazepine receptor staining. Paraffin embedded sections of tumor samples were mounted on silane-covered slides, and subsequently deparaffinized and rehydrated by conventional methods. Antigen retrieval was performed in a heated citrate buffer (pH 6.0) and endogenous peroxidase was blocked by 3% hydrogen peroxide solution in the dark. The sections were incubated overnight (at 4 °C) with a rabbit polyclonal anti-peripheral-type benzodiazepine receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 in a 5% bovine serum albumin, fraction V (BSA) solution in PBS. Immunohistochemical detection was accomplished using streptavidin-biotin-peroxidase (LSAB-kit DAKO, Carpinteria, CA, USA) and the reaction developed by incubation with 0.03% of 3,3′-diaminobenzidine — DAB (Sigma, St Louis, MO, USA). Sections were counterstained with Mayer's hematoxilin and analyzed using the Image Pro Plus® software package.

2.3. Peripheral-type benzodiazepine receptor detection by flow cytometry

Peripheral-type benzodiazepine receptor staining for flow cytometric analysis was performed as proposed elsewhere (Sanger et al., 2000). Briefly, tumor cells were fixed in 0.4% (w/v) paraformaldehyde in PBS for 5 min. Saponin was used for permeabilization (0.1% (w/v) saponin in PBS containing 0.1% (w/v) bovine serum albumin (BSA) for 5 min). The cells were incubated for 30 min at 4 °C with an anti-peripheral-type