Isoquinoline and Peripheral-type Benzodiazepine Binding in Gliomas: Implications for Diagnostic Imaging

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ABSTRACT

Binding of the isooquinoline PK 11195 and of the benzodiazepines Ro5-4864 and flunitrazepam was compared in glioma cells and tissues. In human and rat glioma cell cultures [3H]PK 11195 bound with higher affinity (Kd = 14.01 and 15.76 nM, respectively) than either Ro5-4864 (Kd = 1200 and 84.9 nM, respectively) or flunitrazepam (Kd > 10,000 and = 848 nM, respectively). Autoradiograms of postmortem human brain sections containing glioma revealed that [3H]PK 11195bound specifically to intact tumor cells and not to cells of normal cerebral cortex or necrotic areas of the tumor. Total [3H]Ro5-4864 or [3H]flunitrazepam binding to these sections was indistinguishable from nonspecific binding, and regions of tumor and normal brain could not be delineated. These results support the use of radiolabeled PK 11195 for clinical trials of imaging human gliomas by positron emission tomography.

INTRODUCTION

PBS are ligand accepters of unknown physiological relevance first described in “peripheral tissues” outside the rat central nervous system (1–3). They are anatomically and pharmacologically distinct from central benzodiazepine receptors which are neuronal sites linked to γ-amino butyric acid-mediated Cl– channels (1, 4). PBS are present in high concentrations in most organs of the thoracic, abdominal, and pelvic cavities (2, 5). However, PBS are present only in low concentrations in the brain, primarily on glia (6–8), but are present in high concentrations in malignant gliomas (9–11). Because normal brain has low binding of PBS ligands, the potential exists for imaging of malignant gliomas using radiolabeled PBS ligands and PET or single photon emission tomography.

Diazepam and flunitrazepam bind to both the central benzodiazepine receptor and the PBS. However, only the central benzodiazepine receptor binds clonazepam (1, 4). Traditionally PBS have been defined by their ability to bind Ro5-4864 but not clonazepam (3, 4, 12). An isooquinoline derivative, PK 11195, binds to the same tissues as the benzodiazepine, Ro5-4864, in rodents and is capable of competing with Ro5-4864 for binding in these tissues (13, 14).

Ligands which bind to PBS are capable of binding with high capacity to malignant glioma cells (9–11). Binding to homogenized rat C6 glioma cells with [3H]flunitrazepam (5 nM) was 10 times higher than binding in the rat kidney under the same conditions (9). Rat C6 glioma and human U251 glioma, when grown in the striatum of athymic rats, were successfully imaged against low background binding in normal brain by both in vitro and in vivo methods (10, 11). Radioligands capable of imaging the tumors included the isooquinoline derivative, PK 11195, and the benzodiazepines, Ro5-4864 and flunitrazepam. Based on this animal model, [11C]Ro5-4864 was used to scan brains of patients with gliomas by positron emission tomography. In the PET scans, binding in the tumor by [11C]Ro5-4864 was lower than or equal to binding in normal cerebral cortex (15). The reason for the observed variation in radiolabeled Ro5-4864 binding between rats and humans is unknown, but PBS ligand binding differences between species could possibly be a factor.

The purpose of this study was to investigate the binding of radiolabeled PBS ligands in cultured human glioma cells and in postmortem specimens of human brain containing glioma and to compare this human PBS ligand binding with that of rat glioma.

MATERIALS AND METHODS

Drugs and Chemicals. Unlabeled benzodiazepines were gifts of Dr. P. Sorter, Hoffman-LaRoche, Nutley, NJ. Unlabeled PK 11195 was a gift of Dr. G. Le Fur, Pharmuka, Genevilliers, France. Tritiated PK 11195 (75.2 Ci/mmol) and Ro5-4864 (77.9 Ci/mmol) were purchased from Du Pont-New England Nuclear, Boston, MA. Tritiated flunitrazepam was purchased from Amersham, Arlington Heights, IL.

Cultures and Tissues. Human glioblastoma lines U251 and D54 were obtained from Dr. Darrel D. Bigner of Duke University. Rat glioma line C6 was purchased from the American Type Culture Collection, Rockville, MD. The cells were seeded in 24-well plates and grown to confluency in humidified air supplemented with 5% CO2 at 37°C using minimal essential medium supplemented with 10% fetal bovine serum and 1% Na+ pyruvate (Gibco, Grand Island, NY).

For autoradiographic studies, 1-cm-thick slabs of postmortem human brain containing gliomas were obtained at autopsy from the Department of Pathology at the University of Michigan Medical Center. The tissue was immediately frozen at −70°C.

Binding Assay on Cell Cultures. Medium of exponentially growing cells was replaced with 140 mM NaCl buffered with 10 mM NaH2PO4 (pH 7.4, 4°C), 2.6 mM KCl, and 1.4 mM KH2PO4 for 10 min to remove binding inhibitors which might be present in the medium. Prewash buffer was aspirated and replaced with buffer containing [3H]PK 11195, [3H]Ro5-4864, or [3H]flunitrazepam with 1 mM clonazepam (to block “central benzodiazepine receptors”). Nonspecific binding was determined in the presence of 10 µM unlabeled PK 11195 or 10 µM Ro5-4864. Following the incubation (15 min, 4°C), the ligand mixture was aspirated. Labeled monolayers were postwashed 3 times for 5 min with NaCl buffer. Cells were removed from wells with trypsin, placed into vials with 4 ml of scintillant, and assayed for tritium by liquid scintillation spectrometry.

Binding studies (Fig. 1) were carried out using 1 nM tritiated ligand in the absence of competitor or in the presence of 10 µM unlabeled PK 11195, Ro5-4864, or flunitrazepam. Saturation experiments (Table 1) were carried out using 150 pM to 30 nM [3H]PK 11195. Displacement studies were carried out by adding increasing concentrations (10 pM to 10 µM) of unlabeled Ro5-4864, flunitrazepam, or clonazepam (a benzodiazepine specific for the central benzodiazepine receptor) to mixtures containing 1 nM [3H]PK 11195. Protein concentration was determined by the Bradford assay (Biorad, Richmond, CA).

Autoradiography on Postmortem Human Glioma Samples. Frozen postmortem human brains with glioma were sectioned (40 µM) and mounted on glass slides. Sections were postwashed 3 times for 5 min in

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* The abbreviations used are: PBS, peripheral-type benzodiazepine-binding site(s); PET, positron emission tomography.
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50 mM Tris-Cl. Incubations with 1 nM [3H]PK 11195, 2 nM [3H]Ro5-4864, or 5 nM [3H]flunitrazepam were carried out in the same buffer. Blanks were determined in the presence of 10 μM unlabeled ligand. Sections were postwashed 3 times for 5 min and then dried under a cool stream of air. The slides were placed in an X-ray cassette with radioactive standards. Ultradens II was apposed to the slides for 2 wk at 4°C before being developed. The images produced by the radiolabeled tissue sections were analyzed by computer-assisted densitometry, and the radioactivity of the underlying tissue was determined by a computer-generated polynomial regression analysis which compared film densities produced by the tissue sections to those produced by the radioactive standards (16).

RESULTS

Tritiated ligands capable of labeling tumors implanted in athymic rats were compared in binding assays performed on human U251 glioma and rat C6 glioma cultures (Fig. 1). Total [3H]PK 11195 binding was approximately twice that of [3H]-Ro5-4864 and greater than 10-fold that of [3H]flunitrazepam binding in cultures from both species. Unlabeled PK 11195 or Ro5-4864 was capable of displacing greater than 85% of the [3H]PK 11195 binding in both species. Unlabeled flunitrazepam was capable of displacing 75% of [3H]PK 11195 binding in rat C6 glioma but could displace only 34% of binding in human glioma cells.

Unlabeled PK 11195, Ro5-4864, or flunitrazepam was capable of displacing half of the [3H]Ro5-4864 binding observed in rat C6 cells in the absence of competitor. In contrast, none of the competing drugs could displace more than 5% of [3H]-Ro5-4864 binding in human glioma cells. Total [3H]flunitrazepam binding was not significantly different from nonspecific binding in the presence of any of the three competing ligands in human glioma cultures. Preliminary studies demonstrated that two other human glioblastoma cell lines, U87 and D54,
Fig. 2. a to f, autoradiographic comparison of PBS ligands in glioma tissue, gray matter, and white matter of human postmortem brain. a, total [3H]PK 11195 binding; b, nonspecific PK 11195 binding; c, total Ro5-4864 binding; d, nonspecific Ro5-4864 binding; e, total flunitrazepam binding; f, nonspecific flunitrazepam binding. Nonspecific binding is measured in the presence of 10 µM unlabeled PK 11195, Ro5-4864, or flunitrazepam for [3H]PK 11195, [3H]Ro5-4864, and [3H]-flunitrazepam, respectively. g, cresyl violet stain of area outlined in a. h, autoradiograph of [3H]PK 11195 binding in the same area; i, computer-enhanced color autoradiograph of [3H]PK 11195 binding.
bound PK 11195 with high affinity but had low affinity for Ro5-4864 and flunitrazepam (data not shown).

Scatchard analysis of PK 11195 binding demonstrated that both U251 and C6 cells have high affinity for[^14C]PK 11195 ($K_a = 14.0 \pm 3.4 \text{ nM}$ and $15.8 \pm 1.5 \text{ nM}$, respectively) and similar binding capacities for PK 11195 ($B_\text{max} = 4.1 \pm 1.39 \text{ pmol/mg of protein}$ and $5.6 \pm 0.6 \text{ pmol/mg of protein}$, respectively [Table 1]). To determine whether the low specific binding observed with either Ro5-4864 or flunitrazepam was due to low affinity of the binding sites for these ligands, competition studies were performed in which increasing concentrations of unlabeled Ro5-4864, flunitrazepam, or clonazepam were added to ligand mixtures containing 1 nM final concentration of[^3H]PK 11195 (Table 1). In all cases, the Hill coefficient was near 1.0, indicating that the ligands were competing for binding at a single class of binding sites (Table 1). The affinity for Ro5-4864 binding to rat U251 glioma ($K_i = 84.9 \pm 14.4 \text{ nM}$) was substantially higher than the affinity for Ro5-4864 binding to human U251 glioma ($K_i = 1204 \pm 97 \text{ nM}$, where $K_i$ = 50% inhibitory concentration/$(1 + [\text{radioligand}]/K_0$ of radioligand$)$).

Likewise, the $K_i$ for flunitrazepam binding to C6 cells was 848 $\pm 53.4 \text{ nM}$ while concentrations as high as 10 $\mu\text{M}$ flunitrazepam did not displace 50% of PK 11195 binding (Table 1). Clonazepam did not compete with[^3H]PK 11195 in either rat or human glioma cell cultures.

Autoradiographic analysis was performed on three postmortem human glioma specimens in order to compare PK 11195 binding to that of Ro5-4864 and flunitrazepam (Fig. 2, Table 2). Fig. 2 presents a single specimen in which the total and nonspecific binding of the three ligands is compared in a to/Total[^3H]Ro5-4864 and[^3H]flunitrazepam binding in viable tumor was similar to that in necrotic tumor and was only slightly different from nonspecific binding in tumor. Intense binding by[^3H]flunitrazepam in gray matter could be blocked with clonazepam, indicating that the binding observed represents central benzodiazepine receptors.

[^3H]PK 11195 (1 nM) bound specifically to viable tumor cells (red/orange ring in Fig. 2a) but not to necrotic tumor or normal cerebral cortex. To demonstrate this further, the cresyl violet stain of a 4-mm x 5-mm square of the section (Fig. 2g) is compared to the autoradiograph (Fig. 2h) and color-enhanced digital transformation of the autoradiograph (Fig. 2i) of[^3H]PK 11195 binding. In the cresyl violet stain, dark areas represent viable tumor cells with concentrated nuclei, medium gray areas represent normal cortex (center of bottom edge), and clear areas represent necrotic tumor. In Fig. 2h, increasing levels of binding are represented by increasing “blackness.” In the color-enhanced panels ($a$ to $f$, $i$), a rainbow color scale is used in which highest binding is represented by red/orange followed by yellow, green, blue, and purple.

The results of densitometric analysis of autoradiograms from three postmortem glioma cases are presented in Table 2. The specimen presented in Fig. 2 is the case which most closely matched the mean values presented in Table 2. The two cases which were not presented autoradiographically were similar to the case shown in Fig. 2.

**DISCUSSION**

The benzodiazepine[^3H]Ro5-4864 was capable of imaging rat or human gliomas which had been grown intracranially in athymic rats (10).[^14C]Ro5-4864 failed, however, to distinguish tumor from noninvolved brain in six patients scanned by positron emission tomography (15). In the current study, it was shown that low nanomolar concentrations of[^3H]Ro5-4864 were incapable of specifically labeling PBS in either human glioma cells grown in culture or in postmortem sections of human glioma. Likewise, the affinity of cultured human glioma cells for the benzodiazepine flunitrazepam was such that no specific binding of 1 nM flunitrazepam could be detected. The low affinity for Ro5-4864 of cultured or postmortem human glioma cells offers one explanation for the inability of[^14C]Ro5-4864 to label gliomas in patients. These studies do not, however, rule out other explanations, such as (a) primarily necrotic tumors in all patients scanned, (b) rapid metabolism of[^14C]Ro5-4864 to a metabolite which does not bind to the PBS, or (c) absence of PBS in the tumors of the six patients.

The reason that[^3H]Ro5-4864 (nM) was capable of imaging U87 human glioma that had been grown in athymic rats but was incapable of specifically binding to the same cells or other human glioma cells grown in culture is still unclear. Possible explanations include altered chemical milieu in the microenvironment surrounding the binding sites in rat tissues, altered glycosylation states, altered binding site conformation, or an endogenous modulator of benzodiazepine binding. Studies are currently under way to determine why human glioma cells, when grown in rats, bind PBS ligands with a pharmacological profile similar to rat glioma cells.

Few human tissues have been studied for their ability to bind PBS ligands. Human iris, pineal gland, and brain bind Ro5-4864 and flunitrazepam with relatively low affinity (17, 18). The reported affinity constants are similar to those observed in cultured human glioma cells (Table 1). Human erythrocytes, platelets, and placenta bind benzodiazepines with high affinities, with binding parameters which more closely resemble those observed in rat tissues (3, 19-21). All of these tissues bind PK 11195 with high affinity, with $K_a$ values ranging from 1 to 16 nM (17-22). It is therefore possible that PK 11195 binds with similar affinity to normal and neoplastic tissues of several species, while Ro5-4864 and flunitrazepam binding varies between species, between normal and neoplastic tissues, or even between normal tissues of the same species.

PBS ligands have been shown to affect the growth rate and differentiation state of cultured tumor cells (23-26). In each of these cases, the concentration of ligand needed to observe the effect was in the micromolar range, while binding affinities for PBS ligands in the same cells were reported to be in the nanomolar range (23-26). The binding assays, however, were performed at 4°C in membrane homogenates. Low affinity binding of Ro5-4864 and flunitrazepam to living monolayers of cells in this study provides examples of cultured cells binding benzodiazepines with affinities that resemble the concentrations needed to affect the growth and differentiation of tumor lines.

Binding of PK 11195, Ro5-4864, flunitrazepam, and clona-
zep to human glioma cells in culture was representative of binding of the same ligands to postmortem human glioma specimens and to surgical resection samples of human glioma tissue (surgical resection data not presented). It is possible then that the monolayer binding assay described in this paper will provide a valid, inexpensive, and rapid means for testing potential PBS ligands for binding to human glioma cells.

As observed in intracranial glioma implants in nude rats (10), PK 11195 binding in human postmortem sections was specific for viable glioma cells and was capable of clearly distinguishing small groups of these cells from necrotic tumor cells and normal cerebral cortex. Because of the high specificity of PK 11195 for living tumor cells, fluorochrome-labeled derivatives of this ligand may prove to be useful as neuropathology probes or in guiding neurosurgeons to small rests of tumor cells during debulking operations. Additionally, the ability of [3H]PK 11195 to bind with high affinity and with high specificity to human glioma cells in culture and to surgical and postmortem human glioma specimens supports the use of radiolabeled PK 11195 for PET scanning glioma patients in clinical trials.

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REFERENCES


Table 2. PK 11193, Ro5-4864, and flunitrazepam binding to intact tumor cells, necrotic tissue, and gray matter in autoradiogram of postmortem human brain

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intact tumor (nmol/mg protein)</th>
<th>Necrotic area (nmol/mg protein)</th>
<th>Gray matter (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]PK 11195*</td>
<td>Total: 2067 ± 414</td>
<td>440 ± 80</td>
<td>551 ± 356</td>
</tr>
<tr>
<td>Specific: 1779 ± 335</td>
<td>143 ± 19</td>
<td>281 ± 219</td>
<td></td>
</tr>
<tr>
<td>[3H]Ro5-4864*</td>
<td>Total: 1473 ± 226</td>
<td>1213 ± 26</td>
<td>1314 ± 18</td>
</tr>
<tr>
<td>Specific: -38 ± 136</td>
<td>292 ± 252</td>
<td>294 ± 121</td>
<td></td>
</tr>
<tr>
<td>[3H]Flunitrazepam*</td>
<td>Total: 195 ± 42</td>
<td>163 ± 142</td>
<td>1275 ± 114</td>
</tr>
<tr>
<td>Specific: 72 ± 8</td>
<td>46 ± 56</td>
<td>1222 ± 123</td>
<td></td>
</tr>
</tbody>
</table>

* One nM final concentration.
† Mean ± SEM (n = 3).
‡ Calculated from total binding - binding in the presence of 10 nM unlabeled ligand.
§ Two nM final concentration.
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