Nucl. Med. Biol. Vol. 16, No. 4, pp. 423–429, 1989
Int. J. Radiat. Appl. Instrum. Part B
Printed in Great Britain.

0883-2897/89 $3.00 + 0.00

Synthesis of a High Specific Activity ^{125}I-Labeled Analog of PK 11195, Potential Agent for SPECT Imaging of the Peripheral Benzodiazepine Binding Site

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(*Received* 27 *May* 1988)

The peripheral benzodiazepine binding site ligand PK 11195 has been ^{125}I-labeled by direct displacement of aromatic chlorine under solid-state conditions in 50–76% radiochemical yield and >94% radiochemical purity. Purification by high pressure liquid chromatography increased the specific activity of the product from an initial 15–17 Ci/mmol to a final activity of 260–910 Ci/mmol. To determine the affinity of this [^{125}I]PK 11195 analog for human glioma cells, saturation experiments were performed on monolayers of U_{251} human glioblastoma cells. Scatchard analysis of saturation data demonstrated that the [^{125}I]PK 11195 analog binds to a single class of sites with a K_D of 8.0 ± 1.7 nM and maximal binding of 3.8 ± 0.1 pmol/mg protein. These values are similar to those obtained when [^{3}H]PK 11195 was assayed in U_{251} cells (K_D = 14 ± 3.4, B_{max} = 4.1 ± 1.3) suggesting that iodination does not appreciably alter the binding of PK 11195 to human glioma cells. *In vivo* autoradiographic studies of brain in C_6 glioma bearing rats demonstrate selective binding of the radioligand to the tumor. These results suggest that this [^{125}I]PK 11195 analog may be a useful radiotracer for the study of peripheral benzodiazepine binding sites.

Introduction

Two pharmacologically distinct binding site subtypes, central and peripheral, have been demonstrated for the benzodiazepines. The central benzodiazepine receptor is localized on neuronal tissue and is the site at which benzodiazepine ligands are thought to exert their anxiolytic, anticonvulsant and muscle relaxant effects (Tallman and Gallager, 1985; Tallman *et al.*, 1982; Haefely, 1983). The peripheral benzodiazepine binding site (PBS), so named for its initial identification in peripheral tissues including adrenal cortex, kidney and heart (DeSouza *et al.*, 1985; Davies and Huston, 1981), is present mainly outside the CNS but is also present in olfactory bulb of rat and in low concentrations in brain glial cells (Anholt *et al.*, 1984; Schoemaker *et al.*, 1982). These two binding site subtypes can be pharmacologically distinguished through the use of highly selective ligands. Thus clonazepam, a potent anticonvulsant, binds to the central receptor with nanomolar affinity but to the PBS with less than micromolar affinity (Wang *et al.*, 1984). Conversely, both Ro 5-4864 [7-chloro-1,3-dihydro-1-methyl-5-(4′-chlorophenyl)-2H-1,4-benzodiazepine-2-one] and the non-benzodiazepine PK 11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinoline carboxamide], which exhibit no anxiolytic activity, bind to the PBS in rat tissues with nanomolar affinity but have at least a 1000-fold lower affinity for the central receptor (Wang *et al.*, 1984; Dubroeucq *et al.*, 1982). These binding site subtypes are further differentiated by the lack of effect of γ-amino butyric acid on ligand binding to the PBS, in contrast to its effect on ligand binding to the central receptor (Schoemaker *et al.*, 1982; Marangos *et al.*, 1982).

Recently, C_6 gliomas grown intracranially in rats have been successfully delineated from normal brain by *in vivo* autoradiographic studies using ^{3}H-labeled PK 11195 (Starosta-Rubenstein *et al.*, 1987). High concentrations of the PBS have also been demonstrated in human glial tumors (Starosta-Rubenstein *et al.*, 1987; Olson *et al.*, 1988). Furthermore, [^{11}C]PK 11195 has been used to map heart PBS *in vivo* (Charbonneau *et al.*, 1986). These studies suggested that a radioiodine labeled analog of PK 11195 might have

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clinical utility for single photon emission computed tomography (SPECT) imaging of the PBS including those in human glioma. We report the synthesis of a high specific activity ^{125}I-labeled analog of PK 11195, its *in vitro* affinity for the PBS in human glioblastoma cells, and *in vivo* brain autoradiographic studies in C_6 glioma bearing rats which demonstrate selective binding of the ligand to the tumor.

Experimental

Materials and methods

No-carrier-added sodium [^{125}I]iodide in 0.1 N NaOH was obtained from Dupont/New England Nuclear, Boston, Mass. PK 11195 was a generous gift of Pharmuka Laboratories, Gennevilliers, France. All solvents were HPLC grade and all other reagents were the best available quality. Silica Sep-Paks were obtained from Waters Associates (Milford, Mass.) Two HPLC systems were used. System 1 consisted of a Waters Associates Model 590 programmable solvent delivery module and a Model U6K injection valve. The stationary phase consisted of two μPorasil™ columns in series [(150 + 300) mm × 3.9 mm, 10-μm particle, silica; Waters Associates], and the column was eluted with THF/hexane (15:85) at a flow rate of 2.5 mL/min. Radioactivity in the effluent was monitored with a Model RM-14 geiger counter (Eberline Instrument Co., Santa Fe, N.M.), and radioactivity in the collected fractions was measured in a Model CRC-12 Radioisotope Calibrator (Capintec Inc., Ramsey, N.J.). System 2 consisted of a Waters Associates Model 680 gradient controller, two Model 510 pumps and Model U6K injector and a reversed-phase μBondapak™ column (3.9 = 300 mm, 10-μm particle, C-18; Waters Associates). Column effluent was monitored for both radioactivity (Flo-One™ Model DR/IC radioactivity detector fitted with a 130 μL solid scintillator cell, Radiomatic Instruments, Tampa, Fla.) and u.v. absorbance (Model SF773 UV/VIS detector, Kratos, Ramsey, N.J.). Radio-TLC analyses were performed on silica gel coated glass plates (2 × 20 cm, Whatman K6F); the radioactive product was in all cases spotted over unlabeled PK 11195. The plates were analyzed on a Berthold Model LB 2832 Automatic TLC-Linear Analyzer.

Synthesis of [^{125}I]PK 11195 (1)

The reaction mixture, which consisted of *ca* 6 mg of $(NH_4)_2SO_4$, 0.1 mg of PK 11195 and *ca* 6 mCi of [^{125}I]NaI in 15–30 μL of 0.1 N NaOH, dissolved in 0.3 ml of EtOH/H_2O (1:2), was added to a 5 mL multi-dose vial containing 3 mm glass beads (Fig. 1). The solvent was evaporated by heating the reaction vessel to 230°C. Air (10 mL) was then slowly injected and heating was continued at 230°C for an additional 20 min. The reaction vessel was cooled to room temperature, and the mixture was extracted with ethanol and analyzed by radio-TLC using either EtOAc or hexane:EtOAc (1:2) as eluant. After evaporating the ethanol under an argon stream, the mixture was extracted with hexane and transferred to a silica Sep-Pak to remove free iodide. PK 11195 and **1** were co-eluted with EtOAc. This fraction was then concentrated under an argon stream in preparation for separation by normal-phase HPLC. An aliquot of this product was removed and analyzed for chemical purity, radiochemical purity, and effective specific activity by radio-HPLC, using HPLC System 2 with solvent system consisting of $CH_3CN:H_2O$ (55:45), a flow rate of 2.0 mL/min, and detection at 223 nm. The retention times for PK 11195 and **1** were 8.6 and 9.6 min, respectively, in this system. For the quantitation of the effective specific activity, the concentration of residual, unlabeled PK 11195 was determined by comparing its peak height with that of authentic standard of known concentration, while the amount of radioactive **1** was determined by counting an aliquot of the preparation in an autogamma counter. The product was obtained in 50–76% radiochemical yield, >94% radiochemical purity, and an effective specific activity of 15–17 Ci/mmol.

Purification of 1 by HPLC

HPLC system 1 was used to separate **1** from unlabeled PK 11195. In this system, the retention times for PK 11195 and **1** were 15.0 and 18.5, respectively. During elution of the radioactive peak, 1-min fractions were collected. The radioactivity in each fraction was then assayed and the heart fractions were pooled. An aliquot of this product was removed and analyzed for chemical purity, radiochemical purity, and effective specific activity as described above. After preparative HPLC, the effective specific activity increased to 260–910 Ci/mmol. In other words, each mCi of no-carrier-added **1** contained 0.38–1.36 μg of unlabelled PK 11195. The radiochemical purity was >99%. The radiochemical yield following HPLC purification was 10–20%.

Synthesis of ortho-[^{125}I]Iodobenzylguanidine (2)

The reaction mixture, which consisted of *ca* 4 mg of $(NH_4)_2SO_4$, 0.1 mg of *o*-chlorobenzylguanidine and *ca* 2 mCi of [^{125}I]NaI, in 15–30 μL of 0.1 N NaOH, dissolved in 0.3 mL of H_2O, was added to a 5 mL multi-dose vial containing 3 mm glass beads (Fig. 2). The solvent was evaporated by heating the reaction vessel to a temperature of 190°C. Air (10 mL) was then slowly injected, and heating was continued for an additional 30 min. After cooling the reaction vessel to room temperature, the mixture was extracted with H_2O and analyzed by radio-TLC, using EtOH:EtOAc (1:1) as eluant. The mixture was then transferred to a Cellex D column (acetate form, 0.5 × 2.5 cm; Bio-Rad, Richmond, Calif.) to remove free iodide. The *o*-chlorobenzylguanidine and **2** were co-eluted with 0.005 M sodium acetate buffer, pH 4.5. To determine the regioselectivity of the reaction, the reaction mixture was subjected to radio-HPLC analysis using System 2 with a solvent system consisting of 0.1 M

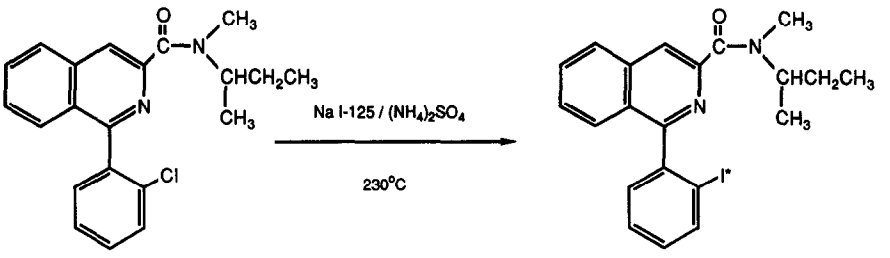


Fig. 1. Synthesis of [^{125}I]PK 11195 analog (**1**) by solid-state interhalogen exchange. Conditions are described in the text.

$NH_4H_2PO_4$/THF (88:12), a flow rate of 2.0 mL/min, and detection at 254 nm. In this system, the retention times for *m*-, *o*-, and *p*-iodobenzylguanidine, which had been synthesized as previously described (Mangner *et al.*, 1982), were 5.1, 7.9 and 9.5 min respectively. The reaction provided **2** in >95% radiochemical yield and >98% radiochemical and chemical purity. Radio-HPLC analysis of **2** spiked with unlabeled *m*-, *o*- and *p*-iodobenzylguanidine demonstrated only one radioactive peak which co-eluted with authentic *o*-iodobenzylguanidine.

Binding to cultured human glioma cells

Human glioblastoma line U_{251} was obtained from Dr Darrel Bigner of Duke University. Monolayers of tumor cells were grown in 24 well plates. Media of exponentially growing cells was replaced with phosphate buffered saline, pH 7.4, for 10 min at 4°C. This prewash buffer was aspirated and replaced with buffer containing **1** (1 pM–25 nM). Nonspecific binding was determined in the presence of 10 μM unlabeled PK 11195. Following a 15 min incubation at 4°C, the ligand mixture was removed by aspiration. The labeled monolayers were washed 3 × 7.5 min with buffer at 4°C. Cells were removed from the wells with trypsin and placed into vials for the measurement of radioactivity by liquid scintillation counting.

In vivo autoradiography

Two to three weeks before the study, male Wistar rats (200–300 g) were innoculated intracerebrally with a suspension of C_6 glioma cells as previously described (Starosta-Rubenstein *et al.*, 1987; Ciliax *et al.*, 1986; Richfield *et al.*, 1988). On the day of the study, the rats were catheterized as previously described (Ciliax *et al.*, 1986) and pretreated i.v. with either 5 mg/kg of PK 11195 dissolved in dimethyl sulfoxide:ethanol (1:1) or vehicle (0.1 mL/kg) 30 min before receiving a bolus injection of 400 μCi of **1** into the contralateral femoral vein. Twenty minutes after administration of **1**, the animals were sacrificed by i.v. injection of 1 mL of saturated aqueous KCl. The brains, containing gliomas, were rapidly removed and frozen on dry ice. Thin ultracryotomy sections (20 μm) through the tumor were thaw mounted onto gelatin coated glass slides and apposed to SB-5 x-ray film (Eastman Kodak Co., Rochester, N.Y.). After 3–7 days exposure, the film was developed in GBX developer to produce autoradiograms. To visualize tumor cell bodies, adjacent sections were stained for Nissl substance by cresyl violet.

Results and Discussion

Studies with [^{3}H]PK 11195 (Starosta-Rubenstein *et al.*, 1987) and [^{11}C]PK 11195 (Charbonneau *et al.*, 1986) suggest that radiolabeled analogs of PK 11195 might make useful agents for both SPECT imaging of glioma and the *in vivo* study of normal PBS pharmacology. While structure–activity relationship studies (Dubroeucq *et al.*, 1982) of the binding of PK 11195 analogs to the PBS have shown that compounds containing chlorine or fluorine in the 2′-position exhibit high affinity for the PBS, they have provided no

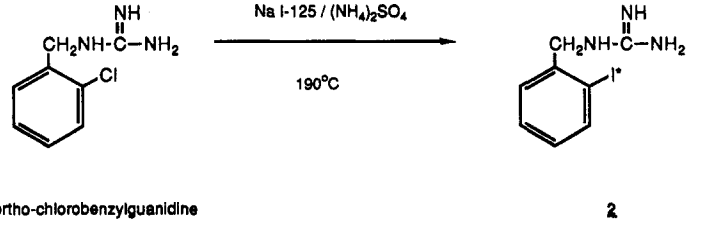


Fig. 2. Synthesis of *o*-[^{125}I] iodobenzylguanidine (**2**) by solid-state interhalogen exchange. Conditions are described in the text.

information on the effect of substitution with bromine or iodine. If iodine could be introduced into PK 11195 without either dramatically reducing the compound's affinity or altering its selectivity for the PBS, then one might be able to develop an agent for SPECT imaging and possibly radiotherapy. Since unlabeled **1** was not available, an ^{125}I-for-^{127}I exchange was not possible. However, the presence of chlorine in the 2′-position of PK 11195 offered the opportunity to radiolabel PK 11195 with ^{125}I by a solid-state interhalogen exchange method.

The synthesis of **1**, shown in Fig. 1, was achieved by the solid-state exchange technique which we have previously described (Mangner *et al.*, 1982), but modified as to temperature, reaction time and the inclusion of 3 mm glass beads to lower iodine volatility. Temperatures from 160 to 230°C were tested, but only at 230°C was **1** produced. At 230°C, the reaction time was varied from 20 to 60 min. The reaction was found to be complete at 20 min, and an increase in reaction time resulted only in a reduced yield. The synthesis of **2** from *o*-chlorobenzylguanidine by this method (Fig. 2) was optimal at 190°C. This lower energy requirement for the interhalogen exchange of *o*-chlorobenzylguanidine than for PK 11195 may reflect greater steric accessibility of chlorine in the benzylguanidine. A previous attempt (Wieland *et al.*, 1982) to synthesize **2** from *o*-chlorobenzylguanidine without glass beads gave poor radiochemical yields. However, subsequent inclusion of 3 mm glass beads to lower iodine volatility has dramatically increased the radiochemical yield of **2** to >95% in 30 min. Synthesis of **1** without glass beads was not attempted.

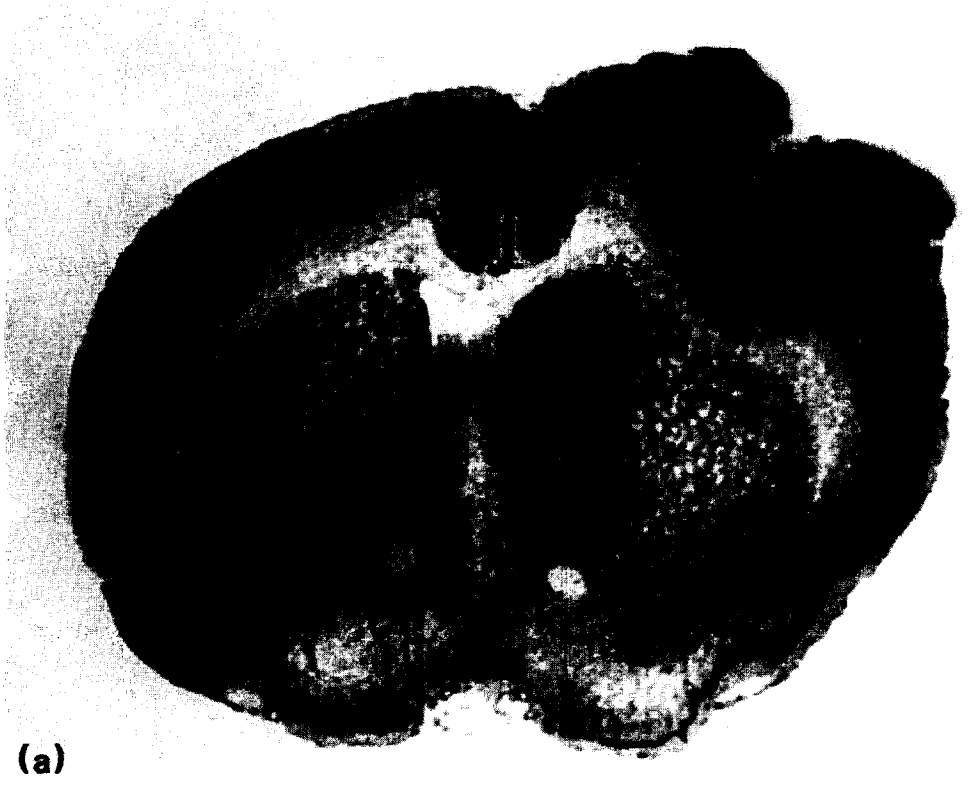
When applied to PK 11195, this modified exchange technique yielded **1** in 50–76% radiochemical yield, >94% radiochemical purity and effective specific activity of 15–17 Ci/mmol. Subsequent normal-phase, preparative radio-HPLC purification provided **1** in >99% radiochemical purity. The effective specific activity was 260–910 Ci/mmol; each mCi of **1** contained 0.38–1.36 μg of unlabelled PK 11195. Because of sacrificial losses incurred in collecting heart fractions, which was necessitated by the anomalous elution order in which the more lipophilic **1** eluted after PK 11195 on normal-phase HPLC, the overall radiochemical yield was reduced to 10–20%. Several other normal-phase HPLC systems were tested but did not reverse the elution order. We believe that this anomalous chromatographic behavior of **1** on normal phase is due to an "*ortho* effect" in which the bulkier iodine atom displaces the 1-phenyl ring from the plane of the isoquinoline ring thus exposing the isoquinoline nitrogen to increased binding to silica.

An attractive feature of this new radioiodide exchange technique is that it can provide a radioiodinated ligand of high effective specific activity, potentially no-carrier-added, even when an appropriate iodinated precursor is not available. The identity of **1** was initially based on three pieces of indirect evidence: (1) the similar chromatographic behavior of **1** PK 11195 on TLC and HPLC, (2) the lack of reactivity of deschloro-PK 11195 under identical radioiodinated conditions, and (3) the regioselectivity of the analogous synthesis of **2** from *o*-chlorobenzylguanidine. The last point was determined by reversed-phase radio-HPLC by spiking **2** with authentic samples of *m*-, *o*- and *p*-iodobenzylguanidines and demonstrating the co-elution of **2** with *o*-iodobenzylguanidine. No radioiodinated *m*- or *p*-iodobenzylguanidine was produced. To confirm the identity of **1**, unlabeled **1** was recently synthesized in our laboratory (Gildersleeve *et al.*, manuscript in preparation) and found to co-elute with **1** on radio-HPLC.

Both Ro5-4864 and the benzodiazepine flunitrazepam bind with high affinity to PBS in rat tissues and are capable of differentiating tumour from normal brain in rat models (Starosta-Rubinstein *et al.*, 1987). In contrast, these ligands bind with low affinity ($<1\ \mu$M) to human glioma cells and are incapable of distinguishing tumor from normal brain parenchyma in autoradiographic studies of post mortem human brain containing glioma (Olson *et al.*, 1988). To determine the affinity of **1** for human glioma cells, saturation experiments were performed on monolayers of U_{251} human glioblastoma cells. Scatchard analysis of saturation data demonstrated that **1** bound to a single class of sites with a K_D of 8.0 ± 1.7 nM and maximal binding of 3.8 ± 0.1 pmol/mg protein. These values are similar to those obtained when [^{3}H]PK 11195 was assayed in U_{251} cells ($K_D = 14 \pm 3.4$, $B_{max} = 4.1 \pm 1.3$) suggesting that the substitution of iodine for chlorine in PK 11195 does not appreciably alter the binding of the ligand to human glioma cells.

The application of **1** to the *in vivo* imaging of brain tumors by autoradiography in C_6 glioma bearing rats is illustrated in Fig. 3(B). A previously reported for [^{3}H]PK 11195 (Starosta-Rubenstein *et al.*, 1987), a dense accumulation of radioactivity was observed in the tumor. A high correspondence between PBS binding of **1** and the distribution of intact tumor cells could be seen when autoradiograms were compared with Nissl stains of adjacent sections [Fig. 3(A)]. Even small foci of infiltrating tumor cells were imaged by **1**. The accumulation of radioactivity in tumor tissue was blocked by pretreatment of the animal with unlabeled PK 11195 (5 mg/kg, i.v.), which strongly suggests that the uptake of **1** reflects specific binding to tumor cell PBS.

This work demonstrates that a radioiodine-labeled analog of PK 11195 can be synthesized in high specific activity by an interhalogen exchange method. Furthermore, after the introduction of iodine at the 2′-position of PK 11195, the ligand is still recognized by the PBS. More detailed autoradiographic and pharmacologic studies of **1**, as well as the synthesis of its ^{123}I-labeled analog, will be reported subsequently. Additionally, this solid-state interhalogen exchange method is being applied to the synthesis of other radioiodinated ligands, including the synthesis of 3-[^{125}I]iodo-MK-801 from 3-bromo-MK-801, an analog



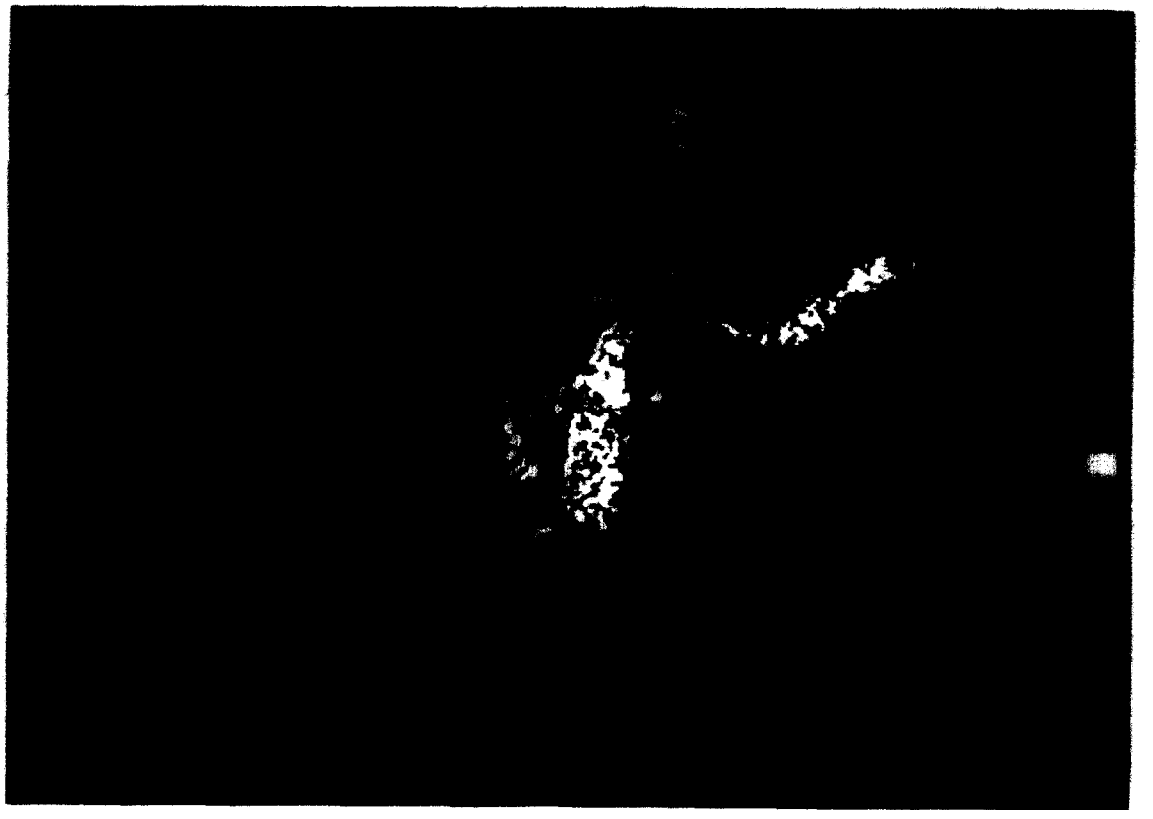


Fig. 3. Panel (a). Cresyl violet stained section through the rat brain bearing a C_6 glioma tumor showing the distribution of C_6 glioma tumor cells (dark stain). Panel (b). Autoradiogram of adjacent section demonstrating the *in vivo* labeling of C_6 glioma with **1**. The animal received an i.v. bolus of 400 μCi of **1** 20 min before death. Little uptake of radioactivity is observed in normal brain; the concentration ratio of radioactivity for glioma to noninvolved cortical tissue was 3:1. Radioactivity concentration increases in the following order: black < violet < blue < green < yellow < orange < red < white.

of the NMDA receptor antagonist MK-801 (Wieland *et al.*, 1988).

These initial results in rat C_6 glioma indicate that ligand **1** is superior to radioiodinated benzodiazepine analogs of the PBS antagonist Ro5-4864 recently studies in our laboratory (Van Dort *et al.*, 1988). The tumor-to-cortex tissue concentration ratio obtained with **1** is higher than that observed with the Ro5-4864 analogs. Also, there is no evidence for the uptake of **1** by white matter as was the case with the radioiodinated benzodiazepines.

These findings lend support to further study of ^{123}I–**1** or a related ^{123}I-labeled analog of PK 11195 as possible tools for the *in vivo* localization and PBS characterization of glioma in humans by SPECT.

Acknowledgements—This work was supported by National Institute of Health Grant No. 3 PO1 NS 15665–08S2. The authors wish to thank Ms Linder Markham for help in preparing the manuscript and the Phoenix Memorial Laboratory and Dr William Mancini, University of Michigan, for use of their facilities.

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