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Photodynamic Tumor Therapy: Mitochondrial Benzodiazepine Receptors as a Therapeutic Target*

Ajay Verma,[1] Stephen L. Facchina,[1] David J. Hirsch,[2] Shi-Yu Song,[5] Larry F. Dillahey,[5] Jerry R. Williams,[5] and Solomon H. Snyder[2,3,4]

[1]Department of Neurology, Uniformed Services University of the Health Sciences, Bethesda, Maryland, U.S.A.
Departments of [2]Neuroscience, [3]Pharmacology and Molecular Sciences, [4]Psychiatry, and [5]Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, U.S.A.

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Abstract

Background: Photodynamic therapy employs photosensitive agents such as porphyrins to treat a variety of tumors accessible to light-emitting probes. This approach capitalizes on the selective retention of porphyrins by cancer cells. Cancer cells also have elevated levels of mitochondrial benzodiazepine receptors which bind porphyrins with high affinity.
Methods: Cultured cancer cell lines were exposed to porphyrin and porphyrin-like compounds and then irradiated with light. Cytotoxicity of this treatment was measured via clonogenic assays. Mitochondrial benzodiazepine receptor pharmacology was studied using [^{3}H] PK11195 binding to cancer cell homogenates and isolated kidney mitochondrial membranes.
Results: We show that therapeutic potencies of porphyrins correlate closely with affinities for mitochondrial benzodiazepine receptors. Sensitivities of tumor cell lines to photodynamic therapy parallel their densities of these receptors.
Conclusion: We propose that porphyrin photodynamic therapy is mediated by mitochondrial benzodiazepine receptors.

Introduction

Photodynamic therapy (PDT) employs the dye-sensitized photooxidation of biological matter to treat various conditions, especially tumors that are accessible to light probes, such as skin, bladder, vaginal, bronchial, and rectal cancers (1–4). Although the molecular mechanism for therapeutic actions for PDT has not been established, substantial evidence indicates a prominent role for mitochondria. Porphyrins, the class of photosensitive dyes most often employed, tend to concentrate in mitochondria (5,6). Moreover, damage to mitochondrial function is one of the earliest events in porphyrin PDT (7–9). We speculated that mitochondrial benzodiazepine receptors (MBR) might play a role in the mechanism of PDT. MBR are distinct from the neuronal "central" benzodiazepine receptors but can bind clinically employed benzodiazepines with high affinity. MBR are localized to mitochondria where they comprise a complex of an 18 kilodalton (kD) receptor protein, the 32 kD voltage-

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Address correspondence and reprint requests to: Dr. Solomon H. Snyder, Department of Neuroscience, Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205, U.S.A. Phone: 410-955-3024; Fax: 410-955-3623.

dependent anion carrier (VDAC), and the 30 kD adenine nucleotide carrier (10–14). Porphyrins represent endogenous ligands for MBR. In tissue extracts, porphyrins are the only substances that bind with nanomolar affinity to MBR (15–17). Porphyrin PDT targets mitochondrial membranes where MBR are localized (18). Incubation of isolated mitochondria with porphyrins and light results in the oxidation of sulfhydryl groups in specific membrane proteins with molecular weights similar to MBR constituents (19). Moreover, loss of ATP/ADP exchange via the adenine nucleotide carrier is a major early step in porphyrin photosensitization of isolated mitochondria (20). In the present study, we show that the relative potencies of porphyrins in eliciting photodynamic killing of tumor cells correlate closely with their affinities for MBR. Moreover, the relative sensitivities of tumor cell lines to PDT parallel their MBR levels.

Materials and Methods

Materials

Cells were obtained from American Tissue Culture Collection (Rockville, MD). [^{3}H] PK11195 was obtained from NEN/Dupont (Boston, MA).

Porphyrins were obtained from Porphyrin Products (Logan, UT). PK11195 was a gift from Pharmuka and R05-4864 was a gift from Hoffman-LaRoche. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Cells were maintained at 37°C in a humidified CO_2 incubator in the following media: mouse L cells were grown in MEM plus 10% horse serum; C6 glioma cells were grown in Ham's F-10 plus 15% horse serum and 2.5% fetal bovine serum (FBS); V79 were grown in MEM plus 10% FBS; HeLa, WiDR, and LS174T cells were grown in MEM plus nonessential amino acids and 10% FBS; and SVEC 4–10 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (low glucose) plus 10% FBS. For clonogenic survival experiments, cells were plated at appropriate densities as suspensions approximately 12 hr before drug/light exposure.

MBR Assays

Rat kidney mitochondria were prepared as described (16). Cell homogenates used for binding studies were prepared by removing culture media, rinsing with phosphate buffered saline (PBS), and homogenizing in 10 mM HEPES (pH, 7.4) using a Dounce B homogenizer with 10 to 15 strokes at 4°C. Protein was determined by Coomassie Blue protein assay (BioRad, Hercules, CA) and adjusted to a final value of 0.5 mg/ml in the binding assay. IC_{50} and K_i values for porphyrins and analogs were determined by displacement of [^{3}H] PK11195 binding to cell homogenates and isolated rat kidney mitochondria, respectively, as described earlier (16). MBR densities of the various cell lines were determined by performing Scatchard analyses on [^{3}H] PK11195 saturation isotherms for each cell line as reported earlier (16). For [^{3}H] PK11195 binding studies in live cells, confluent cultures in 60-mm dishes were used. After medium removal and rinsing with PBS, live cell monolayers were incubated in 2 ml PBS containing 2 nM [^{3}H] PK11195 with and without 10 μM PK11195 or varying concentrations of Photofrin II at 37°C for 1 hr. Cells were scraped from the dishes, collected over GF/C glass fiber filters, and washed three times with ice-cold PBS. The filters were counted for radioactivity in scintillation cocktail.

Phototoxicity Studies

All cultured cells were plated at a density of 300 to 10,000 per dish. Cells were exposed to varying concentrations of porphyrins and analogs in DMEM for 4 hr. After a brief wash with PBS, cells were irradiated for 60 sec with a broad spectrum light from a Kratos lamp at 60 cm distance and cultured in DMEM. Clonogenic assessment for cell survival was performed after 7 to 14 days of cell growth. D37 values represent concentrations that reduce survival to 37% of control.

Results

To explore a role for MBR in porphyrin phototoxicity we first determined whether V79 cell homogenates display high-affinity [^{3}H] PK11195 binding with an MBR pharmacological profile. [^{3}H] PK11195 bound to a single site on V79 homogenates with a Kd of 1.2 nM and B_{max} of 3.9 pmol/mg protein. Binding of 1 nM [^{3}H] PK11195 to V79 homogenates was potently displaced by unlabeled PK11195, R05-4864, and protoporphyrin IX, which are all high-affinity ligands for MBR. Diazepam, a mixed MBR and central benzodiazepine receptor (CBR) ligand,

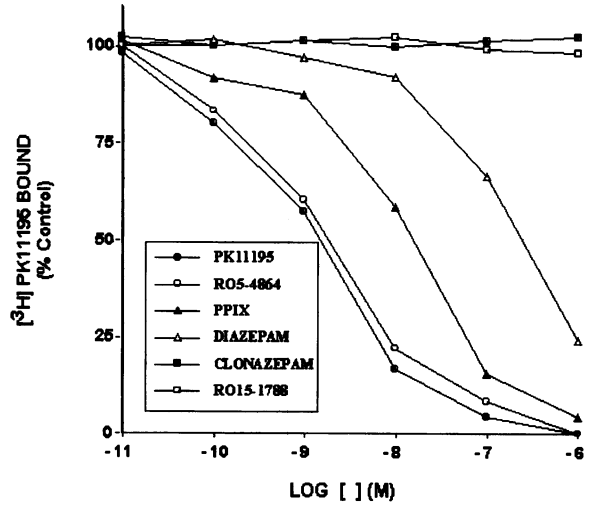

Fig. 1. Pharmacologic profile of [^{3}H] PK11195 binding to V79 cell homogenates. Binding studies performed as described in Materials and Methods demonstrate the presence of MBR in V79 cells. Key for symbols: PK11195 (filled circles), R05-4864 (open circles), protoporphyrin (PP) IX (filled triangles), diazepam (open triangles), clonazepam (filled squares), R015-1788 (open squares).

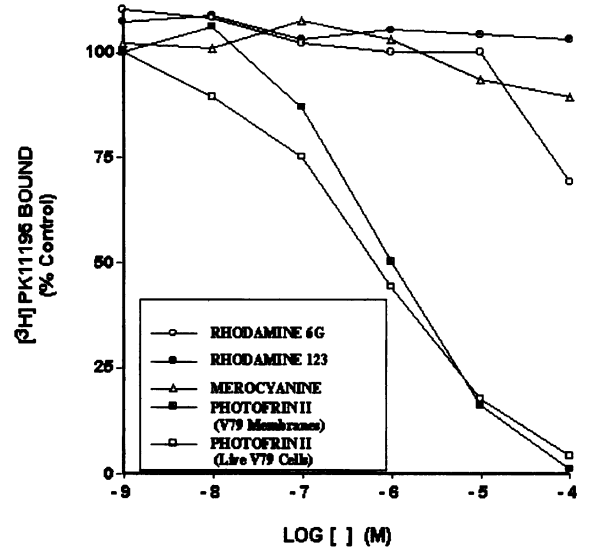

Fig. 2. Inhibition of [^{3}H] PK11195 binding to V79 cell homogenates and live cells (Photofrin only) by photosensitive dyes. Binding studies performed as described in Materials and Methods demonstrate the selective interaction of the porphyrin phototoxic dye, Photofrin II, with MBR in homogenized and live V79 cells. Key for symbols: rhodamine 6G (open circles), rhodamine 123 (filled circles), merocyanine (open triangles), Photofrin II with V79 homogenates (filled squares), Photofrin II with live V79 cells (open squares).

showed intermediate inhibitory potency, whereas the CBR-specific ligands, clonazepam and R015-1788, were without effect (Fig. 1).

[^{3}H] PK11195 binding to V79 homogenates was also potently inhibited by Photofrin II, a clinically employed tumor photosensitizing dye (largely composed of di-hematoporphyrin ether), but not by three other nonporphyrin phototoxic dyes—rhodamine 6G (21), rhodamine 123 (22), and merocyanine (23) (Fig. 2). Photofrin II also inhibited [^{3}H] PK11195 binding to live V79 cells (Fig. 2). Similar K_i values for Photofrin II were found for rat kidney mitochondria (Table 1) and other cell lines (data not shown). In a series of 27 porphyrin derivatives, relative potencies in eliciting photodynamic cell toxicity in the V79 cells correlated very closely with their affinities for MBR ($p < 0.001$).

If PDT involves MBR, then tumor cell lines with higher densities of MBR should be more sensitive. We evaluated the sensitivity to porphyrin photodynamic toxicity in 8 cell lines (Table 2). The relative sensitivity closely paralleled MBR density in these cell lines.

Discussion

Our most striking findings are two strong correlations that together implicate MBR as playing a major role in PDT. First, we observed a close correlation between the potencies of porphyrins to catalyze cellular phototoxicity and their affinity for MBR, establishing a structure–activity profile for porphyrins and porphyrin-like compounds to mediate phototoxicity. Second, we observed that MBR density in tumors parallels their susceptibility to porphyrin-catalyzed phototoxicity. Such data suggest that mitochondrial damage is the proximal event that leads to observed cell death. This conclusion is supported by recent studies by Munday et al. (24), who show that mammalian cells deficient in mitochondria are refractory to porphyrin-catalyzed phototoxicity.

How might MBR mediate PDT? MBR comprises a complex of proteins apparently located at junctional sites of outer and inner membrane of mitochondria (11,12). A molecular complex with such a localization could influence transactions between cytosolic and mitochondrial compartments. For example, hexokinase partitions from cytosol onto the outer mitochondrial surface where it binds to VDAC (25,26). Interactions of VDAC with the inner membrane adenine nucleotide carrier may afford a path for exchange of

Table 1. Relationship of porphyrin phototoxicity and MBR affinity

Porphyrin	MBR K_i (nM)	D37 (nM)
"Neutral" porphyrin	14	20
Protoporphyrin IX	20	60
Mesoporphyrin IX	40	50
Deuteroporphyrin IX	50	60
Fe-Protoporphyrin IX (heme)	50	>32,000
N-methyl mesoporphyrin IX	90	350
N-methyl protoporphyrin IX	95	400
2,4(4,2)Hydroxyethylvinyl-deuteroporphyrin IX	100	125
Photofrin II	100	153
Hematoporphyrin IX	350	1,000
Zn protoporphyrin IX	375	800
Chlorine E6	500	1,000
Diaspartylprotoporphyrin IX	545	480
2-vinyl,4-hydroxymethyl-deuteroporphyrin IX	900	1650
Ditaurylmesoporphyrin IX	1,000	350
Sn-protoporphyrin IX	2,000	12,000
Coproporphyrin III	2,500	>10,000
Biliverdin	2,500	>2,500
Coprotoporphyrin IX	5,000	8,000
Diglutamatylmesoporphyrin IX	7,600	2,400
Hexaporphyrin	24,000	>10,000
Coproporphyrin I	100,000	>10,000
Pentaporphyrin	>100,000	>10,000
Heptaporphyrin	>100,000	>10,000
Bilirubin	>100,000	>2,500
Deuteroporphyrin IX disulfonate	>100,000	>10,000
Deuteroporphyrin IX BBGlycol	>100,000	>10,000

MBR K_i values for porphyrins and analogs were determined by displacement of [^{3}H] PK11195 binding to rat kidney mitochondria as described. Phototoxicity studies using V79 Chinese hamster lung cells were performed as described in Materials and Methods. D37 values represent doses that reduce survival to 37% of control. K_i values are the inhibitory constants of drugs for MBR. As FeProtoporphyrin IX (heme), a potent MBR ligand is not a photosensitive molecule, it does not cause phototoxicity. With the omission of heme, the D_{37} and MBR K_i values correlate with a coefficient of 0.827 ($p < 0.001$). Experiments were performed three times in duplicate and results shown are mean values.

Table 2. Relationship of MBR density and PDT susceptibility in cell lines

Cell Type	MBR Density	PDT Sensitivity
Mouse L	++++	++++
C_6 glioma	+++	+++
V79	+++	+++
$HepG_2$	++	++
Hela	++	++
SVEC 4–10	++	++
WiDR	+	+
LS174T	+	+

MBR density was determined as described in Materials and Methods. K_d and B_{max} values for each cell line were: mouse L = 1.3 nM, 5.5 pmol/mg; C_6 glioma = 1.5 nM, 4.2 pmol/mg; V79 = 1.2 nM, 3.9 pmol/mg; Hela = 1.9 nM, 95 pmol/mg; Hep G_2 = 1.85 nM, 1.9 pmol/mg, SVEC 4–10 = 1.8 nM, 1.7 pmol/mg; WiDR = 1.5 nM, 0.75 pmol/mg; LSI94T = 1.3 nM, 0.9 pmol/mg. PDT sensitivity and cell survival were assessed as described in Materials and Methods. Concentrations of Photofrin II producing 10% survival versus control (Photofrin II without light) for the lines were (μg/ml) mouse L = 0.37; V79 = 0.50; C_6 glioma = 0.57; $HepG_2$ = 0.71; Hela = 0.75; SVEC 4–10 = 0.77; WiDR = 0.92; LS174T = 0.88. Scoring system for this table was as follows: ++++ = B_{max} > 5 pmol/mg protein, 10% survival dose <0.4 μg/ml; +++ = B_{max} 2–5 pmol/mg protein, 10% survival dose 0.4–0.6 μg/ml; ++ = B_{max} 1–2 pmol/mg protein, 10% survival dose 0.6–0.8 μg/ml; + = B_{max} <1 pmol/mg protein, 10% survival dose >0.8 μg/ml. Results are means of two experiments performed in duplicate.

mitochondrial ATP with ADP generated by hexokinase (26). This would provide direct ATP supply to hexokinase and yield improved mitochondrial coupling through kinase-generated ADP. Tumor cells display especially high levels of hexokinase attached to mitochondria (25–27), which might account for the high rate of "aerobic glycolysis" seen in many neoplastic cells. Porphyrin photooxidation of MBR-associated VDAC or adenine nucleotide carrier could thus profoundly affect tumor bioenergetics.

Recent findings have implicated MBR in the transport of porphyrins in and out of mitochondria (28). Porphyrins used in photodynamic therapy may thus enter cells and accumulate on mitochondrial porphyrin transport sites. Porphyrins with the highest affinity for MBR would be expected to accumulate the most in this manner. This may explain the suggested role of MBR in delta-aminolevulinic acid (ALA)–mediated phototoxicity in pancreatoma cells (29), as ALA phototoxicity requires conversion to protoporphyrin IX prior to its phototoxic effects.

The ability of MBR to modulate ion conductances (30) and cholesterol movement across mitochondrial membranes (31) implies a substan-

tial role for MBR in regulating mitochondrial compartmentation. Thus, the adenine nucleotide carrier is believed to mediate the nonspecific increase in mitochondrial inner membrane permeability observed after Ca^{2+} overloading and oxidative injury to mitochondria (32). This protein is labeled by the alkylating benzodiazepine [^{3}H] AHN086 (11) which causes a large release of calcium from isolated mitochondria (33). We hypothesize that porphyrins, when bound to MBR and exposed to light, generate radical oxygen species that oxidize the adenine nucleotide carrier to increase mitochondrial permeability. This would account for the rapid loss of adenine nucleotide exchange and Ca^{2+} release from mitochondria following porphyrin PDT (9,20,34) and associated cytotoxicity. The oxidative sensitivity displayed by the adenine nucleotide carrier (35) supports our hypothesis and indeed may implicate the MBR complex in diverse pathological and physiological phenomena.

Our findings have clear clinical relevance. Some benzodiazepines and related agents have extremely high affinity for MBR in the low nanomolar range and, like porphyrins (36), radiolabeled forms of these agents have been employed to localize neoplasms in vivo (37). Photosensitive compounds with high affinity for MBR may afford more specific PDT agents with fewer side effects. Such compounds may also be readily screened for using binding studies with [^{3}H] PK11195 and isolated mitochondria. Interestingly, MBR levels are increased in several types of tumors (38,39,40).

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