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Characterization of Ligand Binding to Mitochondrial Benzodiazepine Receptors

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SUMMARY

We have evaluated the affinity and density of binding sites for [^{3}H]Ro5-4864 and [^{3}H]PK11195 in intact and fragmented rat kidney mitochondria. These sites are known as peripheral-type or mitochondrial benzodiazepine receptors (MBR) and the preceding paper provided evidence that they function *in vitro* as modulators of mitochondrial respiratory control (1). In this report, MBR density, localization, and ligand specificity were investigated. In intact mitochondria, there were approximately the same number of binding sites for [^{3}H]PK11195 as for [^{3}H]Ro5-4864, and their apparent K_d values were identical. However, in mitochondrial fragments, there were 80% more binding sites for [^{3}H]Ro5-4864 than for [^{3}H]PK11195. Rat kidney mitochondria were fractionated by decompression and digitonin-based methods into outer and inner membrane-containing fractions before and after incorporation of the MBR-specific photoaffinity ligand [^{3}H]PK14105. Assays of selective mitochondrial membrane markers and [^{3}H]Ro5-4864 binding or specifically bound [^{3}H]PK14105 revealed that the receptors were found in the mitochondrial outer membrane. We also evaluated the binding of a large number of structurally and pharmacologically diverse compounds to MBR by studying their ability to inhibit the binding of both ^{3}H-ligands. These compounds had affinities ranging from 0.015 to 100 μM and, with a few exceptions, were similar in their abilities to bind to MBR in intact and fragmented mitochondria. However, there was considerable variation in the ratios between drug potencies at displacing [^{3}H]Ro5-4864 and [^{3}H]PK11195. This represents a new form of evidence that these two radioligands do not label identical sites on the receptor. Thirteen of the drugs, including [^{3}H]Ro5-4864 and [^{3}H]PK11195, were analyzed as to the nature of the inhibition and, with only two exceptions, were competitive inhibitors. One drug, Konig's polyanion, was uncompetitive whereas the other, cyclosporin A, was a noncompetitive inhibitor. These studies revealed several new classes of MBR ligands and suggest that the relationship between ligand structure and binding affinity is highly complex.

In the preceding paper, we reported that BDZ binding sites in the mitochondria of peripheral organs, like kidney and adrenal, are functional receptors that modulate energy coupling (1). These MBR are distinguished from the BDZ receptors in neuronal plasma membranes (2), in that the MBR are located in the mitochondrial outer membrane and are not linked to the action of the neurotransmitter GABA (2, 3). Mitochondrial BDZ receptors have traditionally been defined by their profile of binding affinities for a variety of ligands. For BDZs, the rank order of binding potency is Ro5 > DZ > FLU ≫ CLZ. This is directly opposite to their binding potency rank order at the GABA A/BDZ/Cl^- ionophore receptor complex (2). More recently, the isoquinoline carboxamide PK has been identified as a high affinity MBR-specific ligand (4).

The MBR has been further characterized biochemically and pharmacologically by its interactions with Ro5 and PK (2). Although we found that these drugs had equivalent efficacy at inhibiting mitochondrial respiratory control (1) and it has been shown that these drugs were competitive inhibitors of each other's binding (4), there are data indicating that binding sites for these drugs on the MBR are not identical (5). In particular, results of protein modification experiments using diethylpyrocarbonate indicate that the [^{3}H]PK site can be partially eliminated without affecting the [^{3}H]Ro5 binding site (6, 7). In addition, the densities of their binding sites in different tissues (5) and in kidney mitochondria are not identical (see Results). With this apparent MBR heterogeneity in mind, one aim of the present study was to try to identify ligands that distinguished between Ro5 and PK binding sites on the receptor.

We also wanted to determine the localization of the MBR in

ABBREVIATIONS: BDZ, benzodiazepine; FLU, flunitrazepam; DZ, diazepam; MBR, mitochondrial benzodiazepine receptors; GABA, γ-aminobutyric acid; CLZ, clonazepam; PK, PK11195; Ro5, Ro5-4864; FCCP; *p*-trifluoromethoxyphenylhydrazone; DMSO, dimethylsulfoxide; DEHP, diethylhexylphthalate; DBP, dibutylphthalate; DEP, diethylphthalate; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; MAO, monoamine oxidase; COX, cytochrome oxidase; VDAC, voltage-dependent anion channel; RAB, respiration assay buffer; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone; TDQ, tridecyl-coenzyme Q; PDQ, pentadecyl-coenzyme Q; β-CCE, ethyl β-carboline-3-carboxylate; β-CCM, methyl β-carboline-3-carboxylate; TTFA, thenoyltrifluoroacetone; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

rat kidney mitochondria, because these organelles were used for the vast majority of our respiration modulation experiments. Also, the ability of the MBR to alter respiratory control may be a function of its proximity to the respiratory apparatus in the mitochondrial inner membrane. An inner membrane localization for the receptor would provide opportunities for direct interactions between the MBR and the electron transport chain and/or ATP synthetase components. In contrast, if the MBR were on the outer mitochondrial membrane, one would expect that its effects on respiration would be indirect. In agreement with data for adrenal mitochondria published previously (3), we report that the MBR in kidney mitochondria is on the outer mitochondrial membrane.

The final aim of the present study was to try to identify and characterize the structural features of ligands that are essential for MBR binding. Previous reports suggested that this might be a difficult undertaking, because the structural diversity of ligands binding to these sites is already substantial. It has been reported that BDZs, an isoquinoline carboxamide, a pyrimidopyrimidine, porphyrins, phthalate esters, a cyclic peptide, dihydropyridines, steroid derivatives, and various diuretics bind to MBR (1, 2, 8–10). The present study extends these investigations using a larger number of drugs from a wider variety of classes.

Materials and Methods

The following compounds were purchased from Aldrich Chemical Co. (Milwaukee, WI): DEHP, DBP, DEP, glutathione, valproic acid, chlorophyll a, CCCP, and phthalic acid. Myxothiazol, valinomycin, and oligomycin were purchased from Boehringer-Mannheim (Indianapolis, IN). Methoxatin was purchased from Fluka (Ronkonkoma, NY). The following compounds were obtained from Hoffmann-LaRoche, Inc. (Nutley, NJ): Ro5, DZ, FLU, CLZ, and Ro15-1788. PK was obtained from Rhone-Poulenc Sante (Gennevilliers, France). Sandoz (East Hanover, NJ) was the source of cyclosporin A. Coenzyme Q10 and FCCP were purchased from Serva (Westbury, NY). Unless otherwise noted, all other compounds and reagents were purchased from Sigma (St. Louis, MO).

The authors are grateful to their colleagues at American Cyanamid for providing the following compounds: LL-F42248α, piericidin A, reticulol, ivermectin, CL259,763, nalidixic acid, norfloxacin, ciprofloxacin, ofloxacin, pefloxacin, CL218,872, β-CCE, β-CCM. We also thank B. L. Trumpower (Dartmouth Medical School) for providing UHDBT, DBMIB, TDQ, and PDQ. Konig's polyanion was a gift from M. Columbini (University of Maryland).

[^{3}H]Ro5 (77.9 Ci/mmol), [^{3}H]PK (75.2 Ci/mmol), and [^{3}H]FLU (77.4 Ci/mmol) were purchased from Dupont/NEN (North Billerica, MA). [^{3}H]PK14105 (87 Ci/mmol) was obtained from CEA (Gif-sur-Yvette, France).

Preparation of rat kidney mitochondria. Intact "heavy" mitochondria were prepared from whole Sprague-Dawley (male) rat kidneys as described in the previous paper (1). These mitochondria utilized succinate and malate/pyruvate efficiently and exhibited respiratory control ratios of 3–5 with the former and 2.5–4 with the latter substrates. With succinate, ADP:O ratios ranged from 1.6 to 1.8; ADP:O ratios with malate/pyruvate ranged from 1.9 to 2.1 (1). These mitochondria did not phosphorylate ADP with NADH as substrate (1). For binding experiments, they are referred to as intact mitochondria.

For the majority of ligand binding experiments, all of the mitochondria were recovered by centrifugation of the nuclei and cell debris-free (600 × *g*) kidney homogenate (1) at 15,000 × *g* for 10 min. Mitochondria from four kidneys were then suspended in 30 ml of 50 mM Tris·HCl buffer (pH 7.4) with a Brinkmann Polytron and homogenized three times for 20 sec each at maximum speed. The homogenate was then centrifuged at 36,000 × *g* for 10 min and the pellet was suspended in 30 ml of Tris buffer by Polytron homogenization. This procedure was repeated and the final pellet was suspended to give a protein concentration of 0.2–0.5 mg of protein/ml. This preparation is referred to as mitochondrial fragments.

Binding of [^{3}H]Ro5, [^{3}H]PK, and [^{3}H]FLU. Binding of [^{3}H]Ro5 and [^{3}H]PK to intact mitochondria, fragments, and/or mitochondrial subfractions was performed as follows. The suspension of intact mitochondria or mitochondrial subfraction was diluted in the buffer used for mitochondrial respiration studies (1) [60 mM sucrose, 1 mM EDTA, 5 mM K_2HPO_4, 2.5 mM $MgCl_2$, 20 mM KCl, and 30 mM Tris (pH 7.4) (RAB)] at a protein concentration of 0.3–0.4 mg of protein/ml.

Binding was performed at the final concentrations of [^{3}H]Ro5 and [^{3}H]PK indicated in the legends to the tables and figures. The assays were conducted in triplicate for 10 min at 30.5° in RAB (intact mitochondria and subfractions) or for 90 min at 4° in Tris buffer (mitochondrial fragments) in a final volume of 0.2 ml. In all cases, these incubation conditions were sufficient for ^{3}H-ligand binding to reach equilibrium. Assays were conducted in polycarbonate, U-bottom, nonsterile, 96-well plates and were terminated by rapid vacuum filtration through glass-fiber filters with a Cambridge PHD 24-place cell harvester (Cambridge Technology, Cambridge, MA). Filter strips were washed three times with 0.4 ml of RAB at 30.5° (intact mitochondria and subfractions) or 0.4 ml of ice-cold Tris·HCl buffer (mitochondrial fragments). Each well contained 15–50 μg of mitochondrial protein. Nonspecific binding of [^{3}H]Ro5 and [^{3}H]PK was determined in the presence of 10 μM unlabeled Ro5 and PK, respectively. All drugs were dissolved in 100% DMSO (Mallinckrodt; (analytical grade Mallinckrodt, St. Louis, MO) and diluted with the appropriate buffer immediately before testing. At concentrations <0.1% (v/v), DMSO had no effect on binding.

Binding of 4.2 nM [^{3}H]FLU to rat brain GABA A/BDZ receptors was performed using membrane preparations from whole rat brains as described previously (11). In the present studies, the assays were incubated at 4° for 90 min.

Subfractionation of mitochondria by pressure. Mitochondria were prepared from 22 g of rat kidney as described above, resuspended in 6 ml of 0.4 M sucrose, and pressurized at 5000 psi in a French pressure cell. The suspension was rapidly decompressed, diluted to 12 ml with 0.4 M sucrose, and centrifuged at 12,100 × *g* for 10 min. The supernatant was saved and the pellet was resuspended in 6 ml of 0.4 M sucrose and centrifuged again for 10 min at 12,100 × *g* to yield the mitoplast fraction. The combined supernatants were centrifuged at 27,000 × *g* for 10 min to yield an intermediate pellet fraction. The supernatant was then centrifuged at 207,000 × *g* for 90 min to yield the outer membrane fraction and a final supernatant fraction. All procedures were conducted at 0–4°.

Subfractionation of mitochondria by digitonin extraction. Before photoaffinity labeling with [^{3}H]PK14105, intact mitochondria were prepared as described and resuspended in RAB to a final protein concentration of 133 μg/ml. [^{3}H]PK14105 was then added to a final concentration of 5 nM in the presence or absence of 10 μM unlabeled PK and the mitochondria were irradiated for 70 min on ice with long wavelength UV light (λ_{max} = 366 nm) emitted from a chromatography viewing apparatus. After irradiation, the mitochondria were pooled, recovered by centrifugation, and washed four times with RAB containing 10 μM unlabeled PK. They were then resuspended in fresh RAB and an equal volume of freshly prepared digitonin solution in RAB was added so as to yield a final protein concentration of 0.65 mg/ml and a digitonin/protein ratio of 1.92 mg/mg. This suspension was gently mixed for 15 min and then diluted 3-fold with RAB. The suspension was centrifuged at 10,000 × *g* for 30 min to yield pellet (mitoplast) and supernatant (outer mitochondrial membrane) fractions.

Other methods. COX activity was used as the marker for the inner mitochondrial membrane and was measured spectrophotometrically, with cytochrome *c* as substrate, by the method of Wharton and Tzagoloff (12). MAO activity was used as the marker for the outer mito-

chondrial membrane and was determined, with [^{14}C]tryptamine as substrate, by the method of Wurtman and Axelrod (13). Protein was determined by the method of Bradford (14), with bovine serum albumin as the standard.

Results

The general characteristics of [^{3}H]Ro5 and [^{3}H]PK binding at equilibrium to MBR in rat kidney mitochondrial fragments and intact mitochondria were determined (Table 1). The number of binding sites for [^{3}H]Ro5 and [^{3}H]PK was basically equal in intact mitochondria. However, fragmentation of the mitochondria resulted in marked increases in the B_{max} for both radioligands. There was a 300% increase in the B_{max} for [^{3}H]Ro5 and a 60% increase in the B_{max} for [^{3}H]PK. When the B_{max} values for both radioligands in fragments were compared, there were 78% more [^{3}H]Ro5 binding sites than [^{3}H]PK binding sites (Table 1). Thus, mitochondrial fragmentation led to the production of increased numbers of binding sites for both ligands, with sites for [^{3}H]Ro5 increasing more than [^{3}H]PK. In contrast to these variations in B_{max} values, the affinities of these sites for the two radioligands in either intact or fragmented mitochondria were very similar.

Having established the basic binding parameters for [^{3}H]Ro5 and [^{3}H]PK, we evaluated a large number of drugs for their ability to displace these ligands from mitochondrial fragments and intact mitochondria (Table 2). With both preparations and ^{3}H-ligands, all of the compounds produced maximal displacement when added at sufficient concentrations. However, with both mitochondrial preparations, and with virtually all of the compounds, binding affinities (i.e., IC_{50} values) were higher versus [^{3}H]Ro5 than versus [^{3}H]PK. These differences did not disappear when IC_{50} values are converted into K_i values (15). In general, drug affinity, measured with both ^{3}H-ligands, was lower, by a factor of 2, in intact mitochondria than in fragments (Table 2). This difference cannot be accounted for by the minor differences in ^{3}H-ligand affinities in intact mitochondria versus fragments (see Table 1). Whether or not it reflects a certain asymmetric membrane distribution of these binding sites remains to be clarified.

With mitochondrial fragments, the ratios of affinities for the compounds (i.e., K_i [^{3}H]PK/K_i [^{3}H]Ro5 or selectivity ratio) were not constant but ranged from about 1 for PK to 4–5 for dipyridamole and myxothiazol to 25 and 36 for TDQ and cyclosporin A, respectively. Even though the latter appeared to be the most Ro5-selective drug tested, it will be considered separately because it alone displaced [^{3}H]Ro5 in a noncompetitive manner (see below). In mitochondrial fragments, none of the compounds displaced [^{3}H]PK better than [^{3}H]Ro5, and PK actually differentiated the least. In fact, the range in selectivity was so large that it changed the rank ordering of drug affinity. For convenience, we have used the ranking versus [^{3}H]Ro5 so as to be able to compare these results with previous ones.

There is a very strong correlation between drug IC_{50} versus [^{3}H]Ro5 and [^{3}H]PK both in mitochondrial fragments (r = 0.96; Fig. 1) and intact mitochondria (r = 0.92, data not shown).

TABLE 1

Characteristics of [^{3}H]Ro5 and [^{3}H]PK binding in rat kidney mitochondria

Intact rat kidney mitochondria (*intact*) and mitochondrial fragments (*fragments*) were prepared and binding assays were performed as described in Materials and Methods. The ^{3}H-ligand concentration ranges used were 3.2 to 58 nM for [^{3}H]Ro5 and 0.8 to 58 nM for [^{3}H]PK. These values are means (± standard deviation where indicated) of the number of experiments shown in parentheses.

Characteristic	[^{3}H]Ro5		[^{3}H]PK	
	Fragments	Intact	Fragments	Intact
B_{max} (pmol/mg of protein)	14.2 ± 2.4 (4)	4.7 (2)	8.0 (2)	5.1 (2)
K_d (nM)	8.5 ± 2.7 (4)	6.6 (2)	5.7 (2)	6.6 (2)

TABLE 2

Binding affinities of drugs for MBR

All values shown are means from two to six experiments performed as described in Materials and Methods. Intact mitochondria and mitochondrial fragments were prepared from whole rat kidneys as described. [^{3}H]Ro5 and [^{3}H]PK were used at final concentrations of 3.8–4.2 and 3.7–4.2 nM, respectively, over the course of these experiments. All experiments used six to eight concentrations of each competitor drug. The following compounds (K_i values in parentheses) displaced [^{3}H]Ro5 from mitochondrial fragments with affinities lower than 100 μM: PDQ (115.6 μM), cytosine β-furanoside (170 μM), Ro15-1788 (272 μM), reticulol (275 μM), 2-amino-6-mercaptopurine (339.9 μM), hexamethylacetamide (339.9 μM), 2, 3-dimethyloxy-5-methyl-1, 4-benzoquinone (408 μM), 2,4-dinitrophenol (2.1 mM), and glutathione (13.6 mM). K_i values were calculated as described in Ref. 15 using the apparent K_d values shown in Table 1 and the ^{3}H-ligand concentrations indicated above. Inactive compounds, which inhibited binding by <15% at the concentrations indicated in parentheses, were: TTFA (100 μM), atractyloside (500 μM), carboxyatractyloside (500 μM), nonactin (100 μM), nigericin (100 μM), gramicidin S (100 μM), valinomycin (100 μM), oligomycin (100 μM), sodium azide (1 mM), methoxatin (500 μM), bovine heart hexokinase (1 unit/ml), valproic acid (500 μM), succinate (10 mM), ADP (1 mM), ATP (1 mM), phthalic acid (1 mM), FCCP (100 μM), chloroquine (100 μM), coenzymes Q6, Q7, Q9, and Q10 (100 μM), vitamins K1 and K3 (100 μM), CL218,872 (100 μM), β-CCE (100 μM), β-CCM (100 μM), nalidixic acid (100 μM), norfloxacin (100 μM), ciprofloxacin (100 μM), ofloxacin (100 μM), pefloxacin (100 μM), ivermectin (100 μM), ruthenium red (1 mM), EGTA (1 mM), heparin (5 μM), metyrapone (100 μM), deoxycorticosterone (100 μM), and porphobilinogen (100 μM).

DRUG	K_i					
	Mitochondrial fragments			Intact mitochondria		
	[^{3}H]Ro5	[^{3}H]PK	Ratio[a]	[^{3}H]Ro5	[^{3}H]PK	Ratio[a]
	μM			μM		
PK	0.012	0.011	0.92	0.033	0.051	1.50
Ro5	0.013	0.019	1.46	0.019	0.067	3.50
DZ	0.112	0.187	1.67	0.243	0.455	1.90
FLU	0.138	0.211	1.53	0.356	0.557	1.60
Dipyridamole	0.156	0.679	4.35	0.116	0.295	2.50
Deuteroporphyrin IX	0.252	0.319	1.27	0.374	0.100	0.27
Mesoporphyrin IX	0.578	0.650	1.12	0.340	0.378	1.10
LL-F42248α	0.870	2.54	2.92	—[b]	—	—
Hemin	0.952	1.10	1.16	—	—	—
Cyclosporin A[c]	2.04	73.8	36.2	—	—	—
Protoporphyrin IX	2.14	2.92	1.36	—	—	—
Piericidin A	2.58	—	—	—	—	—
Biliverdin	2.92	5.32	1.82	—	—	—
Antimycin A	3.58	4.78	1.34	—	—	—
DEHP	3.87	5.43	1.40	0.748	6.02	8.00
Myxothiazol	4.42	22.4	5.07	—	—	—
UHDBT	5.03	14.2	2.82	—	—	—
DBP	5.71	7.50	1.31	3.20	4.13	1.30
Dicoumarol	5.71	—	—	—	—	—
CL259,763	7.48	—	—	11.6	13.0	1.10
Rotenone	10.5	20.9	2.00	—	—	—
CCCP	12.2	—	—	—	—	—
Konig's polyanion[d]	14.3	19.5	1.4	—	—	—
TDQ	15.6	384.0	24.6	—	—	—
DBMIB	16.3	212.6	13.0	8.2	153.6	18.7
CLZ	18.7	47.3	2.4	12.9	>25.0	>1.3
Chlorophyll a	20.4	36.6	1.8	—	—	—
Mitotane	27.4	65.0	2.4	—	—	—
Coproporphyrin I	44.9	70.3	1.6	—	—	—
HOQNO	62.5	—	—	—	—	—
DEP	83.6	—	—	—	—	—

[a] Selectivity ratio = K_i [^{3}H]PK/K_i [^{3}H]Ro5.
[b] Not tested.
[c] Noncompetitive inhibitor.
[d] Uncompetitive inhibitor.

That there is such good agreement between these affinities for MBR labeled with either ^{3}H-ligand suggests that both are labeling pharmacologically similar sites.

However, there were several exceptions for which affinities were notably higher in intact mitochondria than in fragments, dipyridamole and DBP with both ^{3}H-ligands, deuteroporphyrin IX with [^{3}H]PK, and DEHP with [^{3}H]Ro5. With intact mitochondria, the selectivity ratios were quantitatively similar to those obtained in fragments and ranking was again dependent upon which of the two ^{3}H-ligands was being displaced. In intact mitochondria, deuteroporphyrin IX and DEHP stood out, because the former was actually the only compound that was more potent versus [^{3}H]PK than [^{3}H]Ro5. On the other hand, between the two MBR preparations, DEHP exhibited the largest difference in selectivity, being about 8-fold more potent at displacing [^{3}H]PK than [^{3}H]Ro5 in intact mitochondria but less than 2-fold more potent in fragments. Most of the other compounds differed by less than 2-fold when such comparisons were made.

As expected, CLZ and Ro15-1788, two compounds that are specific for GABA A/BDZ receptor complexes (2), displaced [^{3}H]Ro5 and [^{3}H]PK from MBR with much lower affinities relative to drugs like Ro5 and PK (Table 2). When CLZ and Ro15-1788 were evaluated along with 17 other drugs for potency at displacing [^{3}H]FLU from GABA A/BDZ receptors in rat brain membranes and for potency at displacing [^{3}H]Ro5 from MBR in mitochondrial fragments, it was clear that the latter are pharmacologically distinct from the brain GABA A/BDZ receptors (Table 3). On the basis of their selectivity ratios (i.e., IC_{50} [^{3}H]Ro5/IC_{50} [^{3}H]FLU) being different by several orders of magnitude, PK and Ro5 were the most MBR-specific ligands and CLZ and Ro15-1788 were the most GABA A/BDZ receptor-specific ligands that we evaluated. Our data also show compounds that differentiate between the GABA A/BDZ receptor and MBR by less than 10-fold and therefore might be considered "cross-over" compounds. Interestingly, in this category were BDZs (i.e., DZ, and FLU) and non-BDZs (i.e., dipyridamole, DBP, and cyclosporin A).

Clearly, there are many types of compounds that exhibit specificity for the MBR over the GABA A/BDZ receptor. The degree of structural diversity of these compounds led us to determine whether the binding of 13 of the most structurally distinct compounds was competitive with respect to [^{3}H]Ro5 (Table 4). Representative Scatchard plots depicting the type of inhibition for Ro5, deuteroporphyrin IX, DBP, UHDBT, Konig's polyanion, and cyclosporin A are shown in Fig. 2. Eleven of the 13 compounds tested were competitive inhibitors of [^{3}H]Ro5 binding to MBR in mitochondrial fragments, even though they apparently exhibit little structural similarity. Cyclosporin A and Konig's polyanion were noncompetitive (i.e., decrease in B_{max}; no change in K_d) and uncompetitive (i.e., decrease in B_{max} and K_d), respectively. These results corroborate earlier difficulties encountered in trying to deduce a single pharmacophore for the MBR.

Many of the drugs that bound to MBR have well known pharmacological activities that are not attributed to MBR occupancy. The largest group is the inhibitors of electron transport and includes piericidin A, antimycin A, myxothiazol, UHDBT, rotenone, DBMIB, and 2-Heptyl-4-hydroxyquinoline *N*-oxide (16). These compounds had IC_{50} values in the 4–100

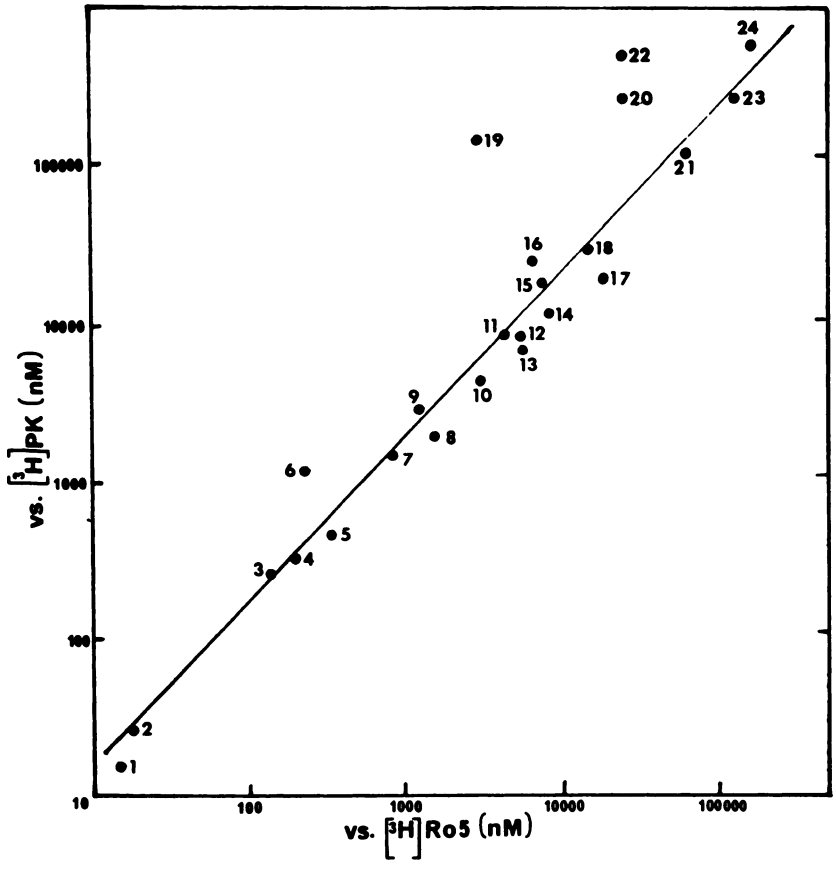

Fig. 1. Correlation of binding affinities versus [^{3}H]Ro5 (*abscissa*) with those versus [^{3}H]PK (*ordinate*). The IC_{50} values used to derive this figure are listed in Tables 2 and 3. The numbers next to the symbols represent: **1** = PK11195; **2** = Ro5; **3** = DZ; **4** = FLU; **5** = deuteroporphyrin IX; **6** = dipyridamole; **7** = mesoporphyrin IX; **8** = LL-F42248α; **9** = hemin; **10** = protoporphyrin IX; **11** = biliverdin; **12** = antimycin A; **13** = DEHP; **14** = DPB; **15** = UHDBT; **16** = myxothiazol; **17** = CLZ; **18** = rotenone; **19** = cyclosporin A; **20** = DBMIB; **21** = coproporphyrin I; **22** = TDQ; **23** = DEP; and **24** = PDQ. The correlation coefficient (*r*) for the *line* in this figure was 0.96.

TABLE 3

Binding affinities of drugs for GABA A/BDZ and MBR

Membranes from whole rat brains and rat kidney mitochondrial fragments, which served as the sources of GABA A/BDZ receptors and MBR, respectively, were prepared as described in Materials and Methods. [^{3}H]FLU and [^{3}H]Ro5 were used at final concentrations of 4.2 and 3.8–4.2 nM, respectively. Results with [^{3}H]Ro5 were taken from Table 2 and converted back to IC_{50} values using the Cheng-Prusoff equation (15). The results obtained with [^{3}H]FLU are means of three independent experiments.

Drug	IC_{50}		IC_{50} ratio [^{3}H]Ro5/[^{3}H]FLU
	[^{3}H]FLU	[^{3}H]Ro5	
	μM		
FLU	0.003	0.203	67.66
CLZ	0.007	29.0	4142.85
Ro15-1788	0.014	400.0	28571.43
DZ	0.018	0.165	9.17
Dipyridamole	2.30	0.230	0.10
Cyclosporin A	22.0	3.00	0.14
DBP	36.0	8.40	0.233
PK	44.7	0.017	0.0004
DEHP	62.0	5.7	0.092
CL259,763	140.0	11.0	0.079
Biliverdin	250.0	4.3	0.017
Ro5	503.0	0.019	0.00004
LL-F42248α	>100	1.284	<0.013
Protoporphyrin IX	>250	3.15	<0.013
Deuteroporphyrin IX	>250	0.37	<0.0015
Mesoporphyrin IX	>250	0.85	<0.0034
Hemin	>250	1.40	<0.0056
Rotenone	>250	15.5	<0.062
Antimycin A	>250	5.27	<0.0211

TABLE 4

Types of inhibition exhibited by ligands for MBR

These experiments were conducted with rat kidney mitochondrial fragments prepared as described in Materials and Methods. The type of inhibition was determined by Scatchard analysis of saturation isotherms using [^{3}H]Ro5 with and without the inhibitor drug (see Fig. 2 for details).

Ligand	Type of inhibition of [^{3}H]Ro5 binding
Ro5	Competitive
PK	Competitive
DZ	Competitive
Deuteroporphyrin IX	Competitive
DBP	Competitive
DEHP	Competitive
Mitotane	Competitive
UHDBT	Competitive
Rotenone	Competitive
LL-F42248α	Competitive
Dipyridamole	Competitive
Cyclosporin A	Noncompetitive
Konig's polyanion	Uncompetitive

μM range, and both UHDBT and rotenone were competitive inhibitors of [^{3}H]Ro5 binding (Table 4). However, the potency of these compounds as electron transport inhibitors did not correlate with their binding to MBR. With the exception of UHDBT, these compounds inhibited electron transport at concentrations approximately 3 orders of magnitude lower than their MBR IC_{50} values[1] (16). Nevertheless, there was a weak potency rank order correlation between inhibition of electron transport and MBR binding (Spearman $rho = 0.76$, $p < 0.05$).

A second subgroup of MBR binding inhibitors is the uncouplers of oxidative phosphorylation represented by CCCP, FCCP, 2,4-dintrophenol, and dicoumarol (16). Generally, these compounds bound weakly or not at all to MBR (Table 2). The chlorinated nitropyrrole antibiotic LL-F42248α (17), which has also been identified as an uncoupler,[1] had the best affinity in this drug subclass. With the exception of dicoumarol, the binding and uncoupling potencies of which were similar (16), the uncoupling potencies of the uncouplers were significantly greater than their binding potencies. Although their action as uncouplers presumably relates to protonophoric activity (16), such an attribute is not known to be required for interaction with MBR or for respiration modulation by these receptors (1). Therefore, it is unlikely that the binding of these drugs to MBR contributes to their uncoupling activity.

Konig's polyanion, which also bound to MBR, was previously reported to inhibit mitochondrial respiration and oxidative phosphorylation[1] (18). These actions are thought to be due, in part, to the ability of the anion to interfere with the VDAC activity of mitochondrial "porin."[2] Konig's polyanion is an uncompetitive inhibitor of [^{3}H]Ro5 binding (Table 4; Fig. 2) and its effects on MBR binding are presumably indirect.

It should also be recognized that many compounds that alter mitochondrial function did not displace [^{3}H]Ro5 significantly at concentrations <100 μM (see legend to Table 2). Among these are the electron transport inhibitors TTFA and sodium azide, the ADP/ATP translocase inhibitors atractyloside and carboxyatractyloside, the ionophores nonactin, nigericin, gramicidin S, and valinomycin, the F1F0ATPase inhibitor oligomycin, the uncoupler FCCP, and the Ca^{2+} uptake inhibitor ruthenium red. In addition, compounds such as succinate, ADP, ATP, several analogs of coenzyme Q, and the enzyme hexokinase did not bind to these receptors. Such data support the notion that inhibition of [^{3}H]Ro5 and [^{3}H]PK binding is not due to nonspecific disruption of mitochondrial membranes.

Another aim of these studies was to establish the distribution of the MBR within mitochondria. Using the same rat kidney mitochondria that were used in the respiration modulation experiments (1), we initially tried to obtain outer and inner mitochondrial membrane-enriched fractions without using detergents. We modified a French pressure cell procedure originally developed for rat liver mitochondria (19) (see Materials and Methods) and were able to partially separate the MAO-containing outer and COX-containing inner membranes of rat kidney mitochondria. The results indicated that about 55% of the MAO-containing outer membrane material was recovered in the outer membrane and supernatant fractions along with 45% of the MBR binding activity. Because only 16% of the COX activity was found in these fractions (Table 5), this suggested that the MBR-specific [^{3}H]Ro5 and the outer membrane-bound MAO activity were co-enriched during the fractionation. There was no similar correspondence between the COX activity in the inner membrane-containing mitoplast fractions and the MBR binding activity in this fraction (Table 5).

To verify this observation, we used a digitonin-based extraction procedure that was reported to extract the receptor from adrenal mitochondria (3). Rather than perform the receptor binding experiments with the fractions after the extraction, we utilized the specific MBR photoaffinity label [^{3}H]PK14105 (20) to place a radioactive tag on the receptor before membrane solubilization with digitonin. Although the extraction of outer

[1] J. D. Hirsch, unpublished observations.

[2] M. Columbini, personal communication.

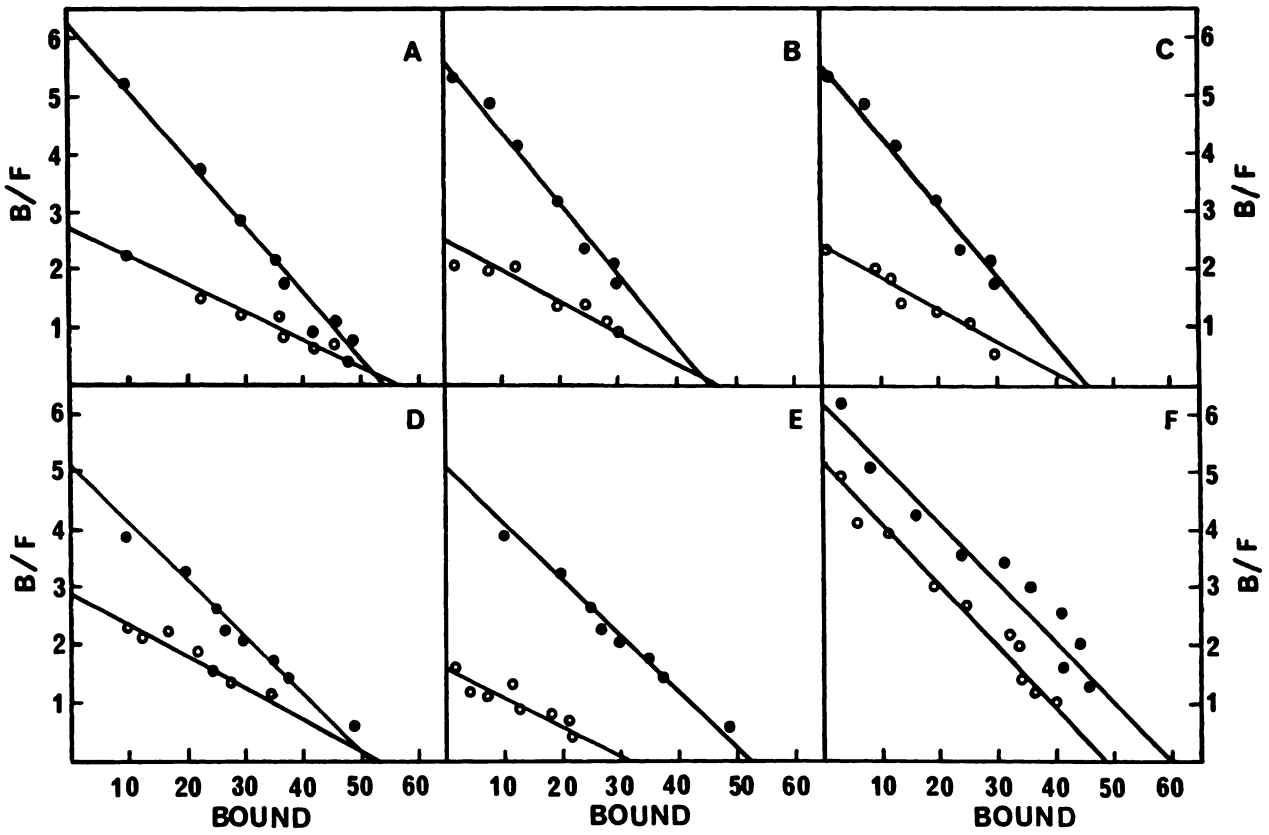

Fig. 2. Type of inhibition produced by selected MBR ligands. Specific binding of [^{3}H]Ro5 to rat kidney mitochondrial fragments was determined over the concentration range of 1.6–39 nM in the presence and absence of the inhibitors. Saturation isotherms that resulted were transformed into Scatchard plots, which are shown in the figure. In each panel, ● = control whereas ○ = inhibitor added. A, 20 nM Ro5-4864; B, 400 nM deuteroporphyrin IX; C, 8 μM DBP; D, 40 μM UHDBT; E, 10 μM Konig's polyanion; F, 2 μM cyclosporin A.

TABLE 5

Localization of MBR in subfractions of rat kidney mitochondria after disruption in a French pressure cell

Rat kidney mitochondria were disrupted by a French pressure cell-based method and subfractionated by differential centrifugation as described in Materials and Methods. Binding of [^{3}H]Ro5 to receptors in the resulting fractions was performed after fractionation. Detailed descriptions of these procedures are provided in Materials and Methods. Assays of the inner membrane marker COX, the outer membrane marker MAO, and protein were performed as described in Materials and Methods. In these experiments, percentage recoveries were based on marker or binding levels in intact mitochondria. The data in the table are from one of two similar experiments. *Intermediate pellet*, the intermediate pellet fraction; *outer membrane*, the outer membrane fraction.

Fraction	Protein	COX	MAO	Binding
		% of starting activity		
Mitoplast	42	73	37	42
Intermediate Pellet	7	13	5	8
Outer Membrane	20	14	23	24
Supernatant	26	2	32	21
		% recovery		
Total	95	98	91	89

membrane from kidney mitochondria by digitonin was not as efficient as expected (Table 6), our data clearly indicated that the [^{3}H]PK14105-labeled receptor segregated with the MAO-containing outer membrane throughout the entire fractionation procedure. In the outer membrane-containing supernatant fraction, 32% of the MAO activity and 34% of the [^{3}H]PK14105 labeled material were recovered. In contrast, there was no inner membrane COX activity recovered in this fraction (Table 6). These results indicate that the MBR in rat kidney mitochondria is associated with the outer mitochondrial membrane.

TABLE 6

Localization of MBR in subfractions of rat kidney mitochondria after extraction with digitonin

Rat kidney mitochondria were photoaffinity labeled with 5 nM [^{3}H]PK14105 before extraction with digitonin. The pellet and supernatant fractions were obtained after centrifugation. Marker assays and all other technical details of this experiment are provided in Materials and Methods. The percentage recovery values were based on marker and radioactivity levels in intact photolabeled mitochondria. The data in the table are from a single experiment. The pellet represents the mitoplast fraction, whereas the supernatant represents the outer membrane.

Fraction	Protein	COX	MAO	[^{3}H]PK14105
		% of starting activity		
Pellet	43	98	59	55
Supernatant	57	0	32	34
		% recovery		
Total	100	98	91	89

Discussion

Our goals in these studies were to characterize the MBR in terms of ligand binding and function *in vitro* and to attempt to understand the basic molecular features of receptor ligands. In the preceding paper on MBR function (1), we reported that the binding affinity for three BDZs, one isoquinoline carboxamide, two porphyrins, one phthalate ester, one pyrimidopyrimidine, one sulfonylphenylacetamide, and one cyclic peptide correlated with their potency at producing a distinctive inhibition of mitochondrial respiratory control. This activity-binding correlation ($r = 0.95$) applied over a drug concentration range of 5–20,000 nM. All of these drugs inhibited respiratory control by stimulating the rate of state IV and inhibiting the rate of state

III respiration (1). In the present paper, we present a detailed characterization of radioligand binding to the MBR as well as binding data on a wide variety of additional MBR ligands.

It is worthwhile to discuss some of the individual ligand types that were identified. Our finding that various porphyrins bound with submicromolar affinities to MBR agrees qualitatively with the original observations made by Verma *et al.* (9), who proposed porphyrins as endogenous ligands for this receptor. The fact that deuteroporphyrin IX and mesoporphyrin IX not only bound to MBR but inhibited respiratory control supports the idea that porphyrins could be endogenous receptor ligands. However, because a large number of naturally occurring substances, including other porphyrins like chlorophyll a, bind to MBR (see Table 2), this line of argument would predict that other biologically active endogenous MBR ligands exist. In fact, both diazepam and cyclosporin A occur naturally (21, 22) and have respiration modulation activity indistinguishable from that shown by porphyrins (1, 23). These results suggest that multiple active endogenous MBR ligands exist.

The data presented here on the phthalate esters (e.g., DBP) are interesting because these compounds had effects on rat liver mitochondrial respiration that were very similar to what we observed, i.e., increased and decreased rates of state IV and III respiration, respectively (24). However, the main difference between our results obtained with kidney and those obtained with liver is that much higher DBP concentrations were necessary to provoke these changes in liver (24). This result is consistent with the findings that liver mitochondria contain many fewer MBR than kidney (25) and that the magnitude of the drug effect on respiration correlates with the density of MBR on mitochondria (1). Although mitochondria appear to be potential intracellular loci for the toxic effects of phthalate esters (24), there is no proof that the pathology caused by these compounds is due to MBR occupation.

Our observations that cyclosporin A binds to MBR are of interest when coupled with the published data of Jung and Pergande (23). These authors reported that cyclosporin A had an IC_{50} of 12 μM in inhibiting rat kidney mitochondrial respiratory control. Using this potency value and our MBR binding affinity value positioned cyclosporin A along the line correlating binding and respiratory control inhibition (see Fig. 3 in Ref. 1). Based on their results, these authors suggested that the nephrotoxicity of cyclosporin A during immunosuppression therapy might be due to its effects on mitochondrial respiration (23) and our findings support this conclusion. However, unlike the majority of MBR ligands we identified, cyclosporin A was a noncompetitive inhibitor of [^{3}H]Ro5 to MBR. Thus, it may interact allosterically with these receptors and perturb the [^{3}H] Ro5 binding site sufficiently to decrease respiratory control. As cyclosporin A is also a known hydrophobic disrupter of biomembrane structure (26), additional studies will be needed before its action on respiration can be assigned solely to its binding to MBR.

Finding receptor binding and respiration modulation activity for the sulfonylphenylacetamide CL259,763 is intriguing because this drug has immunomodulator activity *in vivo* (27). The observation of MBR activity for this compound gains potential significance when one recognizes that three other MBR ligands, Ro5, PK, and DZ, have similar immunomodulator activity *in vivo* and *in vitro* (28, 29). Because very few receptor ligands have been tested as immunomodulators, conclusive data in support of this hypothesis is not available.

Another compound that modulated mitochondrial respiration and bound with relatively high affinity to the receptor is the adenosine uptake blocker dipyridamole. This drug binds with high affinity to adenosine uptake sites and is used clinically as a vasodilator (8). Our results indicate that dipyridamole binds to the MBR with 10-fold higher affinity than it does to GABA A/BDZ receptors, in general agreement with the literature (30). It is unlikely that the vasodilatory effects of dipyridamole are due to its effects on mitochondrial respiration, because its affinity for the MBR is 5–10-fold lower than its IC_{50} for inhibiting adenosine uptake. The latter parameter correlates with vasodilatory potency whereas the former does not (30). The relatively high affinity of dipyridamole for MBR may, however, be related to the caffeine-insensitive sedative effects of this drug (30) or to its reported immunomodulator activity (31).

Two other newly identified MBR ligands are Konig's polyanion and mitotane. The former is a copolymer of methacrylate, maleate, and styrene in a 1:2:3 proportion, with an average molecular weight of 10 kDa, and is a known inhibitor of mitochondrial electron transport and oxidative phosphorylation (18). It reportedly blocks VDAC activity associated with "porin" in the outer mitochondrial membrane,[2] and the mitochondrial uptake systems for succinate and adenine nucleotides (32). We found that Konig's polyanion was an uncompetitive inhibitor of [^{3}H]Ro5 binding, possibly by neutralizing essential positive charges on the mitochondria (32). The interactions of Konig's polyanion with the receptor are presumably nonspecific and do not provide definitive evidence for or against a postulated VDAC-MBR relationship (33).

The last new MBR ligand of interest is mitotane, or 1-(*o*-chlorophenyl-1-(*p*-chlorophenyl)-2,2-dichloroethane. This drug is a structural analog of the insecticide DDT (1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane) and is a selective adrenocorticolytic agent that is used clinically to treat adrenal tumors. After metabolic transformation in adrenal microsomes, mitotane selectively destroys adrenocortical mitochondria and cells (34). This is of interest because the adrenal cortex has the highest density of MBR of all rat tissues (2, 3). Here again, there is the possibility that the cytotoxic action of this drug is related to its affinity for these receptors. It would certainly make sense to evaluate the available mitotane analogs for their effects on the MBR.

In trying to identify a basic pharmacophore for compounds binding to the MBR, one immediately observes the structural and functional diversity that was found. More than half of the compounds with binding affinities higher than 20 μM are natural products. Of these, piericidin A, myxothiazol, antimycin A, and rotenone are highly toxic mitochondrial inhibitors (16), whereas cyclosporin A and dicoumarol have immunosuppressant and anticoagulant activity, respectively (20, 35). The antibiotic activity of the microbial product LL-F42248α has been reported as well (17). The situation is further complicated by the fact that the naturally occurring and synthetic electron transport inhibitors (piericidin A, myxothiazol, antimycin A, UHDBT, HOQNQ, and rotenone) all have well defined loci of action in the mitochondrial inner membrane where they inhibit electron flow (16). Uncouplers like LL-42248α and dicoumarol also act within the inner membrane, although they have no

single specific site of action there (16, 35). Thus, in addition to their well known effects on mitochondrial function, these compounds also bind at much higher concentrations to the MBR on the mitochondrial outer membrane. This binding presumably cannot result in any modulation of respiratory control because, at the drug concentrations required for receptor occupation (see Table 2), respiration has long since been totally inhibited or uncoupled (16, 35). Furthermore, due to their relatively low MBR affinity, there would be little occupation of the receptor at drug concentrations producing complete inhibition of electron transport or uncoupling.

The synthetic compounds that bound to the MBR are also highly structurally and pharmacologically diverse. Three of these compounds are the typical BDZs Ro5, DZ, and FLU, the latter two of which are potent anxiolytics (36). The rest of these compounds are PK, an isoquinoline carboxamide, dipyridamole, a vasodilatory pyrimidopyrimidine, DBP, a toxic phthalate ester, and CL259,763, an immunomodulatory sulfonylphenylacetamide. All of the members of this group of compounds inhibited mitochondrial respiratory control (see Ref. 1) in a manner that correlated extremely well with MBR binding affinity. However, it is clear from our binding and functional results that the ability to inhibit respiratory control via the MBR does not require competitive ligand binding to the MBR sites occupied by [^{3}H]Ro5 and [^{3}H]PK.

One of the intriguing aspects of MBR activity is the fact that the receptor is in the mitochondrial outer membrane whereas the respiratory processes modulated by it reside in the inner membrane. This suggests that the effect of receptor occupation on respiration is indirect and must involve an as yet undefined signal transduction mechanism. At present, it is not understood how the outer and inner mitochondrial membranes communicate with each other. Previous studies have shown that these membranes are in physical contact *in situ* (37) and that activities of outer membrane enzymes can be influenced by processes confined to the inner membrane (38). It has also been shown that the outer and inner membranes cooperate during the process of protein import into the mitochondria (39). However, the details of intermembrane communication have not been worked out. Further studies of the MBR should help to elucidate the interrelationships of the two mitochondrial membrane compartments. Also, once the identity of the MBR has been established, it may be possible to explain the multitude of effects provoked by MBR ligands (2) and assess what physiological role(s) the MBR plays in cells.

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