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Inhibition of the mitochondrial F_1F_0-ATPase by ligands of the peripheral benzodiazepine receptor

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Abstract—Although PK11195 binds to the peripheral benzodiazepine receptor with nanomolar affinity, significant data exist which suggest that it has another cellular target distinct from the PBR. Here we demonstrate that PK11195 inhibits F_1F_0-ATPase activity in an OSCP-dependent manner, similar to the pro-apoptotic benzodiazepine Bz-423. Importantly, our data indicate that cellular responses observed with micromolar concentrations of PK11195, which are commonly attributed to modulation of the PBR, are likely a direct result of mitochondrial F_1F_0-ATPase inhibition.

The best characterized benzodiazepine receptors are the central benzodiazepine receptor (CBR) and the peripheral benzodiazepine receptor (PBR). The CBR is a γ-aminobutyric acid (GABA)-gated plasma membrane chloride channel distributed throughout the central nervous system,[1] while the peripheral benzodiazepine receptor (PBR) is a 18-kDa protein found in the outer mitochondrial membrane in various tissues, including the heart, brain, testes, adrenal glands, liver, muscle, and lymphoid cells.[2] The most well-characterized ligands of these receptors include diazepam, clonazepam, Ro5-4864 (4-chlorodiazepam), and PK11195 (Fig. 1). However, the selectivity of the CBR and PBR for these ligands varies—diazepam binds to both the CBR and PBR, clonazepam binds only to the CBR, and 4-chlorodiazepam (4-Cl-Dz) binds selectively (preferentially) to the PBR.[3] PK11195 is an isoquinoline carboximide that selectively binds the PBR with a higher affinity than 4-chlorodiazepam (9.3 and 23 nM, respectively).[4]

Although the function of the PBR has yet to be clearly defined, PBR ligands are known to positively or negatively regulate cell survival and have immunomodulatory properties.[1–4] We previously described a immunomodulatory 1,4-benzodiazepine, Bz-423, possessing potent apoptogenic and anti-proliferative properties mediated through binding to the oligomycin sensitivity conferring protein (OSCP) of the mitochondrial F_1F_0-ATPase, resulting in inhibition of the enzyme (Fig. 1).[5–8] In response to F_1F_0-ATPase inhibition, cells moderately

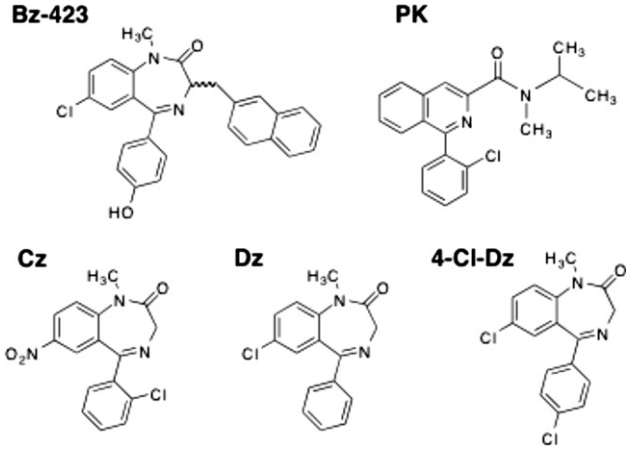

Figure 1. Structures of Bz-423 and related benzodiazepines, PK11195 (PK), clonazepam (Cz), diazepam (Dz), and 4-chlorodiazepam (4-Cl-Dz). Bz-423 does not bind to the CBR and binding to the PBR is between 0.3 and 1 μM.

Keywords: Apoptosis; Mitochondria; Drug.
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decrease ATP synthesis and significantly increase levels of intracellular superoxide, resulting in redox-regulated apoptosis or cell growth arrest.[5,9] The properties of pathogenic immune lymphocytes render these cells particularly sensitive to the actions of this compound and Bz-423 has significant and selective therapeutic effects in animal models of autoimmunity.[5,9]

We have found that micromolar concentrations of certain PBR ligands, 4-Cl-Dz and PK11195, also had anti-proliferative effects in B cells, as observed for Bz-423.[6] PK11195 and 4-Cl-Dz have both anti-proliferative and apoptotic effects in several cell types, including human colorectal cancer,[10,11] esophageal cancer,[12,13] fibrosarcoma,[14] hepatocellular carcinoma,[15] leukemic cells from patients with AML,[16,17] and Jurkat T cells.[18,19] Although many reports have implicated the PBR as the target mediating these effects, the anti-proliferative and apoptotic effects of these compounds occur only at concentrations 1000 times greater than those necessary to saturate the PBR, and there has been an increasing amount of evidence suggesting that these properties are PBR-independent.[20,21] Since the F_1F_0-ATPase is a newly recognized target of benzodiazepines, we were interested in determining if benzodiazepines that bind to the PBR and CBR also possessed activity against this target.

We first assayed the hydrolytic and synthetic activity of bovine F_1F_0-ATPase present in submitochondrial particles (SMPs) in the presence of PK11195, 4-Cl-Dz, clonazepam, and diazepam, as previously described.[7] At drug concentrations ranging from 0 to 300 μM, all four compounds demonstrated modest inhibition of ATP hydrolysis (Fig. 2a). PK11195 was the most potent hydrolysis inhibitor with an EC_{50} of 230 μM (Table 1). For ATP synthesis (the enzymatic direction of the mitochondrial F_1F_0-ATPase that is relevant under most physiological conditions in vivo), 4-Cl-Dz, clonazepam, and diazepam continued to have modest inhibitory effects (Fig. 2b) with EC_{50} values ranging from 120 to 210 μM (Table 1). In contrast, PK11195 was a much more potent inhibitor of ATP synthesis, with an EC_{50} of 33 μM.

To determine if the same subunit is required to inhibit the enzyme for PK11195 as Bz-423, we examined the ability of PK11195 to inhibit F_1F_0-ATPase activity in SMPs reconstituted in the presence or absence of the OSCP, as previously described.[7] Similar to Bz-423, PK11195 was a significantly more potent inhibitor of F_1F_0-ATPase activity in the presence of the OSCP (Fig. 2c), suggesting that this subunit of the F_1F_0-ATPase is important for PK11195-mediated inhibition.

To correlate these in vitro data with effects in whole cells, we incubated Jurkat T cells and Ramos B cells with PK11195, 4-Cl-Dz, clonazepam, and diazepam. Since an early increase in mitochondrial superoxide (O_2^-) signals apoptosis induced by Bz-423, we determined the O_2^- response of cells 1 h following incubation with drug, as well as cell death at 24 h.[5] As expected, PK11195 was the most potent inducer of the early O_2^- response (EC_{50} = 56 and 100 μM in Jurkat and Ramos cells, respectively) and cell death (EC_{50} = 75 and 90 μM in Jurkat and Ramos cells, respectively) (Table 1). Pre-incubation with the antioxidants, Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) and vitamin E, attenuated the O_2^- response and ulti-

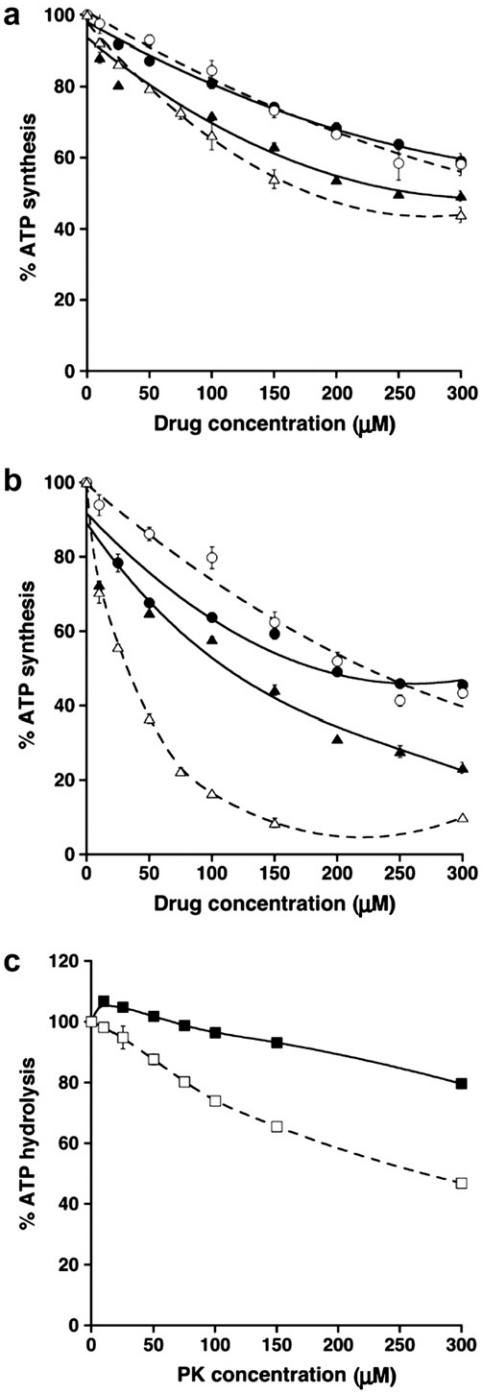

Figure 2. Effect of benzodiazepines on the mitochondrial F_1F_0-ATPase. Rates of ATP hydrolysis (a) and synthesis (b) catalyzed by bovine submitochondrial particles (SMPs) were determined in the presence of PK (△), Cz (▲), Dz (○), and 4-Cl-Dz (●), and plotted in relation to activity in the presence of DMSO vehicle control. (c) Effect of PK on ATP hydrolysis catalyzed by reconstituted SMPs in the presence (□) or absence (■) of the OSCP.

Table 1. Potency of Bz-423 and structurally related ligands of the PBR and CBR

Compound	Bz-423	PK	Cz	4-Cl-Dz	Dz
ATPase activity EC_{50} (μM)					
Hydrolysis	8.9	230	240	>300	>300
Synthesis	5.5	33	120	210	210
ROS EC_{50} (μM)					
Ramos cells	7.3	100	>300	180	170
Jurkat cells	10	56	>300	230	200
Cell death EC_{50} (μM)					
Ramos cells	6.3	90	>300	130	250
Jurkat cells	6.0	75	>300	>300	170

Data are means of three experiments with a standard deviation of ±3%.

Table 2. Inhibition of PK11195-induced signals in Jurkat cells by antioxidants, MnTBAP and Vitamin E[a]

Antioxidant	% ROS+	% Cell death
None	67.9	85.5
MnTBAP	39.7	68.9
Vitamin E	33.9	55.9

Data are means of three experiments with a standard deviation of ±3%.

[a] Data were obtained using 75 μM PK11195.

mate cell death induced by PK11195 (Table 2), consistent with previous observations that the early O_2^- response is essential for apoptosis induced by inhibitors of the F_1F_0-ATPase like Bz-423.

Collectively, our data suggest that the effects of high PK11195 concentrations commonly attributed to modulation of the PBR are consistent with inhibition of the mitochondrial F_1F_0-ATPase. PK11195 has many effects that could not previously be explained solely by its nanomolar binding affinity to the PBR. For example, when mitochondria were stained for binding site densities with the two PBR ligands, PK11195 and 4-Cl-Dz, PK11195 had three times the binding site densities of 4-Cl-Dz,[22] suggesting that PK11195 has more than one mitochondrial binding site. Additionally, when the relative sensitivities of a panel of leukemia cell lines to PK11195-sensitized apoptosis were analyzed, their sensitivities did not correlate with the number of PBR binding sites.[16] Even more specifically, cells thought to lack the PBR still undergo death induced by PK11195.[20] In cells that express the PBR, PBR knockdown via siRNA does not change the concentrations of PK11195 required to inhibit cell proliferation or sensitize cells to apoptosis.[14,21] These observations strongly indicate that PK11195 has another cellular target distinct from the PBR.

From its effects on isolated mitochondria to its effects in whole cells, specific cellular changes induced by high concentrations of PK11195 are consistent with those induced by Bz-423.[7] In isolated mitochondria, not only do both compounds inhibit F_1F_0-ATPase activity in an OSCP-dependent fashion, but they also both induce mitochondrial structural alterations[7,23,24] and inhibit state 3 respiration.[7,25,26] Their mechanisms of inhibiting cell growth and inducing apoptosis are also similar. Inhibition of cell proliferation by Bz-423 and PK11195 is mediated by arrest at the G_1/G_0 phase of the cell cycle.[10,12,15,27] Cells undergoing apoptosis in response to incubation with either agent utilize an intrinsic (mitochondrial) apoptotic pathway, characterized by an early increase in ROS production, mitochondrial permeability transition (MPT), cytochrome *c* release, and caspase activation that collectively lead to cell death.[5,21] Antioxidants protect cells from apoptosis induced by both compounds.[5,24] Cell death can be induced by both agents even in cells overexpressing anti-apoptotic proteins, such as Bcl-X_L and Bcl-2,[19,27–29] and both selectively target activated or transformed cells.[21,30] Taken together, these characteristics strongly suggest that micromolar concentrations of PK11195 induce cellular responses as a direct result of mitochondrial F_1F_0-ATPase inhibition.

Since an endogenous ligand of the PBR remains elusive, PK11195 has been used extensively to characterize the function of the PBR in vivo.[3] The results presented here indicate that conclusions regarding PBR function based on PK11195 should be interpreted with caution. PK111195 and other PBR ligands are currently being developed as drugs. Our data suggest that lead optimization based on these compounds should consider the possibility that the F_1F_0-ATPase is the relevant molecular target.

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