

Toxicity of Alpidem, a Peripheral Benzodiazepine Receptor Ligand, but Not Zolpidem, in Rat Hepatocytes: Role of Mitochondrial Permeability Transition and Metabolic Activation

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ABSTRACT

Whereas alpidem is hepatotoxic, zolpidem is not. Despite closely related chemical structures, alpidem, but not zolpidem, is a peripheral benzodiazepine receptor (PBR) ligand, and is also more lipophilic than zolpidem. We compared their effects in isolated rat liver mitochondria and rat hepatocytes. Zolpidem did not affect calcium-induced mitochondrial permeability transition (MPT) in mitochondria, caused little glutathione depletion in hepatocytes, and was not toxic, even at 500 μ M. At 250 to 500 μ M, alpidem prevented calcium-induced MPT in isolated mitochondria, but caused severe glutathione depletion in hepatocytes that was increased by 3-methylcholanthrene, a cytochrome P4501A inducer, and decreased by cystine, a glutathione precursor. Although cell calcium increased, mitochondrial cytochrome c did not translocate to the cytosol and cells died of necrosis. Cell death was prevented by cystine, but not cyclosporin A, an MPT inhibitor. At low concentrations (25–50

μ M), in contrast, alpidem accelerated calcium-induced MPT in mitochondria. It did not deplete glutathione in hepatocytes, but nevertheless caused some cell death that was prevented by cyclosporin A, but not by cystine. Alpidem (10 μ M) also increased the toxicity of tumor necrosis factor- α (1 ng/ml) in hepatocytes. In conclusion, low concentrations of alpidem increase both calcium-induced MPT in mitochondria, and TNF- α toxicity in cells, like other PBR ligands. Like other lipophilic protonatable amines, however, alpidem inhibits calcium-induced MPT at high concentrations. At these high concentrations, toxicity involves cytochrome P4501A-mediated metabolic activation, glutathione depletion, and increased cell calcium, without MPT involvement. In contrast, zolpidem has no mitochondrial effects, causes little glutathione depletion, and is not toxic.

Despite extensive efforts to predict hepatotoxicity, several new drugs must be withdrawn from the market each year, due to rare but severe hepatic reactions that had not been predicted by toxicological tests and clinical trials. Alpidem was released in 1991 as a highly active anxiolytic agent. However, a few cases of severe hepatitis occurred, mainly during the first 2 months of treatment and in patients receiving other hepatotoxic drugs (Barki et al., 1993). Liver lesions included hepatic necrosis and an inflammatory cell infiltrate, and several patients had a subfulminant course, causing death or requiring liver transplantation (Baty et al., 1994; Ausset et al., 1995). Alpidem was therefore withdrawn from the market in 1995.

On the basis of its ligand properties and chemical structure, several potential mechanisms can be considered for alpidem-induced hepatitis. First, alpidem is a ligand of the

peripheral benzodiazepine receptor (PBR) (Langer et al., 1990) that is located on the outer mitochondrial membrane and interacts with the mitochondrial permeability transition (MPT) pore (Zoratti and Szabo, 1994). Several PBR ligands have been shown to trigger MPT either alone or in combination with other compounds, which also favor MPT; opening of the MPT pore causes the translocation of mitochondrial cytochrome c to the cytosol, thus triggering caspase activation and cell death (Pastorino et al., 1994, 1996; Hirsch et al., 1998; Tanimoto et al., 1999; Zisterer et al., 2000).

Second, alpidem is a lipophilic amine, and several of these molecules (including amiodarone, perhexiline, diethylaminoethoxyhexestrol, and buprenorphine) uncouple and/or inhibit mitochondrial respiration and also inhibit MPT at high concentrations (Fromenty et al., 1990; Deschamps et al., 1994; Berson et al., 1998, 2001).

Last, like many other drugs, alpidem contains unsaturated cyclic structures. Alpidem is transformed, probably by cyto-

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ABBREVIATIONS: PBR, peripheral benzodiazepine receptor; MPT, mitochondrial permeability transition; TNF- α , tumor necrosis factor- α ; DMSO, dimethyl sulfoxide.

chrome P450 1A, into an electrophilic metabolite that forms a glutathione adduct (Durand et al., 1992).

In the present study, the effects of alpidem were investigated in isolated rat liver mitochondria and rat hepatocytes and compared with those of zolpidem. Despite a closely related chemical structure, zolpidem is not a PBR ligand (Romeo et al., 1992); it is less lipophilic than alpidem (Durand et al., 1992), has a different metabolism (Durand et al., 1992), and does not cause hepatitis in humans.

Materials and Methods

Chemicals, Animals, and Treatments. Alpidem was provided by Synthelabo Groupe (Le Plessis-Robinson, France). Zolpidem was purchased from Sigma Chemical (St. Louis, MO). [^{14}C]Palmitic acid (800 mCi/mmol) was from PerkinElmer Life Science Products (Boston, MA). Cyclosporin A was supplied by Novartis (Basel, Switzerland) or purchased from Sigma Chemical. Recombinant rat tumor necrosis factor- α (TNF- α) was purchased from BD Pharmingen (San Diego, CA).

Male Sprague-Dawley Crl:CD(SD)BR rats weighing between 200 and 300 g were purchased from Charles River (Cléon, France). Rats fed ad libitum with an equilibrated standard diet (Autoclavé 113; UAR, Villemaison-sur-Orge, France) until sacrifice by cervical dislocation. Some rats were treated for 3 days with 3-methylcholanthrene (20 mg/kg i.p.) or β -naphthoflavone (40 mg/kg i.p.) and killed 24 h after the last dose of these cytochrome P450 1A inducers (Guengerich et al., 1982).

Mitochondrial Permeability Transition. For permeability transition studies, rat liver mitochondria were isolated in 100 μM EDTA, 500 μM EGTA, 250 mM mannitol, 75 mM sucrose, 10 mM HEPES buffer, pH 7.4, centrifuged, and washed twice in a similar medium, but without EGTA and with 0.5% (w/v) fatty acid-free bovine serum albumin (Berson et al., 2001).

Mitochondria (1 mg of protein/ml) were then resuspended in 215 mM mannitol, 71 mM sucrose, 3 mM HEPES, pH 7.4. After addition of various compounds as indicated, 10 μM CaCl_2 was added, and mitochondrial swelling was monitored by following the absorption of the mitochondrial suspension at 550 nm at room temperature (Berson et al., 2001).

Mitochondrial Oxygen Consumption. For measurements of oxygen consumption and all other mitochondrial studies, mitochondria were isolated in 1 mM EGTA, 300 mM sucrose, 5 mM 3-(*N*-morpholino)propanesulfonic acid, 5 mM KH_2PO_4 , and 0.1% (w/v) bovine serum albumin, pH 7.4 (Berson et al., 2001). Polarographic measurements were performed at 30°C with a Gilson K-IC oxygraph (Middletown, WI) equipped with a Clark electrode. The respiratory medium contained 1 mM EDTA, 225 mM sucrose, 10 mM KCl, 5 mM MgCl_2 , 10 mM tris(hydroxymethyl)aminomethane, and 10 mM KH_2PO_4 , pH 7.4. Mitochondria were energized with either glutamate plus malate (5 mM each) or succinate (10 mM), and respiration was stimulated either by the uncoupler 2,4-dinitrophenol (160 μM) or by ADP (0.2 mM). The respiratory control ratio was calculated as the ratio of the rate of oxygen consumption after addition of 0.2 mM ADP (state 3 respiration) to that after ADP consumption (state 4 respiration). The ADP/O ratio was calculated as the ADP consumed/oxygen atom consumed during the whole state 3 period. NADH-supported respiration was also measured in submitochondrial particles as previously described (Berson et al., 1994).

Mitochondrial Membrane Potential. The fluorescent dye safranin was used to monitor changes in the mitochondrial membrane potential. Mitochondria (1 mg of protein/ml) were incubated at 30°C in 0.38 mM EDTA, 200 mM sucrose, 20 mM HEPES, pH 7.2 (Froymont et al., 1990). After addition of 10 μM safranin and various compounds as indicated, fluorescence was continuously recorded with excitation at 510 nm and emission at 570 nm.

Mitochondrial β -Oxidation. The β -oxidation of [^{14}C]palmitic was assessed as previously described (Berson et al., 1998). Mitochondria (1 mg of protein/ml) were preincubated at 30°C for 5 min, in 0.2 mM ATP, 50 μM L-carnitine, 40 μM CoA, 4.5 mM MgCl_2 , 70 mM sucrose, 54 mM KCl, 9 mM KH_2PO_4 , pH 7.4, with or without 4 mM KCN, and with or without 100 μM alpidem or zolpidem. [^{14}C]Palmitic acid (40 μM) and bovine serum albumin (0.25 mg/l) were added, and formation of acid-soluble β -oxidation products was measured for 10 min. Mitochondrial activity was assessed as the KCN-inhibitable activity.

Studies with Isolated Rat Hepatocytes. Rats were anesthetized with sodium pentobarbital, and in situ liver perfusion was performed as described (Fau et al., 1992). Cells were counted under a microscope. Their initial viability was estimated with trypan blue (0.2%) and averaged 90%. Cells ($3 \times 10^6/\text{ml}$) were incubated at 37°C, under a 95% O_2 , 5% CO_2 atmosphere, in 25-ml Erlenmeyer flasks containing 3 ml of 1 mM CaCl_2 , 0.8 mM MgCl_2 , 116 mM NaCl, 5.4 mM KCl, 5 mM glucose, 0.92 mM NaH_2PO_4 , 25 mM NaHCO_3 , pH 7.4, with amino acids (805 mg/ml), vitamins (8.1 mg/ml), gelatin (15 mg/ml), and either DMSO (0.3%) alone, or alpidem or zolpidem (50–500 μM) added in DMSO.

After incubation, cells were recovered by centrifugation at 4°C. Intracellular glutathione was assessed by measuring nonprotein sulfhydryls after protein precipitation with trichloroacetic acid, followed by measurement of thiols in the supernatant with 5,5'-dithio-bis-(2'-nitrobenzoic acid, as previously described (Fau et al., 1992). Viable cells were assessed by trypan blue exclusion, and lactate dehydrogenase activity in the incubation medium was measured as described (Fau et al., 1992). Cell ATP was assessed by conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate in the presence of phosphoglycerate kinase, followed by oxidation of NADH in the presence of glyceraldehyde 3-phosphate dehydrogenase, as previously described (Fau et al., 1982). Cell calcium was studied with quin2-AM as previously described (Tordjmann et al., 1996).

Cytosolic and Mitochondrial Cytochrome *c*. Cells were homogenized at 4°C in 1 mM EGTA, 0.1% bovine serum albumin, 300 mM saccharose, 5 mM 3-(*N*-morpholino)propanesulfonic acid, 5 mM KH_2PO_4 , pH 7.4. The homogenate was centrifuged at 1,000g for 15 min at 4°C and the supernatant was centrifuged at 10,000g for 15 min to separate cytosol from mitochondria.

Cytosolic and mitochondrial proteins underwent sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis and transfer to nitrocellulose and were exposed to a mouse monoclonal antibody (65981A; BD Pharmingen) recognizing rat cytochrome *c*. Blots were revealed by an enhanced chemiluminescence detection system as previously described (Haouzi et al., 2000).

Ultrastructure of Isolated Rat Hepatocytes. After incubation with alpidem, hepatocytes were centrifuged at 30g for 3 min. Cell pellets were fixed in a solution of 2.5% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, for 1 h at 4°C. After washing with the same buffer, cells were postfixed in a solution of 1% osmium tetroxide buffered with phosphate buffer, for 30 min at room temperature. Pellets were dehydrated by graded ethanol solutions and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Jeol 1010 electron microscope (JEOL, Tokyo, Japan).

Primary Cultures of Rat Hepatocytes. Hepatocytes were isolated by nonrecirculating perfusion with collagenase (Berson et al., 1996). Hepatocytes were cultured at 37°C under a 5% CO_2 , 95% air atmosphere in a Williams' E culture medium, supplemented with fetal calf serum (Invitrogen, Carlsbad, CA), and penicillin (100 U/ml) (Berson et al., 1996). After cell attachment (3 h), the medium was replaced by a new, serum-free medium containing hydrocortisone (70 μM) and either DMSO alone (0.1%), or alpidem or zolpidem (12.5–200 μM) added in DMSO. Lactate dehydrogenase release was measured as the ratio of lactate dehydrogenase activity in the medium to the total activity (Berson et al., 1996).

Statistical Analysis. Comparisons between one control group and several treatment groups were made by analysis of variance followed by a Dunnett's *t* test. Comparison between one control group and a single treatment group was made by the Student's *t* test for independent data. Differences were considered significant for *P* values less than 0.05.

Results

Dual Effects of Alpidem, but Not Zolpidem, on Mitochondrial Permeability Transition. Incubation of mitochondria (1 mg of protein/ml) with sucrose and calcium (10 μ M) caused mitochondrial swelling (Fig. 1). Swelling occurred after a lag time of about 13 min and was prevented by cyclosporin A (Fig. 1). Zolpidem (25 μ M) had no effect on this calcium-induced MPT (results not shown).

Alpidem alone (without calcium) did not cause MPT (results not shown). However, alpidem modulated calcium-induced MPT (Fig. 1). These modulating effects differed at low and high alpidem concentrations.

At a low concentration (25 μ M), alpidem shortened the lag time before the onset of calcium-induced MPT by 43% (Fig. 1). These enhancing effects of alpidem on calcium-induced MPT were not further increased in mice pretreated with β -naphthoflavone (results not shown). β -Naphthoflavone induces a mitochondrial form of cytochrome P450 1A that is activated by the mitochondrial adrenodoxin reductase/adrenodoxin system (Anandatheerthavarada et al., 1997). Failure of β -naphthoflavone to further potentiate the effects of alpidem on MPT may suggest that these effects are due to alpidem itself rather than to cytochrome P450 1A-generated metabolites.

Whereas 50 μ M alpidem still speeded up calcium-induced MPT, this potentiating effect disappeared with 100 μ M alpidem. At still higher concentrations (250 or 500 μ M), alpidem instead prevented calcium-induced MPT (Fig. 1). Several other lipophilic protonatable amines have been shown to

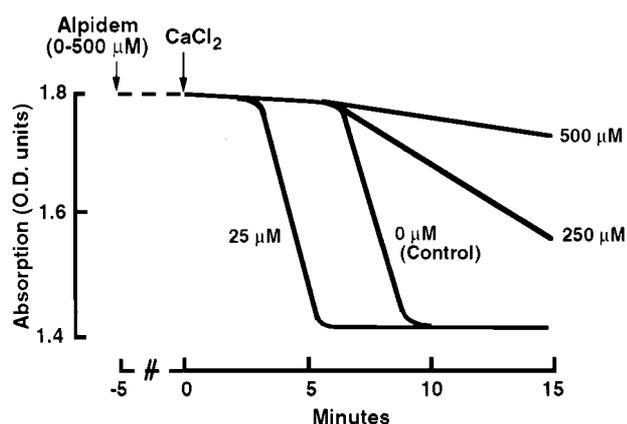


Fig. 1. Effects of alpidem on calcium-induced mitochondrial swelling. Mitochondria were incubated at room temperature in a sucrose medium. After 5 min of preincubation with or without alpidem (25–500 μ M), 10 μ M CaCl_2 was added and mitochondrial swelling was monitored at 540 nm. At a low concentration (25 μ M), alpidem hastened calcium-induced swelling. The lag time between calcium addition and the onset of swelling (mean \pm S.E.M. for three experiments) was 13 ± 3 min without alpidem and 7.4 ± 1.3 with 25 μ M alpidem ($P < 0.05$). This swelling was totally prevented by 2.5 μ M cyclosporin A (results not shown). At high concentrations (250 or 500 μ M), in contrast, alpidem slowed down calcium-induced swelling.

inhibit calcium-induced MPT at high concentrations (Berson et al., 2001).

Alpidem, but Not Zolpidem, Inhibits and Also Uncouples Mitochondrial Respiration. Because the effects of alpidem on MPT require calcium, we could study its other mitochondrial effects in the presence of EDTA, which sequesters calcium and thus prevents MPT. Alpidem inhibited 2,4-dinitrophenol-stimulated respiration in mitochondria energized with glutamate and malate, which feed electrons to complex I of the respiratory chain (Table 1). Alpidem also inhibited the respiration supported by 10 mM NADH, which transfers electrons to complex I in submitochondrial particles: the respiratory rate (mean \pm S.E.M. for three experiments) was 57 ± 5 nmol of O/min/mg of protein without alpidem and $33 \pm 4^*$ with 100 μ M alpidem ($*P < 0.05$). In contrast, alpidem did not inhibit 2,4-dinitrophenol-stimulated respiration in mitochondria energized with succinate, which feeds electrons into complex II and then complexes III and IV (Table 1). These differences indicate that alpidem selectively inhibits complex I.

In addition to inhibiting ADP-stimulated (state 3) respiration, alpidem also had uncoupling effects. Indeed, alpidem increased state 4 respiration in whole mitochondria energized with malate and glutamate, and it decreased both the respiratory control ratio and the ADP/O ratio (Table 2), thus indicating that respiration was partly wasted, without concomitant ATP formation. In contrast, 250 μ M zolpidem, which is less lipophilic than alpidem, had no effect on mitochondrial respiration (Table 2).

Effect of Alpidem on the Mitochondrial Membrane Potential. By transporting protons across the inner membrane, uncouplers can decrease the mitochondrial membrane potential. In the presence of EDTA (blocking MPT), alpidem (50 μ M) decreased the membrane potential of succinate-energized mitochondria by $48 \pm 1\%$; this effect was potentiated by tetraphenylborate (Fig. 2), a lipophilic cation that speeds up the entry of lipophilic amines into the mitochondria (Berson et al., 1998).

No Effect of Alpidem or Zolpidem on Fatty Acid β -Oxidation. Neither alpidem nor zolpidem inhibited the mitochondrial β -oxidation of [U - 14 C]palmitic acid. The formation of acid-soluble β -oxidation products (mean \pm S.E.M. for three experiments) was 21.7 ± 0.3 nmol/min/mg of protein in control incubations, 19.9 ± 4.2 with 100 μ M alpidem, and 23.5 ± 0.8 with 100 μ M zolpidem.

Effects of Alpidem in Isolated Rat Hepatocytes. Alpidem caused dose-dependent cell death in isolated rat hepa-

TABLE 1

In vitro effects of alpidem on respiration stimulated by 2,4-dinitrophenol

Mitochondria were incubated at 30°C with either glutamate and malate (5 mM each) or succinate (10 mM). One minute after alpidem, dinitrophenol (160 μ M) was added and respiration was measured. Results are means \pm S.E.M. for three experiments.

	Oxygen Consumption	
	Glutamate and Malate	Succinate
	nmol O/min/mg of protein	
Control	164 ± 8	221 ± 12
Alpidem (50 μ M)	$114 \pm 10^*$	210 ± 2
Alpidem (100 μ M)	$122 \pm 10^*$	224 ± 39
Alpidem (250 μ M)	$84 \pm 4^*$	193 ± 18

O, atom of oxygen.

* Different from control ($P < 0.05$).

TABLE 2

In vitro effects of alpidem but not zolpidem on ADP-supported respiration sustained by malate and glutamate

Mitochondria were incubated at 30°C with glutamate and malate (5 mM each). One minute after addition of alpidem or zolpidem, ADP (0.2 mM) was added and respiration was measured. Results are means \pm S.E.M. for at least three experiments.

	Oxygen Consumption		RCR	ADP/O
	State 3	State 4		
	<i>nmol O/min/mg of protein</i>			
Control	153 \pm 2	33 \pm 1	4.8 \pm 0.5	2.7 \pm 0.3
Alpidem (50 μ M)	112 \pm 3*	45 \pm 2	2.4 \pm 0.7*	1.9 \pm 0.1
Alpidem (100 μ M)	99 \pm 3*	47 \pm 3*	2.0 \pm 0.5*	1.8 \pm 0.1*
Alpidem (250 μ M)	72 \pm 15*	83 \pm 9*	0.8 \pm 1.0*	1.8 \pm 0.2*
Zolpidem (250 μ M)	144 \pm 14	38 \pm 5	4.1 \pm 0.5	3.1 \pm 0.2

O, atom of oxygen; RCR, respiratory control ratio; ADP/O, ADP formed per O consumed during state 3 respiration.

* Different from control ($P < 0.05$).

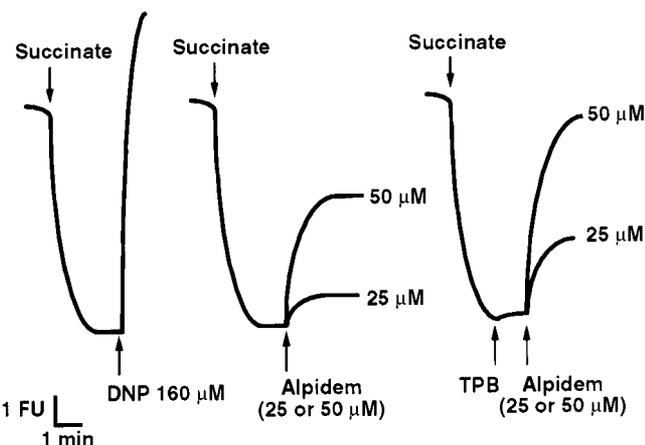


Fig. 2. Effects of alpidem on the mitochondrial membrane potential assessed by the fluorescence of safranin. A decrease in safranin fluorescence indicates an increase in membrane potential. Rat mitochondria (0.7 mg of protein/ml) were incubated with 10 mM succinate, which causes the extrusion of protons from the mitochondrial matrix, increasing the membrane potential. The uncoupler 2,4-dinitrophenol (DNP) was used as a reference compound that causes the reentry of protons into the matrix and decreases the membrane potential. Alpidem (25 or 50 μ M) also caused a dose-dependent decrease in the membrane potential. Tetraphenylborate (TPB) alone (1 μ M) had little effect. When both tetraphenylborate and alpidem were added, there was a marked decrease in the membrane potential. FU, fluorescence unit.

tocytes (Fig. 3). This cell death was not associated with a major decrease in cell ATP. In hepatocytes incubated at 37°C for 2 h with or without alpidem, cell ATP (mean \pm S.E.M. for four to eight determinations) was 17.0 \pm 2.0 nmol/10⁶ hepatocytes without alpidem, 16.4 \pm 1.0 with 50 μ M alpidem, and 12.5 \pm 0.7* with 250 μ M alpidem (* $P < 0.05$ versus control incubation). Thus, even at high concentrations, the inhibitory effects of alpidem on respiration only caused a slight (26%) decrease in cell ATP, which is not enough to cause cell death.

However, alpidem also triggered dose-dependent glutathione depletion (Fig. 3). This depletion was increased by pretreatment of the animals with 3-methylcholanthrene, a cytochrome P450 1A inducer, and prevented by α -naphthoflavone, a cytochrome P450 1A inhibitor (Guengerich et al., 1982; Bourrié et al., 1996). After 90 min of incubation with 50 μ M alpidem, residual cell glutathione was 79% of control in hepatocytes from naive rats, but 48% in 3-methylcholanthrene-treated rats (results not shown). With 500 μ M alpidem, residual glutathione was 13% of control in incubations

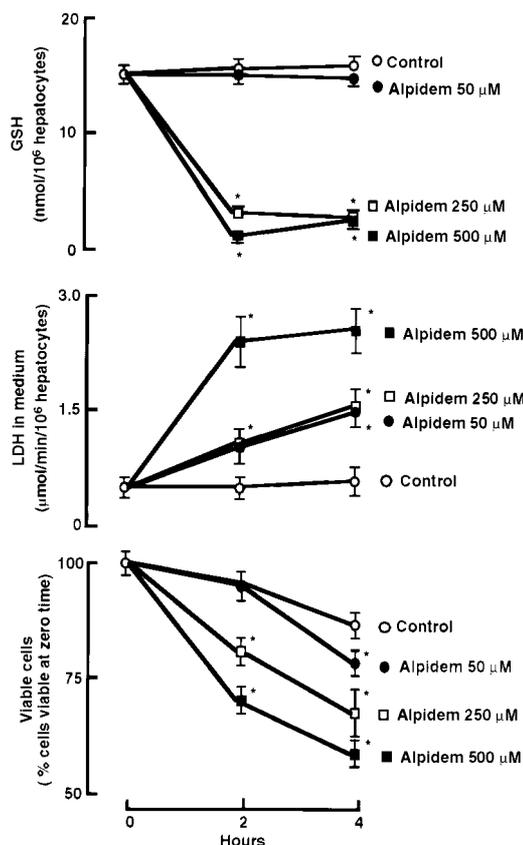


Fig. 3. Effect of alpidem on cell glutathione (GSH), lactate dehydrogenase (LDH) in the incubation medium, and cell survival. Isolated hepatocytes were incubated at 37°C with or without alpidem (50, 250, or 500 μ M) for 2 or 4 h. Results are mean \pm S.E.M. for three to six experiments. *, different from control ($P < 0.05$).

performed without α -naphthoflavone, but 42% of control with 100 μ M α -naphthoflavone (results not shown). These observations support a role of cytochrome P450 1A-generated metabolites in glutathione conjugation (Durand et al., 1992) and depletion.

Glutathione depletion seemed to be the cause of the hepatotoxicity of high alpidem concentrations (250–500 μ M). At these very high concentrations, cell death was preceded by rapid and severe glutathione depletion (Fig. 3). Furthermore, both glutathione depletion and lactate dehydrogenase release were limited by cystine, a glutathione precursor. Indeed, in cells incubated for 4 h with 250 μ M alpidem, residual glutathione (mean \pm S.E.M. for four determinations) was only 6.1 \pm 0.4 nmol/10⁶ hepatocytes in the absence of cystine, but 11.4 \pm 0.8* with 0.5 mM cystine (* $P < 0.05$). Lactate dehydrogenase activity was increased by 122% in the absence of cystine (from 0.9 \pm 0.2 μ mol/min/10⁶ hepatocytes without alpidem to 2.0 \pm 0.2 with 250 μ M alpidem), but by only 44% with 0.5 mM cystine (from 0.9 \pm 0.2 μ mol/min/10⁶ hepatocytes without alpidem to 1.3 \pm 0.1* with 250 μ M alpidem; * $P < 0.05$ versus the incubation without cystine).

As in previous studies on the effects of glutathione-depleting metabolites (Bellomo and Orrenius, 1985; Haouzi et al., 2000), alpidem-mediated glutathione depletion was associated with a marked and early increase in cell Ca²⁺ (Fig. 4). However, whereas this increase in cell Ca²⁺ triggered MPT in a previous study (Haouzi et al., 2000), in contrast, there was no evidence for a role of MPT in alpidem-induced cell

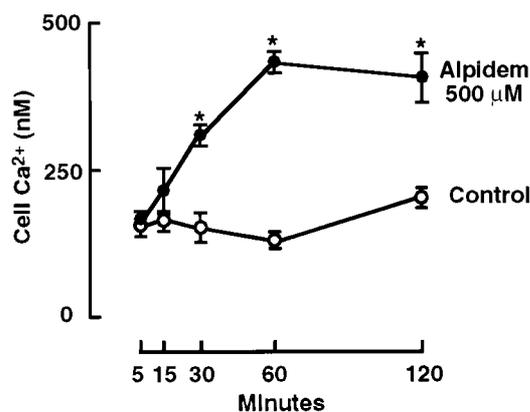


Fig. 4. Effects of alpidem on cell Ca^{2+} . Isolated hepatocytes were incubated at 37°C with or without $500 \mu\text{M}$ alpidem. Cell Ca^{2+} was measured with quin-2 AM for 2 h. Results are means \pm S.E.M. for six determinations. *, different from control ($P < 0.05$).

death. Indeed, alpidem ($500 \mu\text{M}$) did not significantly modify cytosolic or mitochondrial cytochrome *c* (results not shown), and cell death was not prevented by cyclosporin A (Fig. 5), an MPT inhibitor that prevented metabolite-mediated MPT and cell death in a previous study (Feldmann et al., 2000; Haouzi et al., 2000). Furthermore, no apoptotic cells were observed with alpidem, but only necrotic cells (Fig. 6B). These necrotic lesions only affected approximately one-fourth of the hepatocytes, possibly because the abundance of cytochrome P450 1A in centrilobular hepatocytes caused severe glutathione depletion in these cells.

In contrast, glutathione depletion seemed to have no role in the toxicity of lower concentrations of alpidem. With $50 \mu\text{M}$ alpidem, no apoptosis was observed, but approximately 5% of the hepatocytes were necrotic (results not shown). At this low concentration ($50 \mu\text{M}$), alpidem only caused slight glutathione depletion (Fig. 3), which is insufficient to cause cell death. Indeed, cystine (0.5 mM) did not prevent this minimal cell death (results not shown). In contrast, cyclosporin A seemed to be protective, because $50 \mu\text{M}$ alpidem no longer caused any decrease in cell viability in hepatocytes preincubated with this MPT inhibitor (Fig. 5).

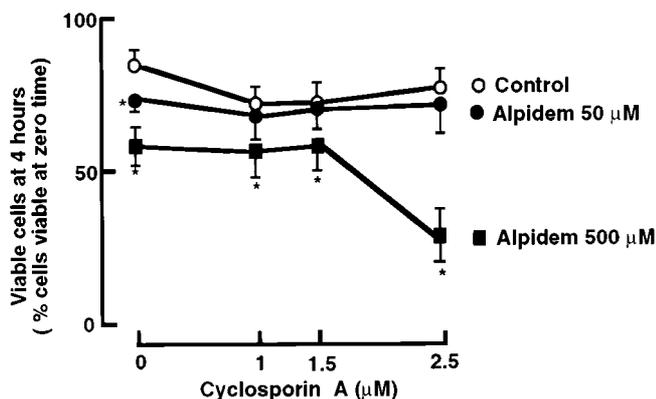


Fig. 5. Effects of cyclosporin A on alpidem-mediated loss of viability in isolated rat hepatocytes incubated with two different doses of alpidem. Hepatocytes were preincubated at 37°C for 30 min with or without cyclosporin A (1, 1.5, or $2.5 \mu\text{M}$). Viable cells (mean \pm S.E.M. for three experiments) were determined by trypan blue exclusion after 4 h of further incubation with or without alpidem (50 or $500 \mu\text{M}$). *, different from control ($P < 0.05$).

Comparison of the Effects of Alpidem and Zolpidem in Isolated Hepatocytes and Cultured Hepatocytes.

In contrast to the severe glutathione depletion caused by $500 \mu\text{M}$ alpidem, cell glutathione was only decreased by 47% in isolated rat hepatocytes incubated for 4 h with $500 \mu\text{M}$ zolpidem; lactate dehydrogenase was not released and viability was unchanged (results not shown). Whereas rat hepatocytes cultured for 24 h with $12.5 \mu\text{M}$ alpidem released lactate dehydrogenase in the medium, $200 \mu\text{M}$ zolpidem caused no lactate dehydrogenase release (Fig. 7).

Potentiation of TNF- α -Induced Toxicity by Alpidem.

Whereas alpidem ($10 \mu\text{M}$) caused no significant lactate dehydrogenase release alone, it increased the toxicity of TNF- α in isolated rat hepatocytes (Table 3).

Discussion

This study shows that alpidem has several cellular effects that may be related to its PBR ligand properties, its lipophilic cationic structure, and its metabolism.

Alpidem is a potent PBR ligand (Romeo et al., 1992). Several such ligands trigger MPT in cells exposed to the PBR ligand alone (Tanimoto et al., 1999; Zisterer et al., 2000) or in combination with other compounds also favoring MPT (Pastorino et al., 1996; Hirsh et al., 1998; Tanimoto et al., 1999). PBR is located in the outer mitochondrial membrane and may modify the activity of the voltage-dependent anion channel, one of the presumed components of the MPT pore at contacts sites between the outer and inner membranes (Krueger, 1995; Zoratti and Szabo, 1995). In the present study, low concentrations of alpidem (25 or $50 \mu\text{M}$) accelerated calcium-induced MPT in isolated rat liver mitochondria (Fig. 1). In a previous study, alpidem enhanced calcium-mediated opening of the mitochondrial megachannel, which is probably the electrophysiological counterpart of MPT (Zoratti and Szabo, 1994).

Other effects of alpidem seem related to its cationic amphiphilic structure. Like several other cationic amphiphilic drugs (Fromenty et al., 1990; Deschamps et al., 1994; Berson et al., 1998, 2001), alpidem both uncoupled and inhibited mitochondrial respiration (Tables 1 and 2). The transfer of electrons along the respiratory chain is associated with the extrusion of protons from the mitochondrial matrix into the intermembrane space, thus creating a large electrochemical potential across the inner membrane, whose energy is secondarily used to generate ATP during the reentry of protons in the matrix through ATP synthase (Fromenty and Pessayre, 1995). Lipophilic tertiary amines interfere with this mitochondrial energy production in the following way (Fromenty et al., 1990; Deschamps et al., 1994; Berson et al., 1998, 2001). They cross the outer mitochondrial membrane in the uncharged form and become protonated in the acidic intermembrane space. The positively charged molecule is then pushed by the mitochondrial membrane potential into the mitochondrial matrix and releases a proton in the alkaline matrix. This protonophoric effect decreases the mitochondrial membrane potential (Fig. 2), thus unleashing the flow of electrons along the respiratory chain and stimulating basal respiration (Table 2). However, the reentry of protons bypasses ATP synthase, so that the increased respiration produces heat instead of ATP (Table 2). Lipophilic amines reaching high intramitochondrial concentrations can also

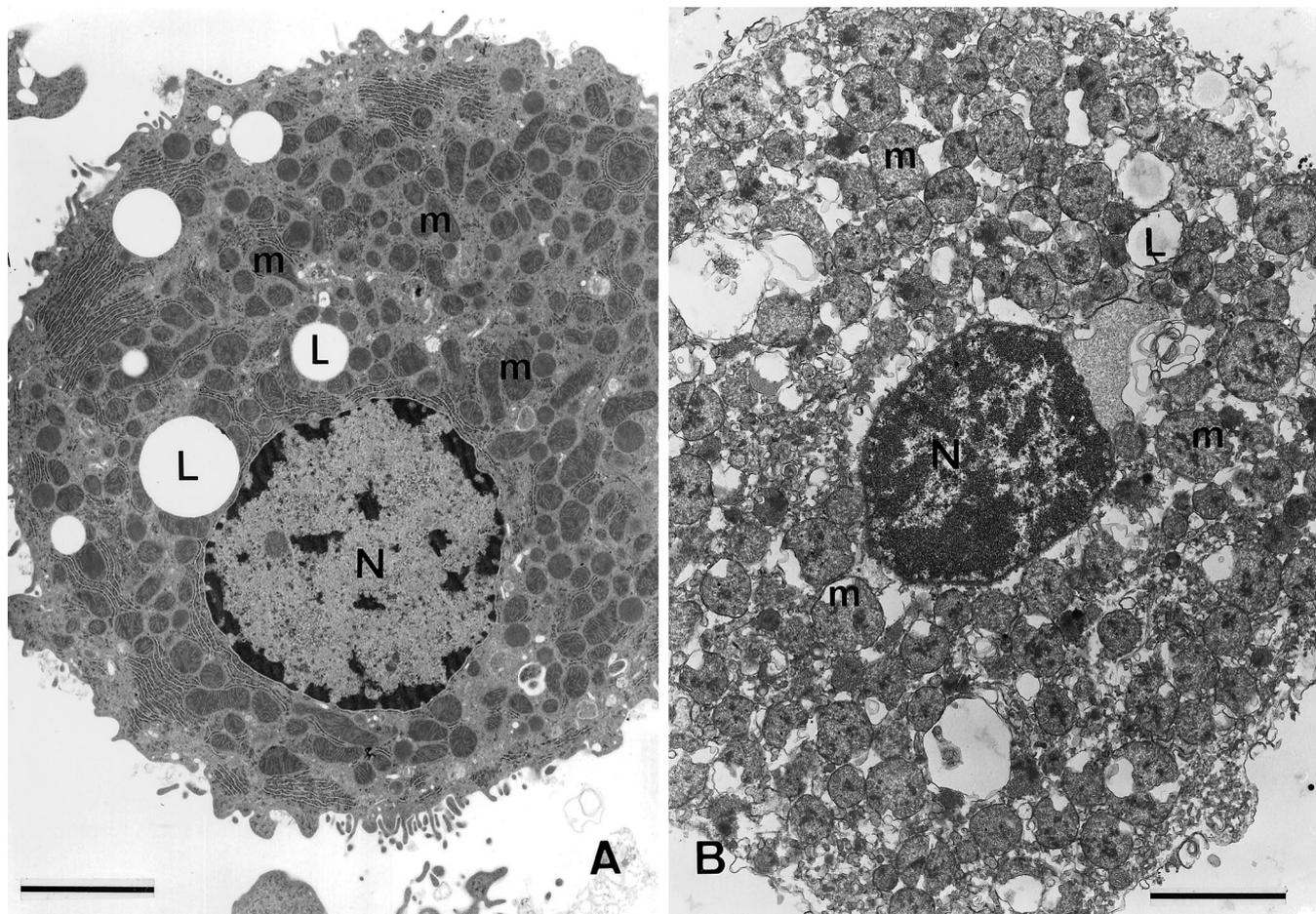


Fig. 6. Necrotic cell death in hepatocytes incubated for 4 h with 500 μM alpidem. A, normal ultrastructural appearance of one hepatocyte incubated without alpidem. B, typical necrotic ultrastructural lesions in one hepatocyte incubated with 500 μM alpidem. A and B, 4000 \times magnification. N, nucleus; m, mitochondria; L, lipid droplet. Scale bar, 2 μm .

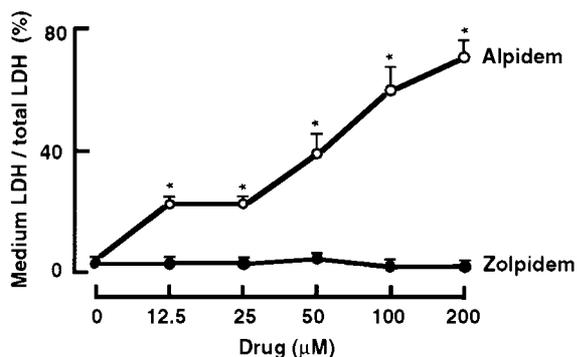


Fig. 7. Comparison of the effects of alpidem and zolpidem in cultured hepatocytes. Hepatocytes were cultured for 24 h with alpidem or zolpidem (12.5–200 μM) and the ratio of lactate dehydrogenase activity in the medium over the total activity was determined. Results are means \pm S.E.M. for three determinations. Note that drug concentrations are plotted on a geometric scale. *, different from hepatocytes incubated without the drug (0 μM), or from hepatocytes incubated with the same concentration of zolpidem ($P < 0.05$).

partially block the flow of electrons in the respiratory chain, particularly within complex I (Fromenty et al., 1990; Deschamps et al., 1994; Berson et al., 1998, 2001). Indeed, alpidem decreased the respiration supported by either NADH or malate and glutamate (which feed electrons into complex I), but did not inhibit the respiration supported by succinate (which provides electrons to complex II and then

TABLE 3

Toxicity of low concentrations of alpidem and TNF- α in isolated rat hepatocytes

Isolated rat hepatocytes were incubated for 2 h with or without alpidem and/or TNF- α , and lactate dehydrogenase (LDH) activity was measured in the incubation medium. Results are means \pm S.E.M. for four to seven determinations.

	LDH in Medium	Increase
	$\mu\text{mol}/\text{min}/10^6$ cells	%
Control	0.16 \pm 0.01	0
Alpidem (10 μM)	0.19 \pm 0.02	19
TNF- α (1 ng/ml)	0.22 \pm 0.02*	37
Alpidem (10 μM) and TNF- α (1 ng/ml)	0.38 \pm 0.02*†	137

* Different from control ($P < 0.05$).

† Different from TNF- α alone ($P < 0.05$).

other complexes) (Table 1; see *Results*), thus indicating selective impairment of complex I by alpidem.

Yet another effect of high concentrations of cationic amphiphilic compounds is to slow down calcium-induced MPT, perhaps due to the decrease in the mitochondrial membrane potential that drives Ca^{2+} uptake (Berson et al., 2001). This effect also occurred with high concentrations of alpidem (Fig. 1). At high alpidem concentrations (250–500 μM), this inhibitory effect now predominated over the enhancing effect of the PBR ligand property of alpidem, and calcium-induced MPT was instead inhibited (Fig. 1).

A last property of alpidem is to be metabolically activated

by cytochrome P450 1A into an electrophilic metabolite that forms a glutathione adduct (Durand et al., 1992). Indeed, alpidem caused dose-dependent glutathione depletion in hepatocytes (Fig. 3), and this depletion was accelerated by 3-methylcholanthrene, a cytochrome P450 1A inducer, and slowed by α -naphthoflavone, a cytochrome P450 1A inhibitor (see *Results*).

The toxicity of alpidem toward isolated rat hepatocytes seemed to involve different mechanisms, depending on the concentrations. At very high alpidem concentrations (250–500 μ M), cell death was preceded by rapid and severe glutathione depletion (Fig. 3), and both this glutathione depletion and the lactate dehydrogenase release were limited by cystine (see *Results*), a glutathione precursor that prevents metabolite-mediated hepatocyte death (Fau et al., 1992, 1994; Lekehal et al., 1996; Haouzi et al., 2000). Thiol depletion and oxidation play a major role in cell death by inactivating plasma membrane calcium translocases (Bellomo and Orrenius, 1985), thus increasing cell Ca^{2+} (Fig. 4).

With some compounds, such as skullcap diterpenoids, these high cell Ca^{2+} concentrations triggered MPT in some mitochondria, causing outer membrane rupture, release of mitochondrial cytochrome *c* into the cytosol, cytosolic caspase activation, and hepatocyte apoptosis, which was prevented by cyclosporin A (Haouzi et al., 2000). However, these mitochondrial effects did not occur with 500 μ M alpidem, probably due to the inhibitory effects of high alpidem concentrations on calcium-induced MPT (Fig. 1). Indeed, mitochondrial cytochrome *c* was not released (see *Results*) and cyclosporin A, an MPT inhibitor, did not prevent cell death (Fig. 5). Furthermore, whereas skullcap diterpenoids caused apoptosis (Haouzi et al., 2000), 500 μ M alpidem caused necrosis (Fig. 6). This necrosis was probably related to the other effects of increased Ca^{2+} , including the activation of tissue transglutaminase (thus cross-linking cellular proteins), proteolytic enzymes (which degrade proteins) and endonucleases (which fragment DNA) (Lekehal et al., 1996).

A different mechanism may be involved in the toxicity of lower alpidem concentrations. Although 50 μ M alpidem only caused very slight glutathione depletion, a slight loss of viability occurred (Fig. 3), and a few necrotic cells were observed on electron microscopy after incubation for 4 h (see *Results*). Cell death was not prevented by cystine (see *Results*), excluding a role of glutathione depletion in this process. However, some isolated hepatocytes may have an altered plasma membrane and may accumulate some calcium. Indeed, average cell calcium (in all hepatocytes) tended to increase after 2 h of incubation in control cells (Fig. 4). The enhancing effects of low alpidem concentrations on calcium-induced MPT (Fig. 1) could trigger massive MPT in these few calcium-loaded cells. Indeed, the toxicity of 50 μ M alpidem was prevented by cyclosporin A (Fig. 5), suggesting MPT involvement. Extensive MPT in these damaged cells could trigger ATP depletion and necrosis in these few cells, even though the average ATP (in all hepatocytes) was not significantly decreased at 2 h (see *Results*).

It is difficult from the present data to suggest a mechanism for alpidem-induced hepatitis in humans. Peak plasma alpidem concentrations are only 115 ng/ml (0.28 μ M) after a 200-mg dose in humans (Jonkman et al., 1991). The direct toxicity of alpidem alone, or of its reactive metabolites seems unlikely at these low concentrations. However, nontoxic con-

centrations of alpidem and several other PBR ligands can cause cell death when combined with nontoxic or minimally toxic concentrations of other compounds that also trigger MPT (Tafani et al., 2000), such as TNF- α (Table 3), ceramide, etoposide, doxorubicin, or γ -irradiation (Pastorino et al., 1996; Hirsch et al., 1998; Bono et al., 1999). Conceivably, the potentiating effect of low alpidem concentrations on MPT could cause cell death in a few hepatocytes already affected by TNF- α or other hepatotoxic drugs, which, indeed, were frequently coadministered in patients with alpidem-associated hepatitis (Barki et al., 1993).

The phagocytosis of these few dead hepatocytes by macrophages and other antigen-presenting cells could then cause the presentation of hepatic peptides modified by the covalent binding of reactive metabolites, thus stimulating helper T lymphocytes and triggering immunization in a few patients (Pessayre, 1995; Pessayre et al., 1999). An immune process would explain the inflammatory infiltrate observed in alpidem-associated hepatitis and the rarity of this adverse effect. Cytotoxic T lymphocytes kill hepatocytes (presenting foreign or modified peptides on their major histocompatibility complex class I molecules) by expressing Fas ligand on their membrane and releasing TNF- α , both of which trigger MPT (Pessayre et al., 2000; Feldmann et al., 2000; Tafani et al., 2000). Whereas Fas ligand only acts on the lymphocyte's target cell, released TNF- α may also kill nonantigen-bearing ("bystander") cells (Ando et al., 1997). Stimulation of TNF- α -induced cell death by alpidem could be involved in the severity of alpidem-associated hepatitis, which caused death or required liver transplantation in several patients (Barki et al., 1993; Baty et al., 1994; Ausset et al., 1995).

Despite a related chemical structure, zolpidem is not a PBR ligand (Romeo et al., 1992) and does not enhance calcium-induced MPT (see *Results*). Although zolpidem is also a tertiary amine, it is less lipophilic than alpidem and does not interfere with mitochondrial function. Furthermore, even at extremely high concentrations (250 μ M), representing 350 times the human plasma concentrations (Salva and Costa, 1995), zolpidem only causes moderate glutathione depletion, which is not sufficient to trigger cell death (see *Results*). These major differences may explain why zolpidem, unlike alpidem, does not cause hepatitis in humans.

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