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Interactions of chloroquine with benzodiazepine, γ-aminobutyric acid and opiate receptors

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Chloroquine, a widely used antimalarial agent, also possesses antiinflammatory properties which are useful for the treatment of rheumatoid arthritis and lupus erythematosus [1]. In adults, therapeutic doses of chloroquine (500 mg once weekly as preventive therapy in endemic areas, or a total of 2.5 g on 3 consecutive days for the treatment of acute attacks of malaria) are generally regarded as safe [2]. However, intoxicating doses of this drug cause convulsions and respiratory disturbances in an experimental rat model [3] or myocardial depression, circulatory collapse, and cardiac arrest in humans [4], with high mortality rates.

In searching for a mechanism to explain the toxic effects of chloroquine, we investigated interactions of this drug with several receptor systems which are involved in the generation of convulsions or in cardiorespiratory depression. These included the γ-aminobutyric acid (GABA*), central benzodiazepine (BZ), peripheral BZ and opiate receptors.

Materials and Methods

[^{3}H]Flunitrazepam (FNZ) (77 Ci/mmol), [^{3}H]PK 11195 (74.3 Ci/mmol), [^{3}H]muscimol (20 Ci/mmol), [^{3}H] [D-Ala^2,D-Leu^5]enkephalin (DADL) (30 Ci/mmol), [^{3}H] [D-Ala^2,*N*-MePhe^4-Gly(ol)^5]enkephalin (DAMGO) (40.8 Ci/mmol), [^{3}H][D-Pen^2,D-Pen^5]enkephalin (DPDPE) (37.1 Ci/mmol), [^{3}H]naloxone (49.7 Ci/mmol) and [^{3}H]ethylketocyclazocine (EKC) (24.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA), and [^{3}H]naloxone benzoylhydrazone (NalBzoH) was prepared from naloxone as previously described [5]. Unlabeled PK 11195 was a gift from Dr. A. Bouvier (Rhone-Poulenc Sante, Vitry-sur-Seine, France). Unlabeled GABA and chloroquine were obtained from the Sigma Chemical Co. (St. Louis, MO). Unlabeled DPDPE, DSLET and DAMGO were purchased from Peninsula Laboratories (Belmont, CA) and Lumax was obtained from Lumac (Schaesberg, The Netherlands).

All binding assays were performed as previously reported. In brief, central and peripheral BZ receptor binding was performed using [^{3}H]FNZ and rat cerebral cortical membranes and [^{3}H]PK 11195 and rat kidney membranes [6]. GABA binding was assessed in fresh rat cerebral cortex as previously described using [^{3}H]muscimol [7]. All opiate assays were performed using techniques reported previously [5, 8]. μ_1 Binding was determined in calf thalamic membranes using [^{3}H]DADL and DPDPE (10 nM) to block residual δ sites; μ_2 binding was determined in calf thalamic membranes using [^{3}H]DAMGO with DSLET (5 nM) to block μ_1 binding. δ Binding was assessed as previously reported [5] using calf frontal cortex membranes and [^{3}H]DPDPE. κ_1 Binding was performed using guinea pig cerebellar membranes and either [^{3}H]EKC (1.4 nM) with unlabeled DAMGO (100 nM) and DPDPE (100 nM) to block μ and δ sites or [^{3}H]NalBzoH in the presence of EDTA (5 mM), as previously reported [5]. Under these conditions [^{3}H]NalBzoH binding is virtually identical to that of [^{3}H]U69,593, the κ_1-selective ligand [9]. κ_3 Binding was determined in calf striatal membranes using [^{3}H]NalBzoH with EDTA (5 mM) [5].

Results are expressed as means ± SEM. Statistical analysis was performed using Student's *t*-test for paired data. Statistical significance was defined as $P < 0.05$. The IC_{50} and IC_{35} values were determined from linear regression analysis of log-logit curves. Saturation curves were analysed by nonlinear regression analysis [5, 8]. K_i values were calculated as previously reported [10, 11].

Results

In competition studies, the K_i values of chloroquine for GABA, peripheral BZ or central BZ receptors exceeded 50 μM, indicating a very low affinity of these receptors for the ligand (Table 1). In contrast, chloroquine blocked opioid binding moderately well. It was most potent against μ_1 and κ_1 binding, with K_i values around 5 μM. Against the other opioid receptor subtypes chloroquine was less active, with K_i values ranging between 20 and 40 μM.

To investigate the nature of chloroquine inhibition on opiate receptor subtypes, full saturation curves of radioligand binding to their corresponding receptors were carried out in the absence or presence of a concentration of chloroquine which blocked binding by 35% in control studies. B_{max} and K_D values were derived from the respective Scatchard analyses. Table 2 presents the affinity (K_D) values and the maximal numbers of binding sites (B_{max}) of the receptor-specific ligands to their corresponding GABA, central BZ, peripheral BZ and opioid receptor subtypes.

Table 1. Inhibition of BZ, GABA and opiate receptors by chloroquine

Receptor	Chloroquine K_i (μM)
Central BZ	>50
Peripheral BZ	>50
GABA	>50
μ_1	5.3 ± 2.2
μ_2	26.9 ± 4
δ	37.3 ± 6.9
κ_1	
[^{3}H]EKC	5.1 ± 0.9
[^{3}H]NalBzoH	6.4 ± 2.5
κ_3	23.8 ± 10.6

Membrane homogenates were incubated with radiolabeled ligands in the absence or presence of 10^{-9} to 10^{-4} M chloroquine. The IC_{50} values were determined and K_i values calculated as described [10, 11]. Results are the means ± SEM of three independent experiments.

* Abbreviations: GABA, γ-aminobutyric acid; FNZ, flunitrazepam; BZ, benzodiazepine; DADL, [D-Ala^2, D-Leu^5]enkephalin; NalBzoH, naloxone benzoylhydrazone; EKC, ethylketocyclazocine; DAMGO, [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; DSLET, [D-Ser^2,Leu^5]enkephalin-Thr^6; IC_{35}, concentrations inhibiting binding by 35%; IC_{50}, concentrations inhibiting binding by 50%; B_{max}, maximal number of binding sites; K_D, dissociation constant; and K_i, dissociation constant calculated from competition studies.

Table 2. Effects of chloroquine on opioid saturation binding studies

Receptor	Control		With chloroquine	
	K_D (nM)	B_{max} (pmol/g tissue)	K_D (nM)	B_{max} (pmol/g tissue)
μ_1	1.29	5.1	2.43	5.9
	1.87	6.3	3.21	6.3
μ_2	1.44	4.8	3.44	4.4
	1.43	5.5	2.87	4.2
δ	1.02	1.8	2.10	2.2
	0.82	1.7	1.6	1.8
κ_1	0.06	5.0	0.102	4.7
	0.07	5.6	0.111	5.13
κ_3	1.7	27.7	3.6	21.7
	2.3	31.8	4.0	28.2

Saturation studies were performed with increasing concentrations of radioligand alone or in the presence of a concentration of chloroquine that inhibited binding by 35% in control studies. The concentrations of chloroquine in the μ_1, μ_2, δ and κ_3 binding assays were 2.5, 40, 35 and 25 μM, respectively. κ_1 Binding was performed with [^{3}H]NalBzoH with or without chloroquine at 6 μM. K_D values and B_{max} determinations were calculated from the saturation curves by nonlinear regression analysis. Results represent two independent determinations. Each of the paired control and chloroquine values were determined at the same time using the same tissue.

The K_D values of these ligands to their respective receptors were in the subnanomolar to nanomolar range. Chloroquine lowered the apparent affinity of the radioligand without appreciably changing the B_{max} values. These data suggest that chloroquine is a competitive inhibitor at these opiate receptor subtypes.

Discussion

Diazepam has been reported to protect patients from chloroquine overdose, reducing mortality in humans [12, 13] and in animal models [14]. The observation that chloroquine overdose is associated with significant convulsant activity and that diazepam can treat acute chloroquine intoxication raised the question of whether the toxic actions of chloroquine might be mediated either through a central or a peripheral BZ receptor. Since the central BZ receptor is a component of the GABA receptor complex and since BZ can influence GABA function significantly [15], a direct effect upon GABA receptors also might explain chloroquine toxicity. On the other hand, the peripheral BZ agonist RO 5-4864 is a potent convulsant in animal models [16], raising the possibility of an association with chloroquine toxicity. When tested directly against both BZ receptors and GABA receptors, chloroquine demonstrated little effect at concentrations up to 50 μM. Thus, diazepam appears to treat the symptoms, such as seizures, rather than altering the basic mechanism through which chloroquine exerts its toxicity.

The ability of chloroquine to inhibit the binding of opiate binding with K_i values in the low micromolar range was unexpected. Therapeutically, blood levels may approach 1 μM [1], and toxic doses would be expected to be higher. Opiate receptors can mediate respiratory depression [17] and have been implicated in seizure activity [18]. Cardiorespiratory depression and convulsions are obtainable at micromolar chloroquine concentrations which are in the range of K_i values of chloroquine at the opiate receptors. Whether these actions account for the toxic effects of chloroquine, and the possible role of naloxone treatment for such intoxication, remain to be investigated in future studies.

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REFERENCES

1. Webster LT, Drugs used in the chemotherapy of protozoal infections. In: *The Pharmacological Basis of Therapeutics* (Eds. Gilman AG, Goodman LS, Rall TW and Murad F), pp. 1029–1048. Macmillan, New York, 1985.
2. Alving AS, Eichelberger L, Craige B Jr, Jones R Jr, Whorton CM and Pullman TN, Studies on the chronic toxicity of chloroquine (SN-7618). *J Clin Invest* **27**: 60–65, 1948.
3. Crouzetto J, Vicaut E, Palombo S, Girro C and Fournier PE, Experimental assessment of the protective activity of diazepam on the acute toxicity of chloroquine. *J Toxicol Clin Toxicol* **20**: 271–279, 1983.
4. Riou B, Barriot P, Timailto A and Baud FJ, Treatment of severe chloroquine poisoning. *N Engl J Med* **318**: 1–6, 1988.
5. Clark JA, Liu L, Price M, Hersh B, Edelson M and Pasternak GW, κ Receptor multiplicity: Evidence for two U50,488-sensitive κ_1 subtypes and a novel κ_3 subtype. *J Pharmacol Exp Ther* **251**: 461–468, 1989.
6. Gavish M, Weizman A, Youdim MBH and Okun F, Regulation of central and peripheral benzodiazepine receptors in progesterone-treated rats. *Brain Res* **409**: 386–390, 1987.
7. Gavish M, Awad M and Fares F, Existence of sites for anions and divalent cations in the solubilized γ-aminobutyric acid/benzodiazepine receptor complex. *J Neurochem* **45**: 760–765, 1985.
8. Clark JA, Houghten R and Pasternak GW, Opiate binding in calf thalamic membranes: A selective μ_1 binding assay. *Mol Pharmacol* **34**: 308–317, 1988.
9. VonVoightlander PF, Lahti RA and Ludens JH, U-50,488: A selective and structurally novel non-*mu* (kappa) opioid agonist. *J Pharmacol Exp Ther* **224**: 7–12, 1983.
10. Cheng Y-C and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
11. Chou T-C, Relationships between inhibition constants and fractional inhibition in enzyme-catalyzed reactions with different numbers of reactants, different reaction mechanisms, and different types and mechanisms of inhibition. *Mol Pharmacol* **10**: 235–247, 1974.
12. Djelardje S, *Intoxications Volontaires par la Chloroquine*. M.D. dissertation, Medical University of Abidjan, Ivory Coast, 1976.
13. Bondurand A, N'Dri K, Coffi S and Saracino E, L'Intoxication a la chloroquine au Centre Hopitalo-Universitaire d'Abidjan. *Afr Med* **179**: 239–242, 1980.
14. Champagne D, *Intoxication Volontaires a la Chloroquine*. M.D. dissertation, Medical University of Paris, France, 1973.
15. Tallman JF, Thomas JW and Gallager DW, GABAergic modulation of benzodiazepine binding site sensitivity. *Nature* **274**: 383–385, 1978.
16. Weissman BA, Bolger GT, Isaac L, Paul SM and Skolnick P, Characterization of the binding of [^{3}H]Ro 5-4864, a convulsant benzodiazepine, to guinea pig brain. *J Neurochem* **42**: 969–975, 1984.
17. Pasternak GW, Multiple morphine and enkephalin receptors and the relief of pain. *J Am Med Assoc* **259**: 1362–1367, 1988.
18. Snead OC III, Opiate-induced seizures: A study of μ- and δ-specific mechanisms. *Exp Neurol* **93**: 348–358, 1986.

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Protection by boswellic acids against galactosamine/endotoxin-induced hepatitis in mice

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In the preceding communication of this series we reported that crude ethanolic extracts of the gum resin exudate from *Boswellia serrata* [1] inhibited by leukotriene B_4 (LTB_4) production from endogenous arachidonic acid by rat peritoneal polymorphonuclear leukocytes *in vitro*, most likely by decreasing the activity of the 5-lipoxygenase (5-LO). We identified boswellic acids as the active principle (manuscript in preparation). However, it is not clear whether such an effect can also be observed in an *in vivo* animal model after oral administration of the drug.

Recent reports showed that administration of endotoxin to rats led to an increase of leukotriene secretion into the bile [2] and that leukotriene synthesis and action inhibitors exerted protective effects against galactosamine/endotoxin-induced lethality [3] and hepatitis [4] in mice *in vivo*. The liver damage biochemically is evidenced by increases in serum sorbitoldehydrogenase (SDH), aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) activities [4].

In the present study, we investigated the *in vivo* effect of the acetyl-boswellic acids (ac-BA) after oral application on the galactosamine/salmonella endotoxin-induced liver damage in mice. The data indicate that pretreatment by ac-BA 1 hr before the intoxication substantially reduced increased levels of serum enzyme activities.

Materials and Methods

D-Galactosamine HCl (GalN) was purchased from Serva (Heidelberg, F.R.G.), endotoxin (*Salmonella abortus equi* lipolysaccharide) from Sebak (Berlin, F.R.G.) and sodium-heparin from Braun (Melsungen, F.R.G.).

The acetyl-boswellic acids (ac-BA) tested in this study were isolated from the gum resin of *Boswellia serrata* according to Winterstein and Stein [5] and characterized by ^{1}H-NMR, MS, i.r. and UV-spectra (manuscript in preparation). The ac-BA mixture applied in the present study was composed of acetyl-β-BA (95%), acetyl-α-BA (2%) and acetyl-11-keto-β-BA (3%).

Male albino mice (han-NMRI) were purchased from the Zentralinstitut für Versuchstiere (Hannover, F.R.G.) and kept on standard diet (Altromin, Lage, F.R.G.) with free access to food and water under 12 hr dark/light rhythm.