Archival Report

Biological Psychiatry

Novel Small-Molecule Inhibitors of Protein Kinase C Epsilon Reduce Ethanol Consumption in Mice

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ABSTRACT

BACKGROUND: Despite the high cost and widespread prevalence of alcohol use disorders, treatment options are limited, underscoring the need for new, effective medications. Previous results using protein kinase C epsilon (PKCε) knockout mice, RNA interference against PKCε, and peptide inhibitors of PKCε predict that small-molecule inhibitors of PKCε should reduce alcohol consumption in humans.

METHODS: We designed a new class of PKCε inhibitors based on the Rho-associated protein kinase (ROCK) inhibitor Y-27632. In vitro kinase and binding assays were used to identify the most potent compounds. Their effects on ethanol-stimulated synaptic transmission; ethanol, sucrose, and quinine consumption; ethanol-induced loss of righting; and ethanol clearance were studied in mice.

RESULTS: We identified two compounds that inhibited PKCε with *K*i <20 nM, showed selectivity for PKCε over other kinases, crossed the blood-brain barrier, achieved effective concentrations in mouse brain, prevented ethanol-stimulated gamma-aminobutyric acid release in the central amygdala, and reduced ethanol consumption when administered intraperitoneally at 40 mg/kg in wild-type but not in *Prkce*$^{-/-}$ mice. One compound also reduced sucrose and saccharin consumption, while the other was selective for ethanol. Both transiently impaired locomotion through an off-target effect that did not interfere with their ability to reduce ethanol intake. One compound prolonged recovery from ethanol-induced loss of righting but this was also due to an off-target effect since it was present in *Prkce*$^{-/-}$ mice. Neither altered ethanol clearance.

CONCLUSIONS: These results identify lead compounds for development of PKCε inhibitors that reduce alcohol consumption.

Keywords: Addiction, Alcohol, Kinase inhibitor, Protein kinase C epsilon

https://doi.org/10.1016/j.biopsych.2017.10.017

Gene targeting, RNA interference, and pharmacological studies support the conclusion that protein kinase C epsilon (PKCε) is a target for development of drugs to treat alcohol use disorder. Compared with wild-type mice, *Prkce*$^{-/-}$ mice consume 50% to 75% less ethanol and show markedly reduced ethanol preference (1), reduced operant ethanol self-administration (2), and increased conditioned place aversion for ethanol (3). Knockdown of PKCε in the amygdala by RNA interference (4) or administration of a peptide that inhibits PKCε translocation in the amygdala or nucleus accumbens (5) reduces ethanol consumption in mice, indicating that these two regions are important sites of PKCε action on ethanol consumption. Consistent with this conclusion, *Prkce*$^{-/-}$ mice show markedly reduced ethanol-stimulated dopamine release in the nucleus accumbens (2) and ethanol-stimulated gamma-aminobutyric acid (GABA) release in the central amygdala (CeA) (6). Furthermore, *Prkce* transcripts are among the most highly expressed in the brains of inbred and selected lines of mice that drink large amounts of ethanol (7). A role for PKCε in human alcohol dependence was recently suggested by a study of lymphoblastoid cell lines, which found that *PRKCE* messenger RNA transcripts were increased by 1.4-fold in 21 alcohol use disorder cases compared with 21 control cases (8).

Recently we generated mutant mice that carry an adenosine triphosphate (ATP) analog-specific gatekeeper mutation in the purine-binding site of PKCε (*AS*-PKCε mice) and found that administration of the selective *AS*-kinase inhibitor 1-naphthyl PP1 reduces their ethanol intake (9). These results predict that small-molecule inhibitors of PKCε should decrease ethanol consumption. Here we report the discovery of compounds that potently inhibit native PKCε. Two prevented ethanol-stimulated GABA release in the mouse CeA and reduced ethanol self-administration in mice. Our results identify lead small-molecule PKCε inhibitors for developing a new class of therapeutics to reduce consumption of alcohol.

ISSN: 0006-3223

METHODS AND MATERIALS

Reagents

Compounds 1.0 and 1.3 were synthesized by Adesis Inc. (New Castle, DE) or by the Center for Innovative Drug Discovery (San Antonio, TX). Compounds were dissolved at 10 mg/mL in 100% dimethyl sulfoxide for kinase assays and for the oral pharmacokinetic study, or in 10% Tween 80 (Sigma-Aldrich, St. Louis, MO) for intraperitoneal (i.p.) administration. Y-27632 (*trans*-4-[(1*R*)-1-Aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide), DNQX (6,7-dinitroquinoxaline-2,3-dione), AP-5 (DL-2-amino-5-phosphonovalerate), and CGP 55845A were from Tocris Bioscience (Bristol, United Kingdom). Ethanol (190 proof) was from Decon Labs (King of Prussia, PA) or Remet Alcohols Inc. (La Mirada, CA). Other chemicals were from Sigma-Aldrich.

Kinase Assay

Human recombinant PKCs (Invitrogen, Carlsbad, CA) were assayed in triplicate (see Supplemental Methods) using Lance Ultra TR-FRET technology (PerkinElmer, Waltham, MA). Compound 1.0 (200 nM) was also screened against a panel of 395 nonmutant kinases by the KINOMEscan Profiling Service from DiscoverX (Fremont, CA).

Animals

Inbred mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Male C57BL/6J wild-type mice, and C57BL/6J × 129S4 wild-type and *Prkce*$^{-/-}$ mice of both sexes, 2 to 6 months of age, were housed under a 12-hour reversed light/dark cycle (lights off 11 AM to 11 PM) with ad libitum access to food (Rodent Diet 5LL2; LabDiet, St. Louis, MO) and water. All procedures were conducted in accordance with the University of Texas at Austin and the Scripps Research Institute Institutional Animal Care and Use Committee policies, and the National Institutes of Health guidelines for the care and use of animals in research.

Pharmacokinetics and Plasma Protein Binding

Procedures used to determine pharmacokinetic parameters and measure protein binding for compounds 1.0 and 1.3 can be found in Supplemental Methods.

Electrophysiology

Whole-cell voltage-clamp recordings of spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were performed in the medial CeA of male C57BL/6J mice (n = 7; 12 to 14 weeks of age), using DNQX (20 μM), AP-5 (30 μM), CGP 55845A (1 μM), and tetrodotoxin (0.5 μM) (6). Results were analyzed using Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) and visually confirmed. Average mIPSC characteristics were determined over a 3-minute period and all drug effects were normalized to their own neuron's predrug baseline.

Ethanol-Induced Loss of the Righting Reflex and Ethanol Clearance

Compounds were first dissolved in 10% Tween 80. Mice received an i.p. injection of vehicle or inhibitor 2 or 6 hours before i.p. administration of ethanol. Studies using C57BL/6J mice used a dose of 3.6 g/kg ethanol and a between-subjects design. Studies with male wild-type and *Prkce*$^{-/-}$ hybrid mice used a counterbalanced, crossover, within-subjects design, in which we administered equally effective doses of ethanol (1) to wild-type (3.6 g/kg) and *Prkce*$^{-/-}$ (3.2 g/kg) littermates. The duration of the loss of the righting reflex (LORR) was measured as described (10). For ethanol clearance, mice were pretreated with vehicle or inhibitor, and 2 hours later were administered 4 g/kg ethanol. Blood samples (20 μL) were collected from the tail vein at indicated times for measurement of blood ethanol concentration (11).

Ethanol Consumption

We followed a published procedure for intermittent 24-hour access, two-bottle choice drinking (12). Compounds were dissolved in 10% Tween 80 in water and administered at 10 mL/kg. After 3 to 4 weeks, when mice were stably drinking 20% (w/v) ethanol (<10% change between consecutive drinking days), they were habituated to daily vehicle (10% Tween 80) injections given three times with 1 day between each injection, before they were administered compounds (0, 10, 20, and 40 mg/kg, i.p.) using a within-subjects design, 2 or 6 hours before presentation of drinking bottles. Ethanol consumption was measured during the subsequent 24 hours. One week later, when drinking had returned to baseline, blood ethanol concentrations were measured in tail blood taken 4 hours after the beginning of a drinking session (11). Continuous access, two-bottle choice drinking was performed as described previously (1). Mice of both sexes were provided ascending concentrations of ethanol at 2%, 4%, and 8% (w/v) over 12 days and then 12% ethanol for the rest of the experiment. After drinking of 12% ethanol was stable, mice were administered vehicle or 40 mg/kg test compound i.p. in a counterbalanced, crossover design with 4 days between injections. Ethanol consumption was measured for 24 hours after each injection.

Sucrose, Saccharin, and Quinine Consumption

Drug and ethanol naïve mice were singly housed and provided one bottle containing 4% (w/v) sucrose and the other tap water for 5 consecutive days. The bottles were weighed daily and positions alternated to account for side preferences. Over the next 6 days, mice were acclimated to three i.p. injections of vehicle (10% Tween 80). Two days later, mice received vehicle or 40mg/kg of compound 1.0 i.p. using a counterbalanced, crossover design with 2 days between injections. Three days later, mice were administered vehicle or 40 mg/kg of compound 1.3 using the same counterbalanced, crossover protocol. Two days later, mice were presented with 0.015-mM quinine or tap water, and the concentration of quinine was increased daily to 0.03 mM and then 0.05 mM. After 3 more days of access to 0.05-mM quinine, mice were administered vehicle or compounds using a counterbalanced, crossover protocol. A second cohort of mice was provided 0.03% (w/v) saccharin or water by the same protocol used for sucrose consumption. After 3 days of acclimation to i.p. injections of vehicle, these mice were administered vehicle or compounds (40 mg/kg) using the same counterbalanced, crossover design.