Stigmasterol, a Soy Lipid-Derived Phytosterol, Is an Antagonist of the Bile Acid Nuclear Receptor FXR

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ABSTRACT: Phytosterols, components of soy-derived lipids, are among the proposed exacerbants of parenteral nutrition–associated cholestasis (PNAC). We investigated whether phytosterols contribute to bile acid (BA)–induced hepatocyte damage by antagonizing a nuclear receptor (NR) critically involved in hepatoprotection from cholestasis, FXR (farnesoid X receptor, NR1H4). In HepG2 cells, stigmasterol acetate (StigAc), a water-soluble Stig derivative, suppressed ligand-activated expression of FXR target genes involved in adaptation to cholestasis (*i.e.* *BSEP*, *FGF-19*, *OSTα/β*). Furthermore, StigAc antagonized BA-activated, FXR target genes *SHP* and *BSEP* in FXR+/+, but not in FXR–/– mouse hepatocytes. Both Stig and StigAc inhibited BA-activated, FXR-dependent reporter gene expression in transfected HepG2 cells, whereas the most prevalent phytosterol in lipids, β -sitosterol, had no inhibitory effect. Finally, among six ligand-activated NR-ligand binding domains (LBDs) tested, antagonism by StigAc was specific to only two (FXR and PXR, pregnane X receptor, NR1I2). We demonstrate that Stig, a phytosterol prevalent in soy-derived PN lipid solutions, is a potent *in vitro* antagonist of the NR for bile acids FXR. (*Pediatr Res* 62: 301–306, 2007)

Since its introduction, PN has revolutionized the care of neonates. Elevated transaminases and bilirubin levels occur often in infants on PN, but with short-term administration, resolution of liver damage is typical. Alternatively, infants necessitating long-term PN are at severe risk of life-threatening complications. These include cirrhosis, liver failure, sepsis, coagulopathy, and death. Those most susceptible to these morbidities are premature infants, those with short bowel syndrome, and those with repeated bouts of sepsis (1). The incidence of PNAC may be as high as 50% in infants who have received PN for as little as 2 m (1). The risk of developing end-stage liver disease is currently estimated at 20%–87% (1–3).

Most PN lipid emulsions are derived from soy and contain numerous phytochemicals, including sterols (phytosterols), structurally similar to both cholesterol and BAs (Fig. 1) (4,5).

In humans, most (>95%) dietary phytosterols are excreted in the feces due to the enterocyte apical ABCG5/G8 transporter, which acts as a gatekeeper to their systemic entry, (6) but is bypassed with i.v. administration and subsequently excreted into bile *via* ABCG5/G8 located on the hepatocyte canalicular membrane (5,6).

There are three principal phytosterols in soy lipid: β -sitosterol, campesterol, and Stig. Several lines of evidence support a potential role for phytosterols as contributors to the development of PNAC: (1) children with PNAC have markedly elevated levels of phytosterols such as Stig in their serum, ranging above 1 mM (normal = 36 μ M); (2) a reduction in PN lipid administration results in a marked reduction in both serum phytosterol and bilirubin levels; (3) i.v. administration of phytosterols in neonatal piglets leads to an increase in serum BAs and reduced BA-dependent bile flow; and (4) newborn piglets provided fish oil–derived lipids i.v. maintain normal bile flow and transaminase levels, whereas piglets given soybean-derived lipids develop cholestasis and impaired bile flow rates (7–9).

Multiple members of the NR superfamily play central roles in the adaptive response to cholestasis, but FXR, NR1H4 has emerged the central BA “sensor” that plays a critical role in maintaining safe intrahepatic BA levels (10). BA-activated FXR mediates hepatoprotection from excess BAs by coordinating a transcriptional reprogramming of hepatocytes: (1) reducing sinusoidal BA import *via* suppression of Na⁺ taurocholate cotransporter polypeptide (NTCP), SLC10A1; (2) reducing BA synthesis *via* repression of CYP7A1; and (3) increasing intrahepatic BA efflux across canalicular and sinusoidal membranes *via* up-regulation of bile salt export pump (BSEP) ABCB11 and organic solute transporter (OST) α/β genes, respectively (10–15). Gene knockout studies demonstrate that FXR–/– mice lack these hepatoprotective mechanisms and are ultrasensitive to BA-induced injury, and treat-

Abbreviations: BA, bile acid; BSEP, bile salt export pump; CDCA, chenodeoxycholic acid; FGF, fibroblast growth factor; FXR, farnesoid X receptor; LBD, ligand-binding domain; LXR, liver X receptor; NR, nuclear receptor; NTCP, sodium taurocholate cotransporting polypeptide; OST, organic solute transporter; PN (AC), parenteral nutrition (–associated cholestasis); PPAR γ , peroxisome proliferator-activated receptor gamma; PXR, pregnane X receptor; RXR, retinoid X receptor; SHP, small heterodimer partner; Stig (Ac), stigmasterol (acetate); VDR, vitamin D receptor

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Figure 1. Structures of selected sterols. The nuclear ring structures of two phytosterol compounds (β -sitosterol and StigAc) are illustrated. StigAc is a commercially available Stig derivative with an acetate group at position three of the Stig backbone. The structural similarity of these phytosterols to the primary bile acid, chenodeoxycholic acid (CDCA) is also illustrated.

ment of rats with FXR agonists protects against cholestasis (16,17).

Although multiple hypotheses have been proposed to explain the pathogenesis of PNAC, a multifactorial model appears to be at play (18). Current thinking supports that prematurity and immature hepatic function, lack of enteral feedings, bacterial translocation (leading to recurrent sepsis), enrichment of the BA pool with toxic BAs, and the prolonged use of lipid emulsions are contributors (1–3). We report that Stig, a principal phytosterol present in soy-derived lipid infusates, is a potent antagonist of the critical NR BA sensor FXR and provides a novel molecular connection between nutritional components and regulation of essential liver gene expression.

MATERIALS AND METHODS

Tissue culture. HepG2 cells were obtained from American Type Tissue Culture (Manassas, VA). Cells for transfections were grown as monolayers to 75% confluence at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in 10 mL of medium that consisted of modified Eagle's medium (MEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine. Transfection treatment medium was identically prepared with the exception that 10% FBS was replaced by 10% charcoal-stripped medium. Cells were subcultured on a routine basis per standard protocol.

Primary mouse hepatocytes were isolated *via* a standardized perfusion protocol and plated in complete William's E medium in six-well dishes at a density of 250,000/mL (19). For quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), mouse hepatocytes were seeded for 3 h in complete William's E medium supplemented with penicillin/streptomycin, glutamine/gentamicin, insulin-transferrin-sodium selenite, glucagon, 10% FBS, and amphotericin B, followed by 24-h treatments in 0.25% charcoal-stripped William's E medium. For qRT-PCR, HepG2 cells were seeded at 500,000/mL for 24 h in complete Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with penicillin/streptomycin, L-glutamine, and 10% FBS followed by 24-h treatments in 0.25% charcoal-stripped DMEM.

Treatments. Phytosterols and derivatives were obtained from Steraloids, Inc. (Newport, RI). StigAc (Fig. 1) has superior solubility and stability compared with its parent compound, allowing for an alternative, more standardized phytosterol treatment protocol and hence its use in the majority of our experiments. Phytosterol treatment medium for transfections was prepared by mixing a 20-mM stock solution of the phytosterol in 10% charcoal-stripped MEM medium. This mixture was vortexed and sonicated (Ultrasonic,

Inc.) for 48 h, then syringe-filtered (0.4 μ m) into aliquots, and refiltered into a sterile 0.2- μ m vacuum flask producing a transparent solution of phytosterol in medium. The precise micromolar quantity of phytosterol in each treatment solution was then determined by gas chromatography mass spectrometry (GCMS) (courtesy of Dr. William E. O'Brien, Department of Genetics, Baylor College of Medicine) (20). StigAc treatment medium for qRT-PCR experiments was similarly prepared in 0.25% charcoal-stripped William's E (primary mouse hepatocytes) or DMEM (HepG2 cells) with subsequent validation of StigAc concentrations in the medium *via* GCMS (20). HepG2 cell viability, as measured by EtBr exclusion, after 24 h of incubation, was unaffected by Stig: vehicle (75%), 10 μ M Stig (75%), CDCA 50 μ M (88%), or CDCA + Stig (92%).

NR ligands were obtained from Sigma Chemical Co., except for the LG100268, which was generously provided by Ligand Pharmaceuticals, Inc. (San Diego, CA). In most cases, these ligands were solubilized in dimethyl sulfoxide before cell treatments (4-pregnen-3 β -ol-20-one carbonitrile, 22-R-hydroxycholesterol, rosiglitazone, LG100268, vitamin D). In the case of CDCA, the sodium salt of this ligand was solubilized in sterile water.

Transfections. HepG2 cells were seeded at a density of 250,000 cells/well in 12-well dishes and transfected with Eugene (Roche) transfection reagent; 0.75 μ g of the indicated luciferase reporter plasmids were cotransfected with 0.1 g hFXR, 0.1 μ g retinoic X receptor (RXR), hRXR α , and 0.05 μ g of pRSVrenilla to monitor for transfection efficiency. For transfections testing phytosterol effect on full-length FXR (Figs. 2 and 3A), HepG2 cells were cotransfected with a luciferase reporter plasmid (pCRELuc) containing five copies of an RXR:FXR binding site and expression vectors for hFXR and hRXR α , along with a pRSVrenilla internal control. Cells were then treated with 0–100 μ M CDCA and 0–10 μ M of various phytosterols. After 24 h, cells were harvested and processed on a luminometer (Luminoscan Ascent), and results normalized to RSVrenilla activity to control for transfection efficiency. Transfections were performed at least six times in triplicate. HepG2 cells were cotransfected with a Gal4 luciferase reporter containing five copies of a Gal4 binding site, an expression plasmid vector for a Gal4-DNA-binding domain NR, ligand-binding domain (LBD) fusion protein, an RXR α expression vector, and a pRSVrenilla internal control (21). Cells were treated with agonist or agonist plus 10 μ M StigAc and 24 h later harvested and processed, and luciferase activities normalized as before. The agonist ligands used were FXR, CDCA (100 μ M); pregnane X receptor (PXR), 4-pregnen-3 β -ol-20-one-16 α -carbonitrile (PCN, 10 μ M); vitamin D receptor (VDR), 1 α , 25-dihydroxyvitamin D₃ (100 nM); liver X receptor (LXR) α , 22-(R)-hydroxycholesterol (10 μ M); RXR α , LG100268 (1 μ M); peroxisome proliferator-activated receptor γ (PPAR γ), rosiglitazone (1 μ M).

qRT-PCR. Primary mouse hepatocytes were cultured as noted above and treated for 24 h with vehicle alone (0.25% charcoal-stripped William's E medium) or 100 μ M CDCA with or without 10 μ M StigAc, and HepG2 cells

Figure 2. Stig and StigAc suppress FXR-mediated gene expression *in vitro*; β -sitosterol does not. A luciferase reporter/FXR response plasmid (pCRELuc) was transfected into HepG2 cells along with FXR and RXR α expression plasmids and then treated with 100 μ M CDCA and 10 μ M of phytosterols. Cells were harvested 24 h later, and results normalized to cotransfected pRSVrenilla. Results in the presence of CDCA are presented as the percentage of activation relative to normalized luciferase expression in the presence of agonist (100%). * p < 0.05 vs CDCA alone. $\square$, CDCA + vehicle; $\blacksquare$, CDCA + phytosterol.

Figure 3. StigAc is a potent *in vitro* antagonist of full-length FXR and FXR-LBD. (A) Inhibition of full-length FXR by StigAc. A luciferase reporter plasmid containing five copies of a full-length RXR:FXR binding site (pECRELuc) was transfected into HepG2 cells along with FXR and RXR expression plasmids, followed by treatment with 100 μ M CDCA and StigAc (0–10 μ M). (B) Inhibition of Gal4-FXR-LBD by StigAc. A luciferase reporter plasmid (GSTKLuc) was transfected into HepG2 cells along with an FXR-LBD construct plasmid and RXR α . * p < 0.05 vs CDCA alone. ■, StigAc; □, β -sitosterol.

were plated for 24 h and then treated for 24 h with vehicle alone (0.25% charcoal-stripped DMEM medium) or 50–100 μ M CDCA with or without 10 μ M StigAc. Total RNA was isolated using the RNeasy kit (Qiagen), cDNA was synthesized from 7.5 or 10 μ g of total RNA using the StrataScript First-Strand synthesis system (Stratagene, La Jolla, CA) and used to determine relative small heterodimer partner (SHP), BSEP, fibroblast growth factor (FGF), FGF-19, OST α , and OST β mRNA expression using an ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems, Inc., Foster City, CA) as reported previously (34). Quantitative expression values were extrapolated from standard curves and normalized to a cyclophilin or 18S standard.

RESULTS

Initial transfections were designed to determine which, if any, of the phytosterols present in soy-derived lipid infusates affected FXR activity by the potent BA ligand CDCA. Stig and StigAc suppress BA-activated, FXR-mediated reporter gene expression (Fig. 2). β -Sitosterol, the most prevalent phytosterol in soy-derived lipids, has no significant inhibitory effect on CDCA-activated, FXR-mediated reporter gene expression, whereas campesterol has minimal and insignificant inhibition (data not shown). Phytosterol treatments alone had no significant effect on FXR reporter gene activation (data not shown).

Because Stig emerged as the most potent FXR antagonist among the three main phytosterols, we next tested the *in vitro* dose-response effect of StigAc on the function of ligand-activated full-length FXR (Fig. 3A). Increasing concentrations of StigAc (0–10 μ M) suppress CDCA-activated FXR with an IC₅₀ of 5–10 μ M, which is well below the physiologic range of Stig levels found in the serum of patients with serological evidence of PNAC (9). β -Sitosterol, once again, does not significantly antagonize CDCA-activated, full-length FXR reporter gene expression. Transfections in CV-1 cells similarly demonstrate that StigAc antagonizes BA-activated full-length FXR in this FXR-null cell line (data not shown).

Whether StigAc-mediated antagonism required the FXR-LBD was addressed with Gal4-FXR-LBD transfections in HepG2 cells (Fig. 3B). StigAc antagonizes CDCA activation of a Gal4-FXR-LBD in a dose-dependent fashion similar to that seen with full-length FXR (65% at 10 μ M) (compare Fig. 3A with 3B). In contrast, β -sitosterol does not inhibit CDCA-activated Gal4-FXR-LBD. Such results suggest that the inhibition of CDCA-activated FXR by StigAc involves competition at the FXR-LBD. Again, this suppression of

CDCA-activated FXR-LBD by StigAc is recapitulated in transfected CV-1 (FXR-null) cells, and StigAc had no significant effect on reporter gene expression (data not shown).

Given the structure of phytosterols and the multiple NRs involved in BA homeostasis, it was important to determine whether StigAc inhibited ligand activation of other liver-enriched NRs (Fig. 4). The specificity of StigAc effect was tested using GAL4-LBD constructs of FXR and five other members of the NR superfamily involved in BA metabolism and inflammation: PXR, LXR α , PPAR γ , retinoid X receptor α (RXR α), and VDR. In addition to suppressing CDCA-activated FXR-LBD activity, StigAc also suppresses ligand-activated PXR-LBD activity (Fig. 4). Except for a slight increase in RXR α LBD activity, StigAc has no discernible effect on any other NR LBD tested.

Because StigAc significantly inhibited expression of FXR-regulated plasmids transfected into HepG2 and CV-1 cells, we sought to determine whether it affected CDCA activation of native FXR-responsive genes (*BSEP*, *FGF-19*, and *OST α / β*) in HepG2 cells (Fig. 5A–D). Each of these genes contains an FXR response element in its promoter region that is up-regulated upon treatment with CDCA, and their gene products

Figure 4. Specificity of StigAc effect on various NR LBDs. HepG2 cells were transfected with the GAL4 luciferase reporter, an RXR expression plasmid, and a series of chimeras in which the GAL4 DNA binding domain is fused to the indicated NR LBD. Cells were treated with the appropriate agonist ligand (□) or agonist plus StigAc (10 μ M, ■). Results in the presence of StigAc are represented as the percent of activation relative to the normalized luciferase expression in the presence of agonist (100%). The ligands used were FXR, CDCA (100 μ M); PXR, 4-pregnen-3 β -ol-20-one-16 α -carbonitrile (PCN, 10 μ M); VDR, 1 α ,25-dihydroxyvitamin D₃ (100 nM); LXR α , 22-(R)-hydroxycholesterol (10 μ M); RXR α , LG100268 (1 μ M); PPAR γ , rosiglitazone (1 μ M). * p < 0.05 vs ligand alone.

Figure 5. StigAc antagonizes CDCA activation of FXR target genes *BSEP*, *FGF-19*, *OSTα*, and *OSTβ* in HepG2 cells. Human hepatoblastoma (HepG2) cells were plated and then treated for 24 h. Cells were then harvested, RNA purified, and qRT-PCR performed to determine FXR target gene [*BSEP* (A), *FGF-19* (B), *OSTα* (C), *OSTβ* (D)] expression relative to an 18s control. * $p < 0.05$ vs CDCA alone.

are involved in the hepatoprotective response to BA overload (21–23). Neither vehicle nor 10 μ M StigAc alone affects *BSEP* RNA expression (Fig. 5A). As expected, treatment with CDCA, up-regulates *BSEP* RNA expression 275-fold compared with vehicle. Exposure to only 10 μ M of StigAc markedly suppresses CDCA-mediated activation of *BSEP* RNA (from 275 to 100 $\times$, 65%), nearly identical to the results noted in transfection experiments (Figs. 2 and 3).

Similarly, *FGF-19* RNA levels are unchanged from basal levels when treated with StigAc (Fig. 5B) and increase 25-fold in the presence of CDCA. StigAc potently suppresses BA-activated *FGF-19* RNA expression by 60%. StigAc also antagonizes two additional CDCA-activated, FXR-mediated, BA homeostatic genes in HepG2 cells: *OSTα* and *OSTβ* (25%–30% suppression, Fig. 5C and D). Taken together, these qRT-PCR experiments indicate that StigAc markedly impairs CDCA activation of native FXR-responsive genes in a human-derived liver cell line, HepG2.

Finally, the effect of StigAc on the FXR target genes *SHP* and *BSEP* was studied in hepatocytes derived from FXR $^{+/+}$ and FXR $^{-/-}$ mice. As expected, CDCA activates native expression of mouse *SHP* and *BSEP* RNA in FXR $^{+/+}$, but not FXR $^{-/-}$ hepatocytes, albeit at levels less than those seen in HepG2 cells (Fig. 6). StigAc has no effect on CDCA-activated *SHP* RNA expression in hepatocytes derived from FXR $^{-/-}$ mice; however, 10 μ M StigAc significantly suppresses CDCA-activated *SHP* expression in FXR $^{+/+}$ hepatocytes. StigAc antagonizes CDCA-activated *BSEP* expression in FXR $^{+/+}$, but not FXR $^{-/-}$ hepatocytes (Fig. 6B),

although this suppression does not reach statistical significance, likely due to interanimal variability in the expression of this gene. Taken together, Figures 5 and 6 and reveal that, in addition to its effect on FXR-regulated gene expression in HepG2 cells, StigAc also inhibits native CDCA-activated FXR target gene expression in cultured mouse hepatocytes.

DISCUSSION

Those most at risk of developing PNAC are premature infants and those with short bowel syndrome, but the actual causes, or mechanisms underlying susceptibility, are unknown (1–3). Because cholestasis usually resolves once infants are weaned off PN, a contribution from a cholestatic component in the PN solution has been proposed, with previous literature supporting that this agent may reside within lipids (8,9,15,24). Herein, we demonstrate that among the phytosterols, which are components of soy-derived lipid infusates, Stig is an antagonist of FXR, an NR responsible for maintaining intracellular BA homeostasis.

Phytosterols accumulate to extremely high serum levels in a rare disease due to mutations in either one of the recently identified ABC (ATP-binding cassette) transporter genes *ABCG5* and *ABCG8*, which have increased intestinal absorption and impaired biliary secretion of phytosterols (25–27). The clinical phenotype includes extensive xanthomas, accelerated atherosclerosis, and premature death due to sterol deposition in coronary arteries (28). Although liver disease is not a hallmark of sitosterolemia, a recent report describes a patient

Figure 6. StigAc antagonizes CDCA activation of native FXR-responsive genes in hepatocytes derived from FXR $^{+/+}$ mice, but not in FXR $^{-/-}$ mice. Primary mouse were treated for 24 h as noted and then harvested, RNA was isolated, and qRT-PCR was performed to determine FXR target gene [*SHP* (A), *BSEP* (B)] mRNA expression compared with a cyclophilin control. * $p < 0.01$ vs CDCA alone. □, FXR $^{+/+}$; ■, FXR $^{-/-}$.

with cirrhosis requiring liver transplantation who was subsequently found to be a compound heterozygote *ABCG8* gene mutation (29). This patient also had serologic evidence of autoimmune hepatitis, suggesting that although sitosterolemia alone may not cause significant liver injury, a second insult to the liver in addition to hereditary phytosterolemia may trigger significant hepatocyte damage and cirrhosis. The apparent pathologic contradiction between the liver damage associated with PN-associated phytosterolemia and the relatively benign liver effects in isolated hereditary sitosterolemia can be reconciled at several levels, mainly due to the differential clinical settings and route of exposure to phytosterols. First, PNAC is a disease of neonates, especially very small neonates, who have an obligate loading of soy lipids *i.v.*, with the potential that the handling of phytosterols may be altered in these infants. Second, genes encoding BA transporter (*i.e.* *BSEP*) and BA conjugating/detoxification enzymes undergo postnatal development expression and extrapolating to premature infants suggest an inherently increased susceptibility to BA loads that resolves with age (30,31). In the rat model, *BSEP* expression is low in fetal life and reaches adult levels by 4 wk postnatally (30). The developmental expression of these transporters in humans has been studied to a lesser degree, but recent data suggest that there is a developmental increase in transporter RNA (*i.e.* *BSEP*, *NTCP*, and multidrug resistance-related protein 2) (32). Third, unlike sitosterolemia, PNAC is a multifactorial disease that is exacerbated by ongoing systemic inflammation and infection, and the response of the liver in this setting is unknown. Previous investigators, including us, have established that lipopolysaccharides and cytokines alter the expression of crucial BA transport genes (*e.g.* *NTCP* and *BSEP*) in animal models and thereby directly contribute to cholestasis (33–35). It is quite possible that elevated levels of phytosterols “tip the balance,” but this remains to be proven.

In human liver-derived HepG2 cells, StigAc antagonizes several BA-activated FXR target genes involved in restoring BA homeostasis: *SHP*, *BSEP*, *Ostα/β*, and FGF-19. Mutations in *BSEP* lead to intracellular BA accumulation and a serious hereditary cholestatic condition, progressive familial intrahepatic cholestasis type 2 (PFIC 2), whereas mutations in the other genes have yet to be described (36). The role of FGF-19 as a signaling molecule in cholestasis is interesting and evolving, but has been recently shown to be expressed in HepG2 cells (22,37,39). There are likely to be species-dependent and variable roles played by FGF-15 (in mice) and FGF-19 (in humans) in cholestasis that have yet to be determined (22,29,37–40).

In addition to FXR, our data reveal that StigAc inhibits the *in vitro* activity of ligand-activated PXR. Previous investigations in both wild-type and knockout animals have elucidated the integral role that PXR plays in “detoxifying” the liver of excess BAs (35). It is intriguing to speculate that StigAc may target genes regulated by PXR, which, in combination with its effects on FXR, would serve to doubly impair the ability of the liver to respond to BA loads.

In conclusion, this study provides evidence that Stig, a component of soy-derived lipids, has antagonist activity against a central NR involved in the adaptive response to

BA-mediated hepatotoxicity, FXR. These data suggest that Stig’s antagonism of FXR target gene expression significantly compromises hepatoprotectant mechanisms that normally act to attenuate cholestasis (*e.g.* activation of *BSEP*, FGF-19, *Ostα/β*, and *SHP*). These *in vitro* data demonstrating Stig’s role as an antagonist of the critical BA “sensor” FXR provides a basis for further exploration of the role of phytosterols in PNAC in appropriate animal models. It is only after the validation of these findings into *in vivo* models that we will be able to confirm what has been postulated for at least a decade: that a component of PN lipid infusates is a potential molecular exacerbant in the multifactorial pathogenesis of PNAC.

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