Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells

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Salinomycin, an antibiotic potassium ionophore, has been reported recently to act as a selective breast cancer cell stem inhibitor, but the biochemical basis for its anticancer effects is not clear. The Wnt/β-catenin signal transduction pathway plays a central role in stem cell development, and its aberrant activation can cause cancer. In this study, we identified salinomycin as a potent inhibitor of the Wnt signaling cascade. In Wnt-transfected HEK293 cells, salinomycin blocked the phosphorylation of the Wnt coreceptor lipoprotein receptor related protein 6 (LRP6) and induced its degradation. Nigericin, another potassium ionophore with activity against cancer stem cells, exerted similar effects. In otherwise unmanipulated chronic lymphocytic leukemia cells with constitutive Wnt activation, nanomolar concentrations of salinomycin down-regulated the expression of Wnt target genes such as LEF1, cyclin D1, and fibronectin, depressed LRP6 levels, and limited cell survival. Normal human peripheral blood lymphocytes resisted salinomycin toxicity. These results indicate that the Wnt signaling induced by salinomycin and related drugs inhibit proximal Wnt signaling by interfering with LRP6 phosphorylation, and thus impair the survival of cells that depend on Wnt signaling at the plasma membrane.

Results

Salinomycin Suppresses Wnt1-Induced Signaling Pathway. An initial screen of known drugs, performed by using the TOPFlash cell reporter system (16, 17), identified the ionophores salinomycin, nigericin, thapsigargin, and ionomycin as inhibitors of the Wnt signaling pathway. To further characterize the Wnt inhibitory effect of salinomycin, HEK293 cells were transfected with the SuperTOPflash reporter and a Wnt1 expression plasmid. Expression of Wnt1 increased the transcriptional activity of the SuperTOPflash reporter more than 400 fold versus basal levels (Fig. 1A). Wnt1 overexpression also caused β-catenin accumulation in HEK293 cells (Fig. 1D). Salinomycin strongly suppressed Wnt1-stimulated reporter activity with an IC50 of 163 nM (Fig. 1A, L4), and reduced β-catenin levels (Fig. 1D). In control experiments, salinomycin did not inhibit the luciferase activity of an API-Luc reporter, and actually enhanced the activity of an NFAT-Luc reporter (Fig. 1B and C).

Effects of Salinomycin on Different Components of Wnt Signaling Cascade. To determine the mechanism by which salinomycin inhibits Wnt/β-catenin signaling, the SuperTOPflash reporter was activated proximally by Wnt1 or Fzd5/LRP6, or more distally by β-catenin, in transient transfection assays. Although nanomolar concentrations of salinomycin inhibited Wnt1-induced signaling, the drug had very little effect on reporter gene activity in cells that overexpressed Fzd5/LRP6 or β-catenin (Fig. 2 B and C). Much higher concentrations (4 μM) of salinomycin were needed to block the Wnt signaling induced by the downstream activators (Fig. 2D). Thus, salinomycin can suppress Wnt signaling by at least two different mechanisms, depending on drug dosage.

Salinomycin Blocks Wnt1-Induced LRP6 Phosphorylation. To test whether salinomycin directly targets the Wnt or LRP6 molecules, Wnt1, Wnt3 or dickkopf-related protein 2 (DKK2) were used to activate Wnt signaling. Although DKK2 can act as an agonist or antagonist of Wnt signaling, it has been shown to activate Wnt signaling when LRP6 is overexpressed (18). Wnt1, Wnt3, and DKK2 each enhanced the activity of the SuperTOPflash reporter, and these effects were antagonized by 250 nM salinomycin (Fig. 2A).

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Salinomycin selectively targets the Wnt/Fzd/LRP complex. (A) Salinomycin selectively inhibited Wnt signaling mediated by Wnt1/Fzd5/LRP6 or Wnt3/Fzd5/LRP6 or DKK2/Fzd5/LRP6. The SuperTOPflash reporter assays were performed on HEK293 cells transfected with the indicated expression vectors. The cells were treated with either vehicle (DMSO) or salinomycin (250 nM and 500 nM) for a further 24 h. The cells were harvested and luciferase activity was measured as described in Materials and Methods. (B) The relative inhibition rate (as percentage) was used to compare the inhibitory effects of salinomycin on Wnt signaling induced by Fzd5/LRP6 or Wnt1/Fzd5/LRP6 or Wnt3/Fzd5/LRP6 or DKK2/Fzd5/LRP6. (C) HEK293 cells were transfected with empty vector and Wnt1 expression vector for 24 h. Then cells were treated with the indicated amounts of salinomycin or vehicle control for 16 h. The phospho-LRP6 (Ser1490) and total LRP6 were detected by Western blot analysis.

Salinomycin can potentially disrupt Na⁺/Ca²⁺ exchange and cause an increase in cytoplasmic calcium levels (16). However, the calcium ionophores thapsigargin (Fig. 4) and ionomycin (Fig. S1) strongly inhibited Wnt pathway activation by Fzd5/LRP6 and, to a lesser degree, β-catenin, at concentrations at which salinomycin was ineffective.

Salinomycin specifically inhibits the Wnt/β-catenin signaling pathway initiated by Wnt1. (A) HEK293 cells were transfected with a SuperTOPflash reporter and empty vector or Wnt1 expression plasmid. (B) An AP1-luciferase reporter was transfected with a vector expressing constitutively active Ha-Ras (RasV12) into HEK293 cells. (C) HEK293 cells were transfected with an NFAT-luciferase reporter along with NFATc expression plasmid. The transfected cells were treated with salinomycin at increasing concentrations (from 4 nM to 1,250 nM) for 24 h. The luciferase activities were measured and values were normalized to β-gal activity. Results are expressed as fold induction compared with empty vector control. (D) HEK293 cells were transfected with empty vector or Wnt1 expression plasmid. Then cells were treated with the indicated amounts of salinomycin for 16 h. The β-catenin and β-actin levels were detected by Western blot analysis.

In contrast, salinomycin had much less effect on Fzd5/LRP6-mediated reporter gene activity in cells overexpressing Fzd5/LRP6 (Fig. 3 A and B).

Previous studies have demonstrated that Wnt pathway activation requires LRP6 phosphorylation by casein kinase-1γ, glycogen synthase kinase-3β, and various other kinases (8, 19). Indeed, nanomolar concentrations of salinomycin blocked Wnt1-induced LRP6 phosphorylation at serine 1490 in HEK293 cells, as assessed by immunoblotting with a specific antibody (Fig. 3C). Furthermore, salinomycin treatment caused degradation of the LRP6 protein in a dose-dependent fashion (Fig. 3C).

Nigericin, Like Salinomycin, Inhibits Wnt Signaling Cascade. Nigericin is a potassium ionophore with structural similarity to salinomycin, which is also toxic to breast cancer stem cells (15). Nigericin, like salinomycin, selectively inhibited Wnt1-mediated signaling in HEK293 cells at nanomolar concentrations, but had little effect on reporter activity induced by β-catenin overexpression (Fig. 4). These results point to a common mechanism underlying the Wnt inhibitory action of the two potassium ionophores.

Effect of Calcium Ionophores. Salinomycin can potentially disrupt Na⁺/Ca²⁺ exchange and cause an increase in cytoplasmic calcium levels (20, 21). It has been established that the canonical Wnt/LRP6-mediated signaling cascade is antagonized by elevating intracellular calcium levels (16). However, the calcium ionophores thapsigargin (Fig. 4) and ionomycin (Fig. S1) strongly inhibited Wnt pathway activation by Fzd5/LRP6 and, to a lesser degree, β-catenin, at concentrations at which salinomycin was ineffective.

Sensitivity of CLL Cells to Salinomycin. Primary CLL cells produce Wnt proteins, have constitutive Wnt activation, and express high levels of the Wnt-regulated transcription factor Lymphocyte Enhancing Factor 1 (LEF1) (22). However, CLL cells do not have known activating mutations or deletions of the β-catenin transcription complex. Hence, CLL cell survival might be sensitive to compounds that inhibit proximal Wnt signaling by interference with LRP6 phosphorylation and stability. To test this hypothesis, primary CLL cells from 13 patients and peripheral blood mononuclear cells (PBMCs) from seven healthy donors were incubated
with increasing concentrations of salinomycin for 48 h. Induction of apoptosis was detected by cyttofluorometry. Incubation of the malignant lymphocytes with salinomycin induced apoptosis within 48 h, with a mean IC_{50} of 230 nM. Under the same conditions, salinomycin failed to induce apoptosis in PBMC at 100-fold higher concentrations (Fig. 5). This result suggests that salinomycin has selective cytotoxicity to CLL cells that are known to depend on Wnt signaling.

**Salinomycin Reduces LRP6 Protein Levels and Down-Regulates Expression of Wnt Target Genes in CLL Cells.** LRP6 expression has been detected in primary CLL cells, albeit at low levels (22, 23). As salinomycin could block Wnt-induced LRP6 phosphorylation and cause the degradation of LRP6 protein in HEK293 cells (Fig. 3C), we also assayed its effect on the LRP6 protein in CLL cells. As predicted, the drug reduced LRP6 levels in the malignant lymphocytes (Fig. 6A).

**LF1, cyclin D1, and fibronectin** are target genes of the Wnt/β-catenin pathway, which are up-regulated in CLL cells compared with normal lymphocytes (22, 24). The CLL cells from four patients were treated with salinomycin for 16 h, and then were analyzed by quantitative PCR for gene expression. At this time point, the CLL cells were still viable, but all three Wnt-dependent transcripts had decreased (Fig. 6B).

**Discussions**

Salinomycin and nigericin are among the first drugs reported to act as selective cancer stem cell inhibitors (15). These polyether ionophores interfere with transmembrane potassium potential and promote mitochondrial and cellular potassium efflux (25, 26). Our experiments demonstrate that salinomycin treatment of cells antagonizes the Wnt signaling cascade. At submicromolar concentrations, the drug inhibits Wnt1-induced LRP6 phosphorylation, and causes the degradation of the LRP6 protein, an essential component of the Wnt receptor complex. At micromolar concentrations, salinomycin also promotes β-catenin degradation.

Considering the importance of Wnt/β-catenin signaling in stem cell biology and in various malignancies, the Wnt antagonistic actions of salinomycin may contribute to its toxicity toward breast cancer progenitor cells.

The phosphorylation of LRP6 is crucial for activation of Wnt/β-catenin signaling. Several protein kinases, including glycogen synthase kinase-3 and casein kinase-1γ, have been shown to phosphorylate this Wnt coreceptor (27). A recent study showed that transmembrane protein 198 can promote LRP6 phosphorylation and Wnt signaling activation by recruitment of casein kinase family proteins (28). Another report showed that salinomycin is able to overcome multidrug resistance mediated by the ATP-binding cassette transporter p-glycoprotein 170 (29). Cruciat et al. demonstrated that LRP6 phosphorylation and internalization requires the activity of the vacuolar H^{+}-adenosine triphosphatase (V-ATPase) (30). The prorenin receptor functions as a specific adaptor between LRP6 and V-ATPase. The V-ATPase protein complex localizes in intracellular organelles and at the plasma membrane, where it pumps protons and acidifies vesicles, thereby promoting endocytosis (31). Inhibition of V-ATPase activity by bafilomycin blocks Wnt/β-catenin signaling (30). Nigericin has been reported to induce intracellular alkalinization (32, 33). It is therefore likely that salinomycin and nigericin may interfere with the proton gradient generated by the V-ATPase, and thus block the phosphorylation and endocytosis of LRP6 that are required for initial Wnt signal transduction.

Previous studies have revealed that salinomycin can also cause an increase in intracellular calcium levels by disrupting Na^{+}/Ca^{2+} exchange (21). The toxicity of salinomycin has been attributed to drug-induced accumulation of intracellular calcium (20). As an increase in intracellular calcium causes inhibition of the canonical Wnt pathway (16), we tested the effect of other intracellular calcium regulators on LRP6-mediated Wnt signaling. Unlike salinomycin, both thapsigargin and ionomycin blocked Wnt signaling downstream of LRP6. Thus, at least at submicromolar concentrations, salinomycin-induced inhibition of Wnt signaling probably does not depend on its calcium mobilizing activity.

The LRP5 and LRP6 coreceptors for Wnt signaling have been revealed as potential oncogenic proteins. Abrupt expression of an LRP5 splicing isoform has been implicated in breast tumor formation (34), and LRP6 is overexpressed in a subset of human breast carcinomas (35). Overexpression of LRP6 in the mouse mammary gland induces mammary hyperplasia, whereas down-regulation of LRP6 inhibits breast cancer tumorigenesis (35, 36). Furthermore, an LRP antagonist, Mesd (mesoderm development), markedly suppresses breast cancer tumor growth in vivo without significant toxicity (35, 36). These studies strongly suggest that LRP6 is a potential therapeutic target in breast cancer. The...
were measured by real-time PCR as described in cyclin D1 primary LEF1 Lu et al. HEK293 cells were maintained in DMEM supplemented with 10% FBS and 100 μg/mL penicillin and 100 μg/mL streptomycin. HEK293 cells were transfected in 12-well plates by using FuGENE (Roche), and 0.5 μg of reporter plasmid, 50 to 100 ng of the control plasmid pCMXgal, and 100 to 200 ng of the various expression plasmids, and carrier DNA pcDNA3 plasmid, for a total of 1 μg per well. After overnight incubation, the cells were treated with the different concentrations of drugs or vehicle. For luciferase assays, cells were lysed in isoeionic potassium phosphate buffer, pH 7.8, containing 1% Triton X-100, and luciferase activities were assayed in the presence of substrate by using a multiple-mode detection reader (Infinite M2000, Tecan). The luciferase values were normalized to the activity of the β-gal, and are expressed as percentage of control luciferase or fold stimulation of luciferase activity compared with the basal level. All of the transfection results are representative of a minimum of three independent transfections.

Fig. 6. Salinomycin reduced the LRP6 protein level and down-regulated the expression of LEF1, cyclin D1, and fibronectin in CLL cells. (A) Primary CLL cells from two patients were treated with indicated amounts of salinomycin for 16 h. The phospho-LRP6 (Ser1490), total LRP6, and β-actin were detected by Western blot. (B) Primary CLL cells from four patients were treated with increasing concentrations of salinomycin for 16 h. The mRNA levels of LEF1, cyclin D1, and fibronectin were measured by real-time PCR as described in Materials and Methods.

Cell Culture and Transfection. HEK293 cells and primary CLL cells were treated with the indicated amounts of salinomycin as described in the figure legends. Cells were transfected twice with the different concentrations of drugs or vehicle. For luciferase assays, cells were lysed in isoeionic potassium phosphate buffer, pH 7.8, containing 1% Triton X-100, and luciferase activities were assayed in the presence of substrate by using a multiple-mode detection reader (Infinite M2000, Tecan). The luciferase values were normalized to the activity of the β-gal, and are expressed as percentage of control luciferase or fold stimulation of luciferase activity compared with the basal level. All of the transfection results are representative of a minimum of three independent transfections.

Western Blot Analysis. HEK293 cells and primary CLL cells were treated with the indicated amounts of salinomycin as described in the figure legends. Cells were treated twice with the different concentrations of drugs or vehicle. For luciferase assays, cells were lysed in isoeionic potassium phosphate buffer, pH 7.8, containing 1% Triton X-100, and luciferase activities were assayed in the presence of substrate by using a multiple-mode detection reader (Infinite M2000, Tecan). The luciferase values were normalized to the activity of the β-gal, and are expressed as percentage of control luciferase or fold stimulation of luciferase activity compared with the basal level. All of the transfection results are representative of a minimum of three independent transfections.

RNA Isolation and Real-Time PCR. Total RNA was isolated from 1 × 10^6 primary CLL cells by TRIzol reagent (Invitrogen). The RNA samples were further purified by using a RNeasy Protect kit (Qiagen). The RNA samples were quantified in triplicate by real-time PCR on a 7900HT Fast Real-Time PCR System (Applied Biosystems) by using the following primer sets to detect the expression of LEF1, cyclin D1, fibronectin, LEF1 sense, 5′-AGGCATCATCCACATTAGCAG-3′; LEF1 antisense, 5′-AGGTCTTGTGGTCCTCTGCT-3′; cyclin D1 sense, 5′-AAGTGCCAAGCAGTCTG-3′; and cyclin D1 antisense, 5′-TCAGTCTGCCCTGAC-3′; fibronectin sense, 5′-ACCTACGAGGTACTGCTGTTCT-3′; and fibronectin antisense, 5′-TTCAAGACATCTGGCACCCTCA-3′. PCR was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. The PCR conditions were 2 min at 50 °C (one cycle), 10 min at 95 °C (one cycle), and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The data were analyzed by using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The ΔCt values from each tissue were obtained by subtracting the values for 18S Ct from the at earlier time points. Interestingly, we observed that 250 nM salinomycin was a high enough dose to block LRP6 phosphorylation induced by overexpressing Wnt1 in HEK293 cells. Considering multiple kinases responsible for LRP6 phosphorylation (27), our results suggest that salinomycin may specifically suppress Wnt-induced LRP6 phosphorylation. Furthermore, we showed that salinomycin down-regulates the expression of Wnt target genes and exerts selective toxicity to primary CLL cells compared with normal PBMCs. Thus, drugs that inhibit LRP6 phosphorylation or stability may be therapeutically active in CLL.

Materials and Methods

Human Samples. Samples were collected by the CLL Research Consortium after informed consent was obtained from patients fulfilling diagnostic criteria for CLL. Institutional review board approval was obtained from the University of California, San Diego, for the procurement of patient samples in this study, in accordance with the Declaration of Helsinki. Buffy coat samples from healthy volunteers were obtained from the San Diego Blood Bank.

Reagents and Plasmids. Salinomycin, nigericin, thapsigargin, and ionomycin were purchased from Sigma-Aldrich. A Gen-plus collection of 960 known drugs was obtained from Microsource. The reporter plasmid TOPflash and the β-catenin expression plasmid were gifts from H. Clevers (University of Utrecht, Utrecht, The Netherlands). The SuperTOPflash reporter construct was from Karl Willert (University of California, San Diego, San Diego, CA). The NFAT-Luc and AP1-Luc reporters were purchased from BD Biosciences. The TOPflash plasmid for human DKK2 was from Origene Technologies. The expression plasmids encoding Wnt1, Wnt3, LRP6, NFATc, H-rasV12, and β-gal have been described previously (22, 42, 43).

Western Blot Analysis. HEK293 cells and primary CLL cells were treated with the indicated amounts of salinomycin as described in the figure legends. Cells were treated twice with the different concentrations of drugs or vehicle. For luciferase assays, cells were lysed in isoeionic potassium phosphate buffer, pH 7.8, containing 1% Triton X-100, and luciferase activities were assayed in the presence of substrate by using a multiple-mode detection reader (Infinite M2000, Tecan). The luciferase values were normalized to the activity of the β-gal, and are expressed as percentage of control luciferase or fold stimulation of luciferase activity compared with the basal level. All of the transfection results are representative of a minimum of three independent transfections.

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sample Ct. One difference of Ct value represents a twofold difference in the level of mRNA.

**Cell Apoptosis Assays.** Apoptosis was determined by the analysis of mitochondrial transmembrane potential (ΔΨm) using 3,3′-dihexyloxocarbocyanine iodine (DiOC6(3)) and by cell membrane permeability to propidium iodide (PI). Primary CLL cells were cultured at a density of 5 × 10^6 cells/mL in RPMI with 10% FBS containing salinomycin at concentrations ranging from 0 to 10 μM. After treatment for 48 h, 100 μL of the cell culture was collected and transferred to FACS tubes containing 100 μL FACS buffer with 60 nM DiOC6 and 10 μg/mL PI. Cells were incubated at 37 °C for 30 min and analyzed with the FACScan flow cytometer (Becton Dickinson). Fluorescence was recorded at 525 nm (FL-1) for DiOC6 and at 600 nm (FL-3) for PI. The apoptotic cells were determined by calculating the percentages of the DiOC6/PI− CLL populations.

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