Aglycone Polyether Nanchangmycin and Its Homologues Exhibit Apoptotic and Antiproliferative Activities against Cancer Stem Cells

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S Supporting Information

ABSTRACT: The potential of the polyether salinomycin as an inhibitory agent against cancer stem cells has attracted interest in this family of compounds. In this study, we found that the aglycone polyether nanchangmycin and its homologues show promising activities against breast cancer stem cells as well as 38 other different types of cancer cells by *in vitro* assays. We found that aglycone polyethers caused elevations in calcium levels, an accumulation of reactive oxygen species and mitochondrial inner membrane permeability to H^+ and K^+ , resulting in the release of cytochrome *c* and apoptosis-inducing factor and the triggering of caspase-dependent apoptosis. Our analyses also indicate that aglycone polyethers are potent Wnt/ β -catenin signaling inhibitors, blocking the Wnt pathway and resulting in reduced cell survival. Notably, the key autophagy-related proteins LC3A/B were also activated by aglycone polyether treatment. Furthermore, nanchangmycin showed inhibitory effects toward somatic tumors developed from MCF-7 paclitaxel-resistant breast cancer cells injected into BALB/c mice. Our study not only provides promising candidates for therapy against cancer stem cells but also provides the groundwork for identifying stronger therapeutic agents among the natural polyether compounds.

KEYWORDS: aglycone polyether, cancer stem cell, mitochondrial dysfunction, Wnt, autophagy

INTRODUCTION

Current cancer treatments, including chemotherapy and radiation therapies, succeed at eliminating masses of cancer cells and inhibiting rapid proliferation but often miss a subset of tumor cells that sustain tumorigenesis and cellular heterogeneity with a tumor-regenerating capacity.¹ The cancer stem cell (CSC) model has been proposed to account for these properties, such as the capability for extensive proliferation and self-renewal.² CSCs are tumor-initiating cells and are responsible for chemo-resistance and distant metastases.^{3–5}

Thus, CSCs may be more effective molecular targets for development of therapeutic strategies.

Salinomycin, a naturally occurring polyether ionophore antibiotic widely used in veterinary medicine, was identified as a selective CSC inhibitor in a high-throughput screen to discover compounds that would prevent epithelial-mesenchymal transition (EMT), a developmental process that mimics behavior found in breast CSCs (BCSCs); salinomycin was over

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Figure 1. Chemical structures of the four alcycone polyether antibiotics.

Figure 2. Comparison of polyether and paclitaxel treatment on BCSCs. The polyethers salinomycin, J1-001-1, J1-001-2, J1-001-3, and J1-001-4 were tested. (A) ALDH activity of adherent cells and mammosphere cells. ALDH activity was measured using the ALDEFLUOR kit with the ALDH substrate BODIPY-amino acetaldehyde. The ALDH inhibitor DEAB (diethylaminobenzaldehyde) was used to control for background fluorescence. +DEAB, negative control; –DEAB, positive control. (B) Ratio of residual MDA-MB-231 mammosphere cells as measured by ALDH activity. Flow cytometry was conducted after exposure of the cells to the same concentration (5 μ M) of reagents (paclitaxel, salinomycin, J1-001-1, J1-001-2, J1-001-3 or J1-001-4) for 72 h, $n = 3$. (C) Population of residual BCSCs after treatment with one of the five polyethers or paclitaxel for 72 h, $n = 3$, $P < 0.001$ compared with control. (D) Microscopic examination of the morphology of MDA-MB-231 mammosphere cells after drug treatment for 72 h.

a 100-fold more effective than paclitaxel at reducing the proportion of CSCs.⁶ Mechanistic studies by the Carson group revealed that salinomycin strongly inhibited phosphorylation of the Wnt coreceptor lipoprotein receptor-related protein 6 (LRP6) in Wnt signaling and downregulated the expression of

several Wnt target genes in chronic lymphocytic leukemia cells.⁷ Further studies on the effects of salinomycin on cancer proliferation, apoptosis, and metastasis have demonstrated that this drug also interferes with the Hedgehog signaling pathway,⁸ activates p38 MAPK⁹ and AMPK-dependent autophagy,¹⁰

Table 1. Screening of the Antiproliferative Activity of Aglycone Polyethers on Different Cancer Cell Lines^a

cell lines	IC ₅₀ (M) ^a				cell lines	IC ₅₀ (M) ^a			
	J1-001-1	J1-001-2	J1-001-3	J1-001-4		J1-001-1	J1-001-2	J1-001-3	J1-001-4
T98G (brain cancer)	2.82 × 10 ⁻⁷	6.56 × 10 ⁻⁸	2.86 × 10 ⁻⁷	5.17 × 10 ⁻⁷	A-673 (rhabdomyosarcoma)	1.72 × 10 ⁻⁷	3.18 × 10 ⁻⁸	1.41 × 10 ⁻⁷	2.68 × 10 ⁻⁷
MeWo (skin cancer)	1.70 × 10 ⁻⁶	4.47 × 10 ⁻⁷	1.20 × 10 ⁻⁶	3.30 × 10 ⁻⁶	DU 145 (prostate cancer)	9.38 × 10 ⁻⁷	2.67 × 10 ⁻⁷	1.38 × 10 ⁻⁶	1.93 × 10 ⁻⁶
A-498 (kidney cancer)	7.11 × 10 ⁻⁷	1.73 × 10 ⁻⁷	5.54 × 10 ⁻⁷	7.48 × 10 ⁻⁷	SK-UT-1 (uterine sarcoma)	3.59 × 10 ⁻⁷	3.74 × 10 ⁻⁸	4.31 × 10 ⁻⁷	2.67 × 10 ⁻⁷
SK-MEL-2 (skin cancer)	1.67 × 10 ⁻⁷	5.45 × 10 ⁻⁸	1.45 × 10 ⁻⁷	3.1 × 10 ⁻⁷	MES-SA/Dx-5 (uterine sarcoma)	1.38 × 10 ⁻⁷	1.96 × 10 ⁻⁸	9.15 × 10 ⁻⁸	2.58 × 10 ⁻⁷
SK-HEP-1 (liver cancer)	3.66 × 10 ⁻⁷	1.16 × 10 ⁻⁷	4.55 × 10 ⁻⁷	6.69 × 10 ⁻⁷	A-204 (rhabdomyosarcoma)	6.58 × 10 ⁻⁸	3.94 × 10 ⁻⁹	4.65 × 10 ⁻⁸	2.97 × 10 ⁻⁸
MG-63 (bone cancer)	6.21 × 10 ⁻⁸	8.50 × 10 ⁻⁹	1.12 × 10 ⁻⁷	1.39 × 10 ⁻⁷	G-361 (Melanoma)	2.74 × 10 ⁻⁷	1.42 × 10 ⁻⁸	3.19 × 10 ⁻⁷	5.48 × 10 ⁻⁷
ACHN (kidney cancer)	1.73 × 10 ⁻⁶	5.17 × 10 ⁻⁷	1.45 × 10 ⁻⁶	2.20 × 10 ⁻⁶	HCT 116 (colon cancer)	2.90 × 10 ⁻⁷	7.58 × 10 ⁻⁸	2.67 × 10 ⁻⁷	5.31 × 10 ⁻⁷
SW 982 (synovial sarcoma)	7.86 × 10 ⁻⁷	4.36 × 10 ⁻⁷	7.46 × 10 ⁻⁷	4.96 × 10 ⁻⁷	SJSA-1 (bone cancer)	2.45 × 10 ⁻⁷	7.64 × 10 ⁻⁸	3.88 × 10 ⁻⁷	2.80 × 10 ⁻⁷
SK-OV-3 (ovary cancer)	1.39 × 10 ⁻⁶	3.9 × 10 ⁻⁷	9.09 × 10 ⁻⁷	1.50 × 10 ⁻⁶	COLO 205 (colon cancer)	2.90 × 10 ⁻⁷	8.21 × 10 ⁻⁸	1.95 × 10 ⁻⁷	1.29 × 10 ⁻⁷
PC-3 (prostate cancer)	6.68 × 10 ⁻⁷	1.24 × 10 ⁻⁷	4.28 × 10 ⁻⁷	7.83 × 10 ⁻⁷	MKN-45 (gastric cancer)	4.85 × 10 ⁻⁷	1.38 × 10 ⁻⁷	3.75 × 10 ⁻⁷	4.58 × 10 ⁻⁷
A549 (lung cancer)	3.21 × 10 ⁻⁷	8.49 × 10 ⁻⁸	2.88 × 10 ⁻⁷	5.57 × 10 ⁻⁷	DOHH2 (lymphoma)	2.61 × 10 ⁻⁹	<4.40 × 10 ⁻¹⁰	2.09 × 10 ⁻⁹	3.1 × 10 ⁻⁹
OVCAR-4 (ovary cancer)	2.20 × 10 ⁻⁶	1.85 × 10 ⁻⁶	3.16 × 10 ⁻⁶	4.25 × 10 ⁻⁶	K562 (leukemia)	2.81 × 10 ⁻⁸	1.28 × 10 ⁻⁸	3.99 × 10 ⁻⁸	1.06 × 10 ⁻⁷
Calu-3 (lung cancer)	3.48 × 10 ⁻⁷	1.13 × 10 ⁻⁷	3.62 × 10 ⁻⁷	3.59 × 10 ⁻⁷	PAC (pancreas cancer)	6.81 × 10 ⁻⁷	9.33 × 10 ⁻⁸	3.99 × 10 ⁻⁷	3.06 × 10 ⁻⁷
U-87 MG (brain cancer)	2.25 × 10 ⁻⁷	8.63 × 10 ⁻⁹	2.25 × 10 ⁻⁷	8.33 × 10 ⁻⁸	NUGC-4 (gastric cancer)	1.49 × 10 ⁻⁷	1.11 × 10 ⁻⁸	7.71 × 10 ⁻⁸	9.68 × 10 ⁻⁸
Hep 3B2.1-7[Hep3B] (liver cancer)	6.49 × 10 ⁻⁸	8.68 × 10 ⁻⁹	3.32 × 10 ⁻⁸	6.17 × 10 ⁻⁸	Hep G2 (Liver Cancer)	2.42 × 10 ⁻⁷	5.51 × 10 ⁻⁸	2.77 × 10 ⁻⁷	2.21 × 10 ⁻⁷
A375 (melanoma)	5.36 × 10 ⁻⁷	6.16 × 10 ⁻⁸	2.21 × 10 ⁻⁷	4.90 × 10 ⁻⁷	MIA PaCa-2 (pancreas cancer)	3.10 × 10 ⁻⁸	3.45 × 10 ⁻⁹	2.58 × 10 ⁻⁸	2.45 × 10 ⁻⁸
MD (spleen macrophage)	8.45 × 10 ⁻⁹	1.47 × 10 ⁻⁹	6.83 × 10 ⁻⁹	1.89 × 10 ⁻⁸	Jurkat (lymphoma)	2.88 × 10 ⁻⁷	7.81 × 10 ⁻⁸	1.96 × 10 ⁻⁷	3.88 × 10 ⁻⁷
AHH-1 (lymphoid)	1.44 × 10 ⁻⁶	2.83 × 10 ⁻⁷	1.37 × 10 ⁻⁷	2.37 × 10 ⁻⁶	*MDA-MB-231 (breast cancer)	2.80 × 10 ⁻⁷	2.63 × 10 ⁻⁷	7.10 × 10 ⁻⁷	1.41 × 10 ⁻⁶
MV-411 (leukemia)	1.56 × 10 ⁻⁸	7.21 × 10 ⁻¹⁰	6.8 × 10 ⁻⁹	4.79 × 10 ⁻⁹	*MCF-7 (breast cancer)	3.85 × 10 ⁻⁷	1.65 × 10 ⁻⁸	2.86 × 10 ⁻⁷	3.71 × 10 ⁻⁷

^aIC₅₀ values represent the half-maximal inhibitory concentration compared to control and were determined after exposure of the cells to different drug concentrations for 72 h. Experiments were conducted using the CellTiter-Glo reagent except for those marked by “*”, which were performed using the MTT cytotoxicity assay. All compounds were evaluated in at least three independent experiments. The percentage inhibition was calculated using the following equation: Cell growth inhibition % = $[1 - \text{RLU}_{\text{Compound}} / (\text{RLU}_{0.3\% \text{ DMSO}})] \times 100\%$. Experiments were performed by Genescript Biological Technology Co., Ltd. Bars denote the standard error with $n = 3$ for each treatment combination. Quality control criteria: doxorubicin on BEL-7402 cells. For more details, see the [Supporting Information](#).

Figure 3. Effects of aglycone polyethers on calcium and mitochondrial function. (A and B) Proteome analysis of the (A) calcium signaling pathway and (B) mitochondrial signaling pathway after J1-001-2 treatment of MDA-MB-231 cells. (See the [Supporting Information](#) for experimental details.) The dashed red line means no changes (ratio = 1) between treated cells and untreated cells. (A) CAPN2, calpain-2 catalytic subunit, calcium-regulated nonlysosomal thiol-protease; CAPNS1, calpain small subunit 1, regulatory subunit of the calcium-regulated nonlysosomal thiol-protease; VDAC1–3, voltage-dependent anion-selective channel protein 1–3; ATP2B1, plasma membrane calcium-transporting ATPase 1; SLC25A4–6, ADP/ATP translocase 1–3; EGFR, epidermal growth factor receptor; PPIF, peptidyl-prolyl cis–trans isomerase F, mitochondrial. (B) AIFM1 (apoptosis-inducing factor 1, mitochondrial), CYCS (cytochrome c), UQCRB (Cytochrome *b*-c1 complex subunit 7), CYC1

Figure 3. continued

(Cytochrome c1, heme protein, mitochondrial), UQCRC1–2 (Cytochrome *b*-c1 complex subunit 1–2, mitochondrial). (C and D) Effects on calcium and ROS. Flow cytometry analyses of (C) intracellular calcium levels and (D) intracellular ROS levels. MDA-MB-231 cells were seeded in 6-well plates at a density of 1×10^5 cells/well and incubated for 24 h before treatment with J1-001-1 and J1-001-2 at the indicated concentrations. MDA-MB-231 cells were stained with (C) Fluo-3 M after 16 h or (D) DCFH-DA after 24 h. (E–H) Effects on H^+ and K^+ . Permeabilization of the mitochondrial inner membrane to H^+ and K^+ following exposure to J1-001-1 (E and G) and J1-001-2 (F and H). Effects of J1-001-1 (I) and J1-001-2 (J) on mitochondrial ROS levels. ROS levels in liver mitochondrial were measured by flow cytometry using the probe DCFH-DA ($\lambda_{ex} = 495$ nm, $\lambda_{em} = 520$ nm). Isolated mitochondrial fractions were incubated in buffer G (containing 100 mM sucrose, 10 mM Tris-HCl, 50 mM KCl, 10 mM MOPS, 5 mM K_2HPO_4 , 2 μ M rotenone, pH ~ 7.4) with DCFH-DA (20 μ M) at 37 °C for 20 min in the dark. Then, the pellets were incubated with (I) J1-001-1 or (J) J1-001-2 at the indicated concentrations for 30 min, $n = 3$. The fluorescence in the FL-1 channel was detected for at least 100 000 events with BD AccuriTM C6 (BD, USA) and analyzed with BD AccuriTM C6 System software.

reduces Akt and NF- κ B proteins,^{11,12} and increases expression of P-glycoprotein (P-gp)¹³ and ABC transporter proteins,¹⁴ in addition to other activities.¹⁵ Another important feature of salinomycin is its ability to overcome multidrug resistance when administered in combination treatments.¹⁶

In addition to salinomycin, several other polyether antibiotics have also shown promising and unexpected antitumor activity, including inostamycin,¹⁷ ionomycin,¹⁸ and monensin,¹⁹ which have been used as ruminant growth promoters as well as coccidiostatic agents. However, these compounds are only a small fraction of the naturally occurring polyether antibiotics. So far, more than 120 compounds of this family have been discovered,²⁰ but most of them have not been tested for antitumor activity. In the present study, we identified a new series of aglycone polyethers that shows promising toxicity for BCSCs as well as for 38 different types of tumor cells.

■ RESULTS

Identification of Polyether Candidates More Effective than Salinomycin. The therapeutic potential of several nonaglycone polyethers, such as salinomycin, inostamycin, ionomycin, lasalocid acid, and monensin, has been previously investigated.²¹ However, there exists a class of promising and characteristic aglycone polyethers with similar polyketide backbones, but with different aglycone groups, that has not been tested against tumors.²⁰ To date, 52 aglycone polyethers have been discovered,²² which are classified into five types according to their chemical characteristics (Figure S1), and, of their associated aglycone groups, 4-*O*-methyl- β -*D*-amycetose is the most predominant and is found in 34 of the 52 known natural aglycone polyethers. We chose salinomycin as the representative of nonaglycone polyethers, and marduramycin and nanchangmycin as representatives of types I and II aglycone polyethers, respectively, for evaluating their anticancer effects (Figure S2A). The results revealed that the anticancer activity of nanchangmycin ($IC_{50} = 0.28$ μ M) was greater than that of salinomycin ($IC_{50} = 2.85$ μ M) and marduramycin ($IC_{50} = 4.54$ μ M), indicating that type II aglycone polyethers may be a promising library for drug development.

In previous studies, we reported the method for preparation of nanchangmycin and deaglycone-nanchangmycin.^{23–25} Here, we compared the anticancer activity and IC_{50} values of deaglycone-nanchangmycin, salinomycin, and nanchangmycin (Figure S2B). Results of an MTT cytotoxicity assay using several cancer cell lines indicated that nanchangmycin is a promising anticancer candidate, with greater effectiveness than that of salinomycin, and that the aglycone group in nanchangmycin plays an important role in the anticancer activity of this polyether. Through a literature search, we

identified four aglycone polyethers that shared the same backbone with nanchangmycin, and for simplicity, we named this compound family J1-001, with the individual compounds named as follows: nanchangmycin as J1-001-1; A-130-A as J1-001-2; endusamycin as J1-001-3; and CP-80,219 as J1-001-4 (Figure 1). All four compounds were isolated from their respective *Streptomyces* hosts following fermentation, and their anticancer activities were evaluated.

Aglycone Polyethers Decreased Cancer Stem Cell Populations from Breast Cancer Cells. The development, validation, and therapeutic use of novel compounds and drugs that effectively eradicate CSCs is an important research goal, but currently there are no reagents better than salinomycin with the potential capacity for eliminating CSCs.²⁶ To establish an *in vitro* CSC model for investigating the behavior of BCSCs, we used three growth factors (B27 at 2 mL/100 mL, hEGF at 20 μ L/100 mL, and insulin at 40 μ L/100 mL) to promote tumor stem cell growth and differentiation for the formation of mammospheres.^{27,28} Aldehyde dehydrogenase (ALDH) has been identified as an effective biomarker for BCSCs,²⁹ so we selected ALDH as our target marker. MDA-MB-231 breast cancer cells were seeded into 6-well low-attachment plates followed by incubation for 10–12 days as a suspension culture. The mammosphere formation assays showed a 6.3-fold increase (from 3.9 to 25.1%) in ALDH-positive cells in suspension cultures versus adherent cultures (Figure 2A).

Subsequently, this method was used to investigate the effects of the four aglycone polyethers on the proportion of BCSCs, using salinomycin and paclitaxel as controls. Compared to the untreated cells, salinomycin treatment decreased the proportion of BCSCs by 2-fold. In contrast, paclitaxel treatment increased the proportion of BCSCs by 2-fold (up to 48.2%) (Figure 2B) compared with control samples. The effect of J1-001-4 on BCSCs was similar to that of salinomycin. Interestingly, the inhibitory effect on BCSCs was significantly lower for the other three aglycone polyethers (6.1% for J1-001-1, 2.3% for J1-001-2, and 3.3% for J1-001-3) were significantly lower than that observed for the salinomycin group (12.6%) (Figure 2B,C), indicating that these three aglycone polyethers had a greater inhibitory effect. In Figure 2D, the effects of the J1-001 series aglycone polyethers on CSC inhibition are clearly demonstrated by microscopy.

Antitumor Activity Screening of Aglycone Polyethers in Cancer Cell Lines. Salinomycin has exhibited promising biological activity against different tumors.¹⁵ To investigate the antitumor activity of the aglycone polyethers, a panel of cancer cell lines, representing 38 typical cancers, was selected (Table 1). Overall, the aglycone polyethers showed cytotoxic activity against the majority of the cancer cell lines, in micromolar to

Figure 4. Aglycone polyether J1-001-2 inhibits MDA-MB-231 cell proliferation through the Wnt signaling pathway and by promoting cell autophagy. (A and B) Proteome analysis of (A) the Wnt signaling pathway and (B) autophagy and apoptosis after J1-001-2 treatment of MDA-MB-231 cells. The dashed red line means no changes (ratio = 1) between treated cells and untreated cells. (See the [Supporting Information](#) for experimental details.) (C–F) Western blot analysis of MDA-MB-231 cells treated with J1-001-2. (C and D) Cells were exposed to J1-001-2 for 16 h, and blots were probed for (C) Wnt signaling pathway proteins [Wnt, P-LRP6 (S1490), β-catenin, and P-catenin (S552)] or for (D) Wnt-targeted proteins (P-GSK3β, cyclin D1, CD44, c-myc, survivin). GAPDH and β-actin were used as reference proteins. (E) Cells were exposed to J1-001-2 for 3–48 h, and blots were probed for LC3-A/B, P-catenin(S552), caspase 3 and GAPDH. (F) Cells were exposed to different concentrations of J1-001-2 for 16 h, and blots were probed for LC3-A/B, caspase 3 and β-actin.

nanomolar concentrations (Table 1). For example, J1-001-2 showed dramatically high cytotoxicity toward lymphoma (DOHH2, 4.4×10^{-10} M), leukemia (MV-4-11, 7.2×10^{-10} M), rhabdomyosarcoma (A-204, 3.94×10^{-9} M), bone cancer (MG-63, 8.5×10^{-9} M), liver cancer (Hep3B, 8.68×10^{-9} M), and brain cancer (U-87, MG 8.63×10^{-9} M) cells. Additionally, J1-001-1 and J1-001-2 demonstrated better anticancer activity than did J1-001-3 or J1-001-4, as shown in Table 1, with broad anticancer effects.

Aglycone Polyethers Affect Intracellular Calcium Levels and Mitochondrial Function. Polyethers as ionophore antibiotics with selectivity for alkali ions can increase intracellular calcium levels by disrupting $\text{Na}^+/\text{Ca}^{2+}$ exchange.³⁰ To obtain a comprehensive view of signaling pathways induced by aglycone polyethers, MDA-MB-231 cells were treated with J1-001-2 for 24, 48, and 72 h, and then, the

proteomic and phosphoproteomic profiles were recorded and compared with those of control cells to evaluate changes in the proteins. We quantified changes in 2039 proteins, with 773 proteins showing obvious alterations. Proteomic analysis results revealed that 11 proteins associated with calcium signaling were enhanced after J1-001-2 induction (Figure 3A). Given that increased intracellular Ca^{2+} is one of the initiating factors in apoptosis,³¹ Fluo-3 AM reagent was used to evaluate the level of intracellular Ca^{2+} in MDA-MB-231 cells after exposure to aglycone polyethers (J1-001-1, J1-001-2) for 16 h. As shown in Figure 3B, the fluorescence intensity of intracellular Ca^{2+} rapidly increased by 2–3 folds relative to levels in control cells.

Various factors in the intermembrane space of mitochondria, including cytochrome c, apoptosis-inducing factor (AIF), and reactive oxygen species (ROS), regulate mammalian cell

Figure 5. Aglycone polyether J1-001-1 inhibits paclitaxel-resistant MCF-7 tumor growth *in vivo*. BALB/c mice were injected with paclitaxel-resistant MCF-7 breast cancer cells prior to exposure to the compounds. Each group was given by corresponding sterile drugs through intraperitoneal injection by every 3 days of a 20 day cycle. (A) Effect of J1-001-1 on tumor burden. J1-001-1-L (0.25 mg/kg), J1-001-1-M (0.5 mg/kg), J1-001-1-H (1 mg/kg), paclitaxel (10 mg/kg), or vehicle control was injected intraperitoneally. Tumor volumes were measured at the indicated times. (B) The inhibitory effect on tumor weight of paclitaxel, J1-001-1-L, J1-001-1-M, and J1-001-1-H. ***, $P < 0.01$; ****, $P < 0.001$. (C) The inhibitory effect of nanchangmycin (J1-001-1) on paclitaxel-resistant MCF-7 tumor growth compared with the control.

apoptosis,^{32,33} and our proteomic results showed that cytochrome *c* (CYCS gene) and AIF (AIFM1 gene) were increased by approximately 1.6- and 1.2-fold, respectively, after J1-001-2 treatment for 48 h (Figure 3C). Additionally, we found that aglycone polyethers trigger, in a concentration-dependent manner, a marked increase in ROS levels in a breast cancer cell line after 16 h exposure (Figure 3D). We also evaluated the effect of aglycone polyethers on mitochondria isolated from mice. In the presence of J1-001-1 and J1-001-2, mitochondrial inner membranes exhibited significantly enhanced permeability to H⁺ (Figure 3E,F) and K⁺ (Figure 3H,I) when compared with the membranes of untreated mitochondria. Furthermore, higher levels of mitochondrial ROS were induced by the aglycone polyethers following treatment at the indicated concentrations for 30 min (Figure 3J,K). These findings strongly suggest that the mitochondria are one of the main targets for the J1-001-type polyethers.

Aglycone Polyethers Inhibit the Wnt/ β -catenin Pathway and Activate Apoptosis. In previous studies, salinomycin was found to induce death in cancer cells via multiple pathways, such as Wnt,⁷ AMPK,³⁴ MAPK,³⁵ autophagy,³⁶ NF- κ B,³⁷ and others pathways.¹⁵ Results of our proteomic analyses suggest that the Wnt pathway was inhibited and that the autophagy and apoptosis pathways were activated (Figure 4A,B).

Wnt3a, Wnt5a, and phosphorylated LRP6, which functions as a coreceptor for Wnt pathway, are highly expressed in breast cancer cells.³⁸ When the Wnt pathway is triggered, β -catenin is released and then moves into the nucleus to activate its targets.

J1-001-2 reduced the expression of Wnt3a and Wnt5a in a dose-dependent manner (Figure 4C), and P-LRP6 and LRP6 were also reduced in a dose- and time-dependent manner (Figure 4C,D). P- β -catenin was inhibited at all doses of J1-001-2 starting 3 h after exposure to the drug (Figure 4E); however, the expression of β -catenin did not change (Figure 4C). Cyclin D1, CD44, c-MycMya, survivin, and Wnt signaling-activated targets were highly expressed after long-term stimulation of Wnt signaling. However, our analyses suggest that induction of these genes can be reversed by treatment with J1-001-2, likely through blocking of Wnt signaling. For example, cyclin D1 expression was not detected at a J1-001-2 concentration of 4 μ M, and c-Myc expression was blocked at an even lower dose (2 μ M) (Figure 4D). CD44 and survivin expression were also inhibited at all doses of J1-001-2 (Figure 4D).

In addition to the Wnt pathway, J1-001-2 markedly upregulated LC3 and caspase-3 protein levels in a dose-dependent manner, a phenomenon that was observed within 3 h after J1-001-2 treatment, and the level of apoptosis-related protein caspase-3 was also markedly increased (Figure 4E,F).

Aglycone Polyethers Inhibit the Growth of Paclitaxel-Resistant MCF-7 (MCF-7/TAX) Breast Cancer *in Vivo*. Traditional chemotherapeutic drugs induce multidrug resistance (MDR) in cancer cells,³⁹ and the CSC-enriched cancer cell population reportedly has increased resistance to chemotherapeutic agents.⁴ To determine whether the aglycone polyethers exhibit anticancer activity *in vivo*, we subcutaneously injected 1×10^7 MCF-7/TAX cells, which are

paclitaxel-resistant breast cancer cells, into BALB/c mice to form grafted tumors, using paclitaxel as a positive control. After palpable tumor formation, mice were injected with paclitaxel (10 mg/kg), J1-001-1-L (0.25 mg/kg), J1-001-1-M (0.5 mg/kg) or J1-001-1-H (1 mg/kg), and 3 weeks later, tumors were isolated and weighed (Figure 5 A and B). Results showed that the levels of inhibition by the four treatments, respectively, were 24.4, 39.9, 66.3, and 79.3% (Figure 5C). Notably, tumor size was significantly smaller in J1-001-1-H-treated mice than in vehicle- or paclitaxel-treated mice.

DISCUSSION

Owing to the robust survival mechanisms of tumors, CSCs remain viable and lead to disease relapse;⁴⁰ therefore, drugs capable of compromising CSC proliferation and self-renewal are urgently required. In the CSC mammosphere model used in this study, the inhibitory effect of salinomycin on BCSCs was significantly better than that of paclitaxel treatment, which is consistent with a report by Gupta et al.⁶ showing that salinomycin reduced the proportion of CSCs by over a 100-fold more than did paclitaxel.

Aglycone polyethers represent a very important and interesting group of natural polyether substances with a characteristic structure. They are usually used as antibacterial and antiparasitic agents, and they interfere with transmembrane potassium potential and promote mitochondrial and cellular potassium efflux.^{21,41} The present study demonstrated that removal of the aglycone group resulted in loss of antiproliferative activity against cancer cells, although the backbone was similar to the skeleton of salinomycin. Therefore, we hypothesize that the 4-*O*-methyl- β -*D*-amicetose of the J1-001 compound series contributes to their toxicity toward cancer cells. There is a relationship between the aglycone position and anticancer activity of the four J1-001 aglycone polyethers in that an increasingly shorter distance between the aglycone position and carboxyl terminal of the polyketide backbone was associated with a greater reduction in the BCSC population (J1-001-2 < J1-001-3 < J1-001-1 < J1-001-4) (Figure 2), suggesting that the aglycone unit appended in the vicinity of the carboxyl group of the polyketide backbone is vital to the reduction of BCSCs.

Mitochondria dysfunction regulates cell death by apoptosis. The mitochondrial permeability transition (MPT) is the key mechanism by which mitochondria regulate cell apoptosis and involves the release of specific proteins [cytochrome *c*, apoptosis-inducing factor (AIF), and others] from the mitochondria.³⁷ Many factors can induce MPT, including ROS, the collapse of mitochondrial membrane potential, and an increase in matrix H⁺ and K⁺ concentrations.^{42,43} Our results reveal that aglycone polyethers *in vitro* can increase permeabilization for H⁺ (Figure 3E,F) and K⁺ (Figure 3G,H) and enhance ROS levels in isolated mitochondria to affect MPT function. However, we found that J1-001 aglycone polyethers did not induce changes in mitochondrial membrane fluidity (Figure S7), mitochondrial membrane potential (Figure S8), or mitochondrial swelling (Figure S9) when compared with controls with no drugs. In contrast, intracellular Ca²⁺ overload was affected by J1-001 polyethers through disrupting Na⁺/Ca²⁺ exchange,³⁰ which activates MPT, allowing cytochrome *c* and AIF to pass from the matrix to the cytosol, finally inducing caspase-dependent apoptosis.^{44,45} Furthermore, *in vitro* cell assays demonstrated that intracellular Ca²⁺ flux and ROS levels (Figure 3C,D) can disrupt

mitochondrial function, resulting in release of caspase-activating proteins, such as cytochrome *c* and AIF (Figure 3B). Accumulated ROS can also be damaging to both DNA and proteins.³² Additionally, apoptosis-related protein caspase-3 was hyperactivated upon J1-001-2 treatment in a time- and dose-dependent manner (Figure 4E,F), suggesting that aglycone polyethers may induce cell death not only by interfering with the intracellular ionic balance but also by disrupting mitochondrial homeostasis.

Salinomycin, a nonaglycone polyether, was identified as a potent inhibitor of the Wnt/ β -catenin signaling pathway,⁷ and also confers radiation susceptibility to drug-resistant CSCs.^{46–49} The Wnt/Fzd/LRP complex initially activates phosphorylation of LRP5 or LRP6 leading to β -catenin accumulation in the nucleus, expression of Wnt target genes, and cell proliferation.^{50,51} Previous studies have demonstrated that many polyethers, such as salinomycin, ionomycin, nigericin, and monensin, can inhibit the Wnt signaling pathway by blocking the phosphorylation of LRP6.¹⁵ Our analyses indicated that the Wnt signaling pathway was blocked by J1-001-2 through Wnt and LRP6 degradation and β -catenin reduction. The Wnt/ β -catenin targets cyclin D1, CD44, survivin, and c-Myc also were inhibited (Figure 4D), which could cause tumors to lose proliferative ability.⁴² Although the activation of GSK3 β by salinomycin is associated with the inhibition of mTORC1 and Wnt/ β -catenin signaling in breast and prostate cancer cells,^{7,52} we found that phosphorylated GSK3 β was unchanged in MDA-MB-231 cells after J1-001-2 treatment (Figure 4D). Therefore, aglycone polyethers might not modulate mTORC1 and Wnt/ β -catenin signaling through GSK3 β . Autophagy is the bulk degradation of proteins and organelles, a process essential for cellular maintenance and viability,⁵³ and the autophagy-related proteins LC3A/B were hyperactivated upon J1-001-2 treatment. These findings suggest that J1-001-2 could both limit cancer cell proliferation and induce autophagy.

Treatment with J1-001-2 was found to be effective against many types of cancer cells even at nanomolar doses. For BCSCs and most of the other tumor cell lines, there was a positive correlation between the antitumor activity of the four tested compounds and a reduced distance of the aglycone group from the carboxyl group of the polyketide backbone. Although we observed that J1-001-2 had promising biological effects on different types of cancer *in vitro*, the compound failed *in vivo*, likely due to its poor solubility and side effects on liver and kidney tissue. To avoid these actions, antibody-drug conjugates (ADC) may be a tool to solve the above problem. Fortunately, the *in vivo* experiments with MCF-7/TAX cells confirmed the anticancer properties of J1-001-1. Thus, aglycone polyethers are potential new clinical drugs for treating many different kinds of cancers, and the diversity in the structure of polyether antibiotics suggests the need for comprehensive studies on the therapeutic potential of these compounds.

MATERIALS AND METHODS

Reagents and Antibodies. The compounds J1-001-1, J1-001-2, J1-001-3, and J1-001-4 were isolated and purified in our laboratory. All drugs were dissolved in DMSO (Sigma-Aldrich) to make a 10 mM stock solution. DMEM high-glucose, DMEM-F12, penicillin, streptomycin, and B27 supplements were purchased from Thermo Fisher. Fetal bovine serum was purchased from BI (Kibbutz Beit Haemek, Israel), and low-

endotoxin bovine serum albumin was obtained from Sigma-Aldrich. Human epidermal growth factor (hEGF) was purchased from BD Biosciences, and CellTiter-Glo reagent was obtained from Promega. Anti-Wnt3a, anti-Wnt5a, anti-LRP6, anti-P-LRP6 (Ser1490), anti- β -catenin, anti-phosphor- β -catenin (Ser552), anti-caspase-3, anti-survivin, anti-cyclin D1, anti-c-Myc, anti-phosphor-GSK-3 β (Ser9), anti-LC3A/B, anti- β -actin, anti-GAPDH, anti-mouse IgG, and anti-rabbit IgG antibodies were purchased from Cell Signaling (Beverly, MA, USA). The ALDEFLUOR kit was obtained from StemCell Technologies (Vancouver, Canada). Insulin was purchased from Novo Nordisk (Bagsværd, Denmark).

Identification of Four Aglycone Polyethers. J1-001-1 was identified and obtained in our previous studies.^{23,25} J1-001-2, J1-001-3, and J1-001-4 were extracted, respectively, from the American Type Culture Collection (ATCC) *Streptomyces* strains ATCC 21840, ATCC 39574, and ATCC 53626 after fermentation in SSYC medium (soluble starch 30 g/L, soybean powder 10 g/L, yeast extract 2.5 g/L, and calcium carbonate 3 g/L) at 30 °C for 7 days. These three compounds were purified using preparative reversed-phase high-performance liquid chromatography.

Aglycone polyethers (J1-001-2, J1-001-3, and J1-001-4) have been identified by IR, NMR and X-ray methods,^{54–57} and our data (NMR, LRMS) were consistent with literature. The most characteristic chemical shift of these analogues was observed in ¹H NMR spectra for α,β -unsaturated ketone (6.7–7.36 ppm) and sugar moiety (3.32–3.35 ppm for OCH₃), in ¹³C NMR spectra for α,β -unsaturated ketone (206.2–207.3 ppm for C=O bond, 133.6–146.0 ppm for C=C bond), carboxyl group (181.6–183.9 ppm), spiroketals (104.1–111.1 ppm for O–C–O bond), and sugar moiety (101.5–102.6 ppm for O–C–O bond). In addition, structural identification of aglycone polyethers was also verified by the low-resolution mass spectrometry; further information has been provided in the [Supporting Information](#).

J1-001-2. From ATCC 21840, also called A-120-A. C₄₇H₇₈O₁₃ ¹H NMR (400 MHz, CDCl₃) δ : 7.36 (d, *J* = 10.0 Hz, 1H), 4.53 (t, *J* = 10.0 Hz, 1H), 4.47–4.34 (m, 2H), 4.12–4.06 (m, 1H), 4.02 (d, *J* = 12.0 Hz, 1H), 3.82 (t, *J* = 9.2 Hz, 1H), 3.57 (s, 1H), 3.44 (s, 1H), 3.32 (s, 3H), 3.28 (dd, *J* = 9.0, 6.6 Hz, 2H), 3.16 (d, *J* = 12.0 Hz, 1H), 2.91 (td, *J* = 10.6, 4.2 Hz, 1H), 2.63–2.44 (m, 3H), 2.38 (td, *J* = 11.8, 6.8 Hz, 1H), 2.19–2.06 (m, 2H), 2.06–1.91 (m, 6H), 1.90–1.75 (m, 6H), 1.73 (d, *J* = 10.6 Hz, 3H), 1.66–1.55 (m, 4H), 1.49–1.39 (m, 8H), 1.30–1.17 (m, 8H), 1.13 (t, *J* = 7.0 Hz, 4H), 1.10–1.00 (m, 6H), 0.96 (d, *J* = 7.0 Hz, 3H), 0.90 (d, *J* = 6.2 Hz, 4H), 0.84 (t, *J* = 5.4 Hz, 4H). ¹³C NMR (101 MHz, CDCl₃) δ : 207.34, 181.61, 146.00, 134.06, 111.09, 108.86, 102.57, 98.51, 85.76, 80.87, 79.36, 79.32, 73.13, 72.98, 67.97, 64.16, 60.37, 56.74, 41.11, 40.97, 39.59, 39.25, 37.60, 36.35, 35.61, 35.07, 32.95, 32.11, 31.89, 29.97, 29.75, 29.66, 29.62, 28.01, 27.43, 26.99, 26.01, 20.08, 18.24, 17.77, 17.15, 16.92, 15.27, 14.62, 13.93, 13.91, 11.21.

J1-001-3. From ATCC 39574, also called Endusamycin. C₄₇H₇₈O₁₄ ¹H NMR (600 MHz, CDCl₃) δ : 6.70 (d, *J* = 10.0 Hz, 1H), 4.63 (d, *J* = 10.4 Hz, 1H), 4.46 (dd, *J* = 10.8, 7.6 Hz, 1H), 4.35 (dt, *J* = 5.4, 3.8 Hz, 2H), 3.98 (d, *J* = 12.2 Hz, 1H), 3.92–3.80 (m, 2H), 3.48 (dd, *J* = 11.6, 2.0 Hz, 2H), 3.33 (s, 3H), 3.30–3.23 (m, 2H), 2.84–2.77 (m, 1H), 2.64–2.54 (m, 2H), 2.47 (ddd, *J* = 11.6, 6.8, 2.2 Hz, 1H), 2.31–2.25 (m, 2H), 2.20–2.15 (m, 1H), 1.99–1.78 (m, 4H), 1.78–1.69 (m, 7H), 1.58 (d, *J* = 3.0 Hz, 3H), 1.52–1.41 (m, 6H), 1.40–1.31 (m,

2H), 1.31–1.20 (m, 6H), 1.12–1.04 (m, 6H), 1.04–0.98 (m, 6H), 0.98–0.92 (m, 4H), 0.91–0.86 (m, 4H), 0.85–0.78 (m, 4H), 0.78–0.67 (m, 4H). ¹³C NMR (151 MHz, CDCl₃) δ : 206.19, 183.56, 144.62, 133.61, 110.57, 104.15, 101.51, 98.53, 85.09, 84.27, 79.91, 79.01, 78.56, 74.62, 72.97, 70.42, 69.83, 65.36, 56.85, 44.88, 37.64, 36.65, 35.94, 35.84, 35.80, 35.57, 33.76, 32.89, 30.49, 30.03, 29.87, 29.67, 27.08, 26.87, 24.82, 19.55, 19.51, 18.26, 17.52, 16.83, 16.69, 14.80, 14.33, 13.13, 11.16, 9.86.

J1-001-4. From ATCC 53626, also called CP-80,219. C₄₇H₇₈O₁₄ ¹H NMR (400 MHz, CDCl₃) δ : 6.69 (d, *J* = 10.0 Hz, 1H), 4.66 (d, *J* = 10.4 Hz, 1H), 4.47 (dd, *J* = 10.8, 7.6 Hz, 1H), 4.36 (d, *J* = 7.8 Hz, 2H), 3.99 (d, *J* = 12.2 Hz, 1H), 3.88 (d, *J* = 11.2 Hz, 2H), 3.53–3.43 (m, 2H), 3.35 (s, 3H), 3.28 (t, *J* = 9.7 Hz, 2H), 2.82 (td, *J* = 10.0, 4.5 Hz, 1H), 2.67–2.46 (m, 3H), 2.34–2.23 (m, 4H), 2.19 (dd, *J* = 8.8, 3.7 Hz, 1H), 1.97 (dd, *J* = 8.8, 4.9 Hz, 1H), 1.88 (dd, *J* = 6.8, 3.8 Hz, 3H), 1.83–1.67 (m, 8H), 1.59 (s, 4H), 1.55–1.43 (m, 8H), 1.38–1.18 (m, 8H), 1.07 (ddd, *J* = 8.6, 5.4, 4.6 Hz, 8H), 0.97 (t, *J* = 7.0 Hz, 6H), 0.91–0.81 (m, 4H), 0.72 (t, *J* = 8.8 Hz, 4H). ¹³C NMR (101 MHz, CDCl₃) δ : 206.21, 183.89, 144.44, 133.91, 110.60, 104.17, 101.53, 98.66, 85.17, 84.31, 79.94, 78.94, 78.56, 74.62, 72.91, 70.11, 69.85, 65.24, 56.85, 44.96, 39.03, 37.39, 36.48, 36.14, 36.04, 35.78, 35.29, 33.86, 32.90, 30.50, 30.09, 29.92, 29.66, 27.10, 26.87, 25.03, 24.81, 19.40, 19.30, 18.26, 17.55, 17.08, 16.57, 14.79, 14.54, 14.10, 9.99.

Screening Cell Lines with Polyethers. Screening of polyether compounds in various cell lines was performed with the antitumor evaluation platform of Genescript Biological Technology Co., Ltd. Cells (800 per well) were seeded in 384-well flat-bottomed clear white microplates (Corning). The peripheral wells were filled with PBS. After 24 h, polyethers were added to the wells at 8–9 different concentrations (between 100 μ g/mL and 0.084 ng/mL) in triplicate replicates. Negative control wells were used for normalization; quality control criteria was used doxorubicin on BEL-7402 cells within 5.45×10^{-7} (± 3 folds shift). All cell lines were also treated with DMSO alone (DMSO < 0.3%). CellTiter-Glo reagent was added to each test well after 72 h incubation, and plates were shaken for 2 min on an orbital shaker. Luminescence signals were detected on a PHERAstar Plus multimode reader. For more details, see the [Supporting Information](#).

Measurement of Intracellular ROS and Ca²⁺ Levels. Intracellular ROS production and Ca²⁺ levels were measured using DCFH-DA fluorescent dye (Beyotime Biotechnology) and Fluo-3 AM (Beyotime Biotechnology), respectively. MDA-MB-231 cells were cultured in 6-well plates at a density of 2×10^5 cells/well. After treatment with aglycone polyethers for 16 h, cells were incubated with 10 μ M DCFH-DA at 37 °C for 20 min and then washed twice with PBS. Cells were then analyzed for fluorescence using the flow cytometer mentioned below. The ROS levels in the treatment groups were normalized to that of the control group. Experiments were repeated three times to calculate the mean.

Cell Culture. The human triple-negative breast cancer cell line MDA-MB-231 was obtained from ATCC and cultured in high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO₂ atmosphere incubator at 37 °C. The mammosphere culture was performed as reported by Sun et al.⁵⁸ MDA-MB-231 cells (1000 cells/mL) were cultured in suspension in serum-free DMEM-F12 supplemented with B27, 20 ng/mL hEGF, 0.4%

low-endotoxin bovine serum albumin, and 4 mg/mL insulin. The mammospheres were collected by centrifugation (800 g, 5 min), dissociated into single cells to stimulate propagation, and used to generate the next generation.

Cancer Stem Cell Identification. To identify the cancer stem cells in the breast mammospheres, an ALDEFLUOR kit was used to analyze ALDH activity following the manufacturer's protocol. First, MDA-MB-231 breast cancer cells were seeded into 6-well low-attachment plates followed by incubation for 10–12 days as a suspension culture, then cells were harvested using Accutase cell detachment reagent, and after cell counting, each tube containing 200 000 cells in suspension culture was cocultured with each compound for 72 h. After centrifugation, cells were resuspended in assay buffer and divided into two test tubes; one of the test tubes was used as a negative control and received the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB). Subsequently, the ALDH substrate BODIPY-amino acetaldehyde was added to both tubes, and the tubes were incubated for 45 min at 37 °C. The cells were collected and detected by FACSCalibur flow cytometer (BD Biosciences), and the ratio of stem cells of each tube was analyzed FlowJo Software (TreeStar, San Carlos, CA, USA).

Cell Viability and Cytotoxicity. Cells were plated in a 96-well plate at a density of 5×10^3 cells/well and incubated for 24 h. Subsequently, drugs were added in triplicate at each concentration for each cell line, and plates were incubated for 72 h. Cell viability and cytotoxicity were measured by the MTS assay (Promega) as described in the manufacturer's instructions. Briefly, 20 μ L of the MTS solution was added to each well, and the plates were incubated for another 3 h. The absorbance was measured using the multimode plate reader at 490 nm.

Measurement of Mitochondrial H⁺ and K⁺ Inner Membrane Permeabilization. Liver mitochondria from Wistar rats (150–200 g) were isolated according to standard differential centrifugation procedures. Mitochondrial inner membrane permeabilization to H⁺ and K⁺ was detected, respectively, by passive osmotic swelling of mitochondria suspended in potassium acetate and potassium nitrate medium.⁵⁹ The potassium acetate medium contained 135 mM potassium acetate, 5 mM HEPES, 0.1 mM EGTA, 0.2 mM EDTA, 2 μ M rotenone, and 1 μ g/mL valinomycin (pH 7.1), and the potassium nitrate medium contained 135 mM KNO₃, 5 mM HEPES, 0.1 mM EGTA, 0.2 mM EDTA, and 2 μ M rotenone (pH 7.1).

Animal Experiments. BALB/c mice (weight: 20 ± 2 g) were obtained from the Animal Center (No. 43004700004943), Wuhan University, Wuhan, China. Animal study followed ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and was approved by The Institutional Animal Care and Use Committee (IACUC), Wuhan University Center for Animal Experiment, Wuhan, China (AUP No. S2013014). For xenograft tumor-seeding studies, the indicated numbers of MCF-7/TAX cells were suspended in 100 mL of DMEM (containing 10% FBS) and injected subcutaneously into BALB/c mice. MCF-7/TAX cells were treated for 1 week and allowed to recover in the absence of drug for 2 weeks prior to injection into mice. Tumor incidence was monitored for 14 days after injection. Animals were administered normal saline (vehicle), J1-001-1-L (0.25 mg/kg), J1-001-1-M (0.5 mg/kg), J1-001-1-H (1 mg/kg), or

paclitaxel (10 mg/kg) every 2 days by intraperitoneal injection for 3 weeks.

Western Blotting. Cells were lysed in 1× RIPA buffer (Sigma) and centrifuged. The protein in the supernatant was collected, and its concentration was determined by the Bradford assay using BSA (Sigma) as the standard. Equal amounts of protein (50 μ g) were subjected to 4%–20% gradient gel electrophoresis (Genescript, Nanjing, China) and transferred to PVDF membranes. Primary antibodies were used at a dilution of 1:1000, and secondary antibodies were used at a dilution of 1:2000. β -Actin or GAPDH was used as the reference protein.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsptsci.8b00007.

Additional materials and methods; raw data for immunoblotting (PDF)

Screening cell lines (XLSX)

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Notes

The authors declare the following competing financial interest(s): J1 Biotech Co. Ltd. has applied for several patents base on this work.

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