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Anti-cancer activity of the cell membrane-permeable phytic acid prodrug

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ABSTRACT

Phytic acid (IP6) is an ingredient in cereals and legumes, and limited amounts of this compound are considered to enter the cell and exert anti-cancer effects. These effects have been seen by studying cells treated with around 1–5 mM IP6. However, such a large amount of IP6 chelates metals and changes the pH in cell culture medium. To overcome this problem, we synthesized a prodrug of IP6 (Pro-IP6) and elucidated generation of IP6 from Pro-IP6 in cells. Cellular experiments using Pro-IP6 demonstrated selective anti-cancer effects including apoptosis and inhibition of Akt activation. Furthermore, an *in vivo* study using mice with adult T-cell leukemia also showed that Pro-IP6 reduced the size of the cancer. Taken together, Pro-IP6 is a useful biological tool and may lead to development of new anti-cancer drugs.

1. Introduction

Humans have eaten cereals and legumes as foods for centuries. Components of these foods include not only major nutrients such as carbohydrates, proteins, and fats, but other miscellaneous molecules as well. One such molecule is phytic acid (i.e., *myo*-inositol-1,2,3,4,5,6-hexaphosphate: IP6), a small molecule in which the six hydroxy groups on the *myo*-type inositol ring are fully phosphorylated (Fig. 1a). High levels of IP6 are present in plant seeds [1,2]. Although this molecule is highly negatively charged, data suggest that it is taken into the human body [3,4], and limited amounts of the molecule may be internalized in cells by mechanisms such as endocytosis [5,6]. Notably, IP6 is biosynthesized in a step-wise process from PI(4,5)P2 [7,8], the origin of which is glucose 6-phosphate, an important intermediate of glycolysis [9].

The significance of IP6 as a dietary component is not completely clear, although the protective effects of IP6 against cancer and its anti-cancer effects have been widely studied [1,10]. These effects were demonstrated using mice and rats [1,10], and an epidemiological survey showed a negative correlation between IP6 intake and development of cancer [11,12]. Various functions of IP6 have been reported regarding a possible mechanism by which the molecule suppresses cancer. IP6 chelates iron, suppresses iron-catalyzed production of hydroxyl radicals [13,14], and reduces the pH in the colon [1]. These functions occur outside the cell. Many other functions occur inside the cell, i.e., induction of cell cycle arrest/apoptosis, DNA repair, and regulation of signal transduction related to malignancy such as the Akt pathway [1,10]. These phenomena were shown using cells treated with around 1–5 mM IP6. However, such a large amount of IP6 likely changes the characteristics of the cell culture medium, i.e., chelation of metals and reduction in the pH. Although a cell membrane-permeable prodrug of IP6 is useful for overcoming this problem (Fig. 1a), no such prodrug has been developed. In this study, we synthesized a prodrug of IP6 and evaluated its anti-cancer activity.

2. Results and discussion

2.1. Chemistry

Acyloxymethyl ester is the most commonly used prodrug of phosphates [15]. Addition of a butyryloxymethyl group is effective when designing a prodrug of inositol triphosphates [16]. Thus, a butyryloxymethyl group was selected, and the dodeca(butyryloxymethyl) ester of phytic acid (Pro-IP6) was synthesized from IP6 in one step (Fig. 1b).

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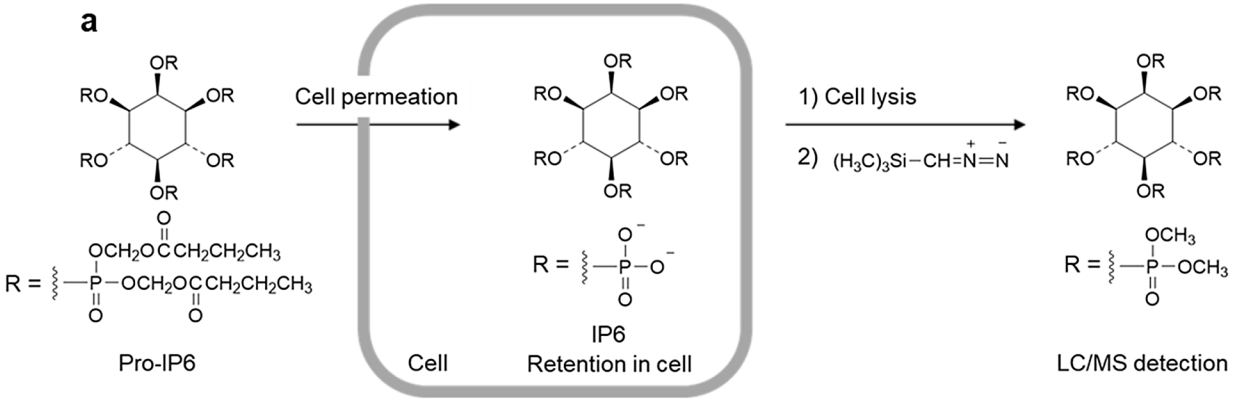

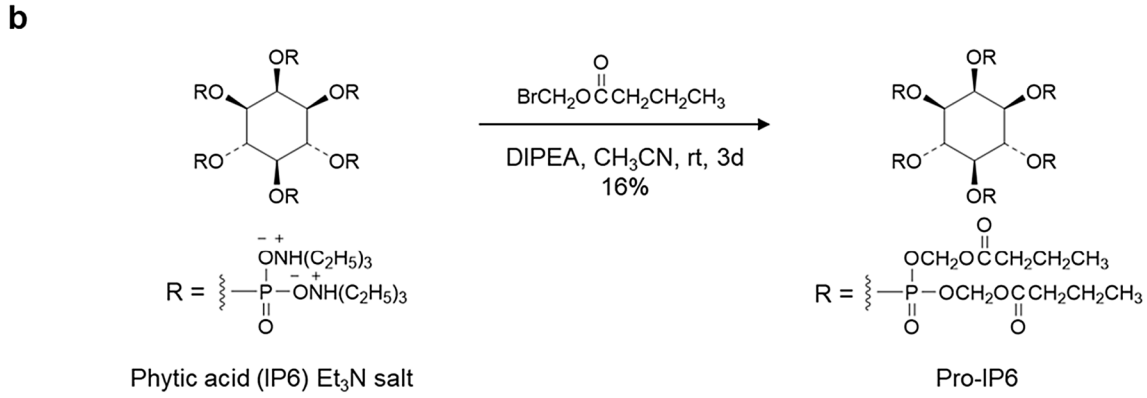

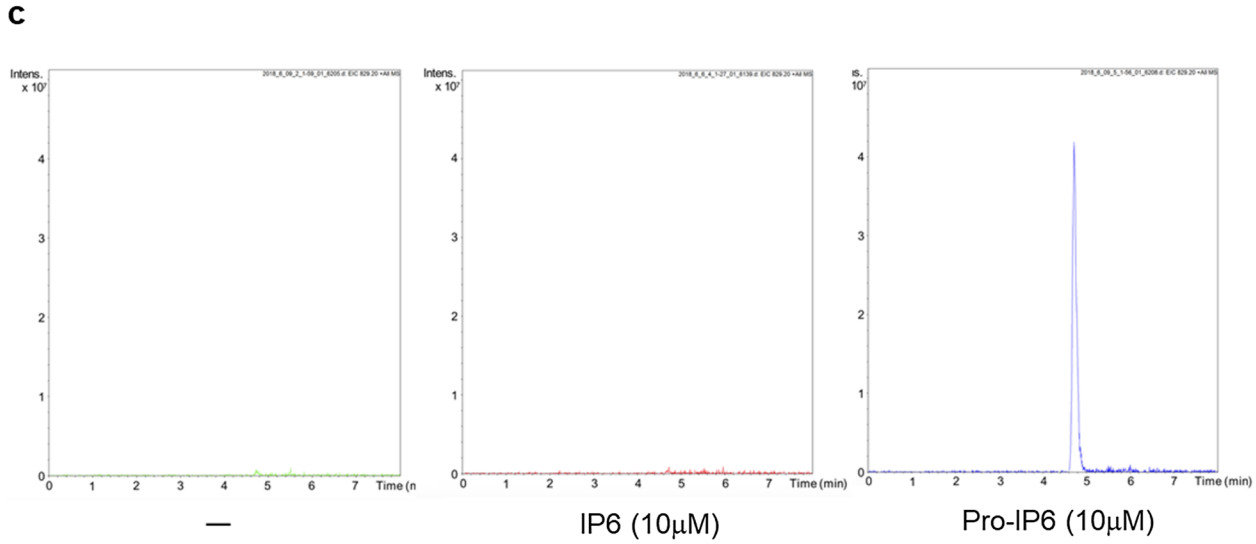

Fig. 1. Generation of IP6 inside a cell treated with Pro-IP6 and detection of IP6 after methylation. (a) Schematic representation. (b) Synthesis of Pro-IP6. (c) Amount of IP6 inside cells treated with IP6 or Pro-IP6 (10 μM). HeLa cells were incubated with dimethylsulfoxide (DMSO) (control), IP6 in water, or Pro-IP6 in DMSO, and incubated for 30 min. Cell lysates were mixed with reverse phase-weakly basic anion exchange resin and eluted. The eluates were analyzed with LC/MS after methylation as shown in (a).

The compound for esterification, bromomethyl butyrate, was synthesized as previously reported [17].

We next examined whether IP6 is generated in cells treated with Pro-IP6. For this purpose, we established a method to detect IP6 in cells using liquid chromatography-mass spectrometry (LC/MS). HeLa cells were treated with Pro-IP6 and lysed. The lysate was mixed with reverse phase-weakly basic anion exchange resin, and the absorbates were eluted. Because the highly negatively charged phytic acid was difficult to separate clearly with LC and detect with high sensitivity with MS, IP6 was methylated for analysis. The eluates from the resin including IP6 were reacted with trimethylsilyldiazomethane, and IP6 methyl ester that was produced was analyzed with LC/MS (Fig. 1a). Notably, IP6 methyl ester was confirmed to not have formed from Pro-IP6 and trimethylsilyldiazomethane (Fig. S1 in supplementary information).

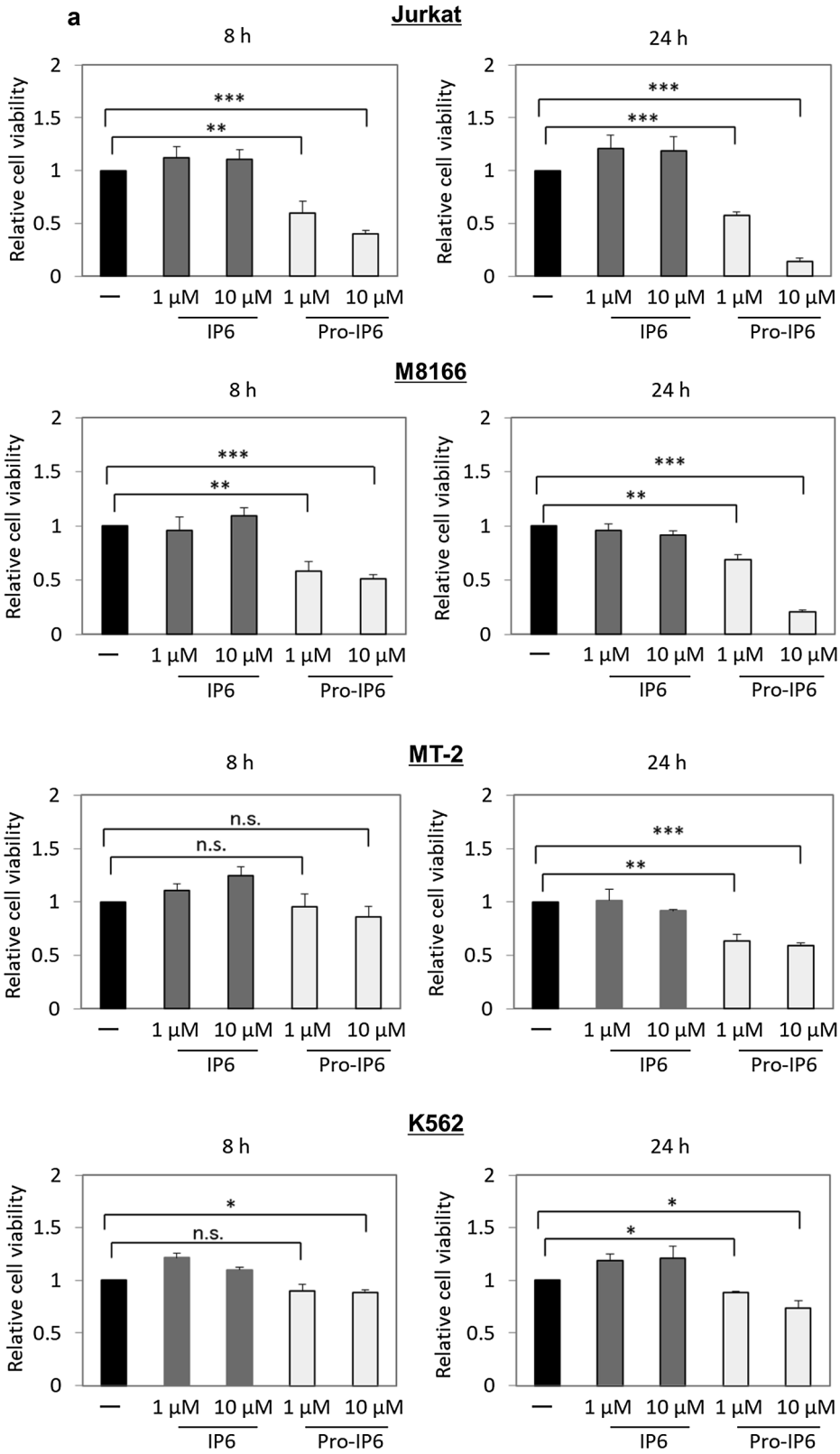

Fig. 2. Cytotoxicity of IP6 and Pro-IP6 against T-cell lines, Jurkat, M8166, MT2 and K562, and peripheral blood mononuclear cells (PBMC). The cells were incubated with DMSO (control), IP6 in water, or Pro-IP6 in DMSO, and incubated for 8 or 24 h. Then, the MTT assay was performed. Relative cell viability is shown. (a) Effect of IP6 or Pro-IP6 (1 or 10 μM) on T cell lines. (b) Effect of IP6 (2 or 5 mM) on T cell lines. (c) Effect of Pro-IP6 (10 μM) on PBMC.

2.2. Biological evaluation

2.2.1. Cellular study

Using established method, we determined the amount of IP6 in HeLa cells at 30 min after treatment with IP6 or Pro-IP6 (10 μM). The results are shown in Fig. 1c. Intact HeLa cells have a concentration of 38 μM endogenous IP6 inside the cell [8], but this concentration was below the detection limit in this system. When IP6 was added, it was

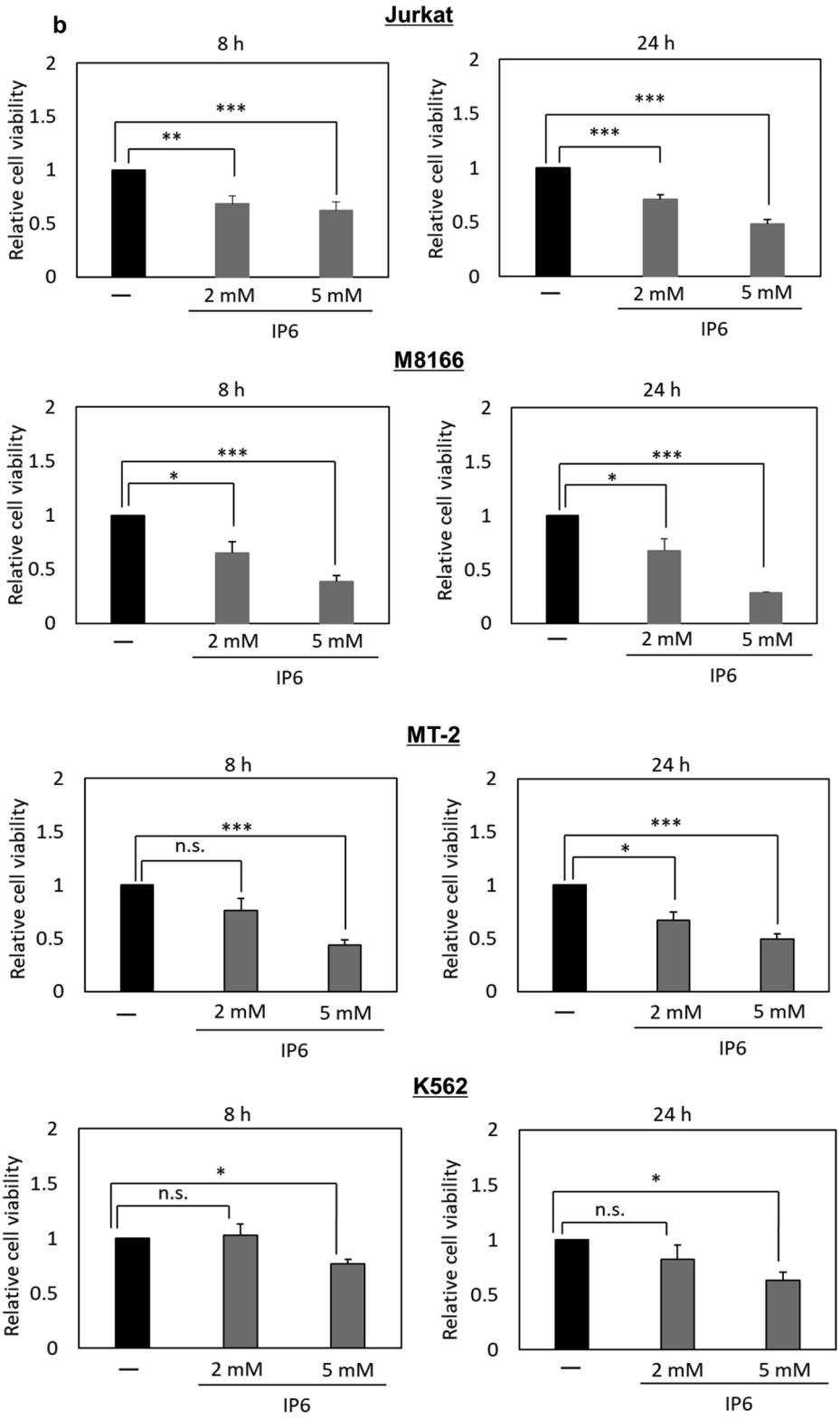

Fig. 2. (*continued*)

still undetectable. On the other hand, after treatment of the cells with Pro-IP6, the amount of IP6 in the cells clearly increased. These results suggest that Pro-IP6 is converted to IP6 by enzymatic hydrolysis inside the cell. Then, the highly charged IP6 was likely retained in the cell due to its inability to exit the cell, thus showing a much higher amount of IP6 than outside the cell.

Next, cytotoxic effects of Pro-IP6 against cancer cells were examined. The human leukemia cell line Jurkat utilizes the Akt pathway. Other leukemia cell lines, M8166, MT-2, and K562, were also used. The cells were treated with IP6 or Pro-IP6 (1 or 10 μM) and incubated for 8 or 24 h. The cell viability was evaluated with the MTT assay. As shown in Fig. 2a, IP6 did not show clear cytotoxicity. In contrast, Pro-IP6 reduced cell viability in a concentration- and time-dependent manner. Among the four cell lines, Jurkat and M8166 cells

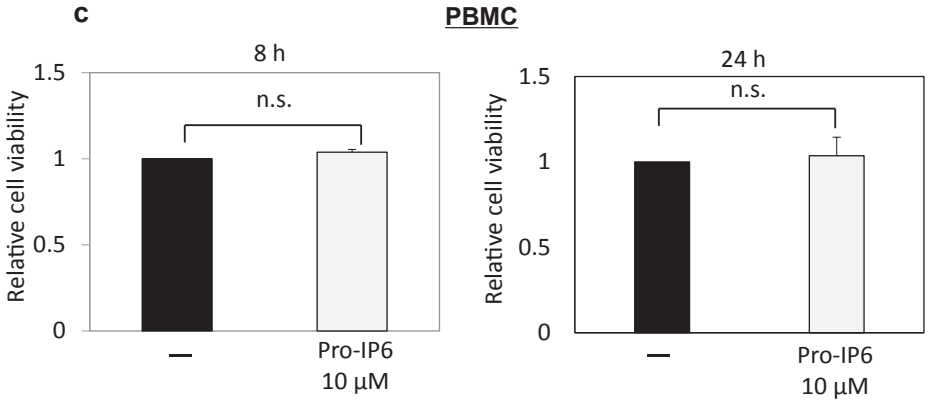

Fig. 2. (*continued*)

were sensitive to Pro-IP6. The same experiments using 2 or 5 mM IP6 showed similar cytotoxicity (Fig. 2b). For example, 10 μM Pro-IP6 showed stronger toxicity than 5 mM IP6 in Jurkat cells. On the other hand, Pro-IP6 did not show cytotoxicity against non-cancerous peripheral blood mononuclear cells (PBMC) (Fig. 2c). These results demonstrated strong and selective anti-cancer activity of Pro-IP6. The Pro-IP6 should be converted to IP6 in these leukemia cell lines and PBMC, since the enzyme to cleave acyloxymethyl ester works in various cells including T cells [15].

We then examined whether Pro-IP6 induces apoptosis in Jurkat cells. The cells were treated with IP6 or Pro-IP6 (10 μM), incubated for 24 h, and analyzed by fluorescence-activated cell sorting (FACS) after reaction with fluorescein isothiocyanate (FITC)-conjugated annexin V and 7-aminoactinomycin D (7-AAD). The results are shown in Fig. 3a. In the absence of compounds and presence of IP6, most cells were not stained with the two reagents. In contrast, in the presence of Pro-IP6, around 90% of cells were FITC positive, and nearly half of them (41.7% of total cells) were 7-AAD negative. FITC- and 7-AAD-double positive cells are thought to be in the late phase of apoptosis or necrosis. Thus, at least 41.7% of the cells underwent apoptosis in the presence of Pro-IP6. Next, the effect of Pro-IP6 on cellular proteins was analyzed by immunoblotting. As shown in Fig. 3b, cleavage of poly(ADP-ribose) polymerase 1 (PARP-1) and Caspase-3 in the presence of Pro-IP6 (10 μM) is consistent with induction of cellular apoptosis.

We further examined the effect of Pro-IP6 on Akt activation (Fig. 3b). In the presence of 1 μM and 10 μM Pro-IP6, phosphorylation of Thr308 was clearly inhibited. The inhibitory effect of Pro-IP6 (1 μM and 10 μM) on Ser473 phosphorylation was also seen, although the effect was not drastic. Inhibition of Akt is one mechanism of induction of apoptosis. Also in a previous study, the inhibitory effect of IP6 (2 mM) on phosphorylation of Thr308 and Ser473 in PC-3 cells was shown [18]. IP6 (10 μM) did not show an obvious inhibitory effect on phosphorylation of Akt (Fig. 3b).

2.2.2. In vivo study

As described above, we developed the IP6 prodrug, Pro-IP6, and tested its use as a biological tool. From another point of view, Pro-IP6 may be useful as an anti-cancer drug. Because Pro-IP6 had cytotoxic activity against the adult T-cell leukemia (ATL) cell lines, M8166 and MT-2 (Fig. 2a), its *in vivo* anti-ATL activity was examined using mice transplanted with ATL S1T cells. After transplantation, Pro-IP6 (20 mg/kg) or IP6 (the same number of moles/kg) was administered intraperitoneally to each mouse every day for 28 days, and the tumor volume was continuously measured. On the 28th day, mice were sacrificed, and the tumor size was measured after complete removal. As shown in Fig. 4 (a and b), Pro-IP6 suppressed the tumor size in contrast to IP6. The effect was not drastic, and a different method of administration may improve the effect. We are currently testing this possibility.

3. Conclusion

Taken together, we developed a cell membrane-permeable prodrug (Pro-IP6) of the worldwide food component, IP6. Prodrugs of inositol phosphate (IP), inositol diphosphate (IP2) and inositol triphosphate were already synthesized [19], but prodrug of IP6 was synthesized for the first time as far as we know. Using this compound, we demonstrated that IP6 induced apoptosis and inhibited Akt phosphorylation. Furthermore, its *in vivo* anti-cancer effects were shown. Another prodrug, that of anti-hepatitis B virus called adefovir pivoxil with an acyloxymethyl group as its phosphate group, entered the market [20,21]. Pro-IP6 is useful not only as a biological tool but also as a drug. The target diseases of Pro-IP6 would include not only cancer, but also various other diseases such as atherosclerosis, neurodegenerative diseases, and diabetes, which are related to Akt activation [22]. The details are currently being investigated.

4. Experimental section

4.1. Chemistry

4.1.1. Synthesis of myo-inositol hexaphosphates dodecakis (butyryloxymethyl) ester (Pro-IP6)

Phytic acid (IP6) sodium salt (1.00 g, 1.51 mmol) purchased from Sigma-Aldrich (St. Louis, MO, USA) was treated by cation exchange resin Dowex 50WX8 (100–200 mesh, Wako, Osaka, Japan), and mixed with triethylamine (3.0 mL) to form phytic acid Et_3N salt. After azeotropic removal of water from this salt (50.0 mg, 0.0269 mmol) by acetonitrile (5.0 mL × 3), the salt and *N,N*-diisopropylethylamine (DIPEA, 0.20 mL) in acetonitrile (5.0 mL) were stirred under argon atmosphere at room temperature for 16 h. Further azeotropic removal of water by acetonitrile (5.0 mL × 3) from this mixture, the residue was dissolved in acetonitrile (5.0 mL), mixed with bromomethyl butyrate (0.240 g, 1.35 mmol) synthesized as previously reported [17] and DIPEA (0.2 mL), and stirred under argon at room temperature for 3 d. The reaction mixture was separated by HPLC LC-10AT (Shimadzu, Kyoto, Japan) with Discovery BIO Wide PoreC18 column (25 cm × 10 mm, 10 μm, Sigma-Aldrich) using MeOH:H_2O = 96:4 (Flow rate: 3 mL/min; Retention time: 6.3 min) to afford colorless syrup Pro-IP6 (8.20 mg, 16%). NMR spectra by AVANCE 600 MHz (Bruker, Billerica, MA, USA) were referenced to TMS. High-resolution mass spectra (HRMS) were recorded by JMSDX303HF (JEOL, Akishima, Japan) by using positive fast atom bombardment (FAB) with 3-nitrobenzyl alcohol as the matrix.

^{1}H NMR (600 MHz, CD_3CN) δ 0.97–0.99 (m, 36H, CH_3), 1.65–1.67 (m, 24H, $CH_2\underline{CH_2}CH_3$), 2.41–2.43 (m, 24H, $\underline{CH_2}CH_2CH_3$), 4.55 (q, J = 9.6 Hz, 1H, $\underline{CH}$), 4.63 (t, J = 9.6 Hz, 2H, $\underline{CH}$), 4.75 (q, J = 9.6 Hz, 2H, $\underline{CH}$), 5.29 (m, 1H, $\underline{CH}$), 5.65–5.71 (m, 24H, $O\underline{CH_2}O$).

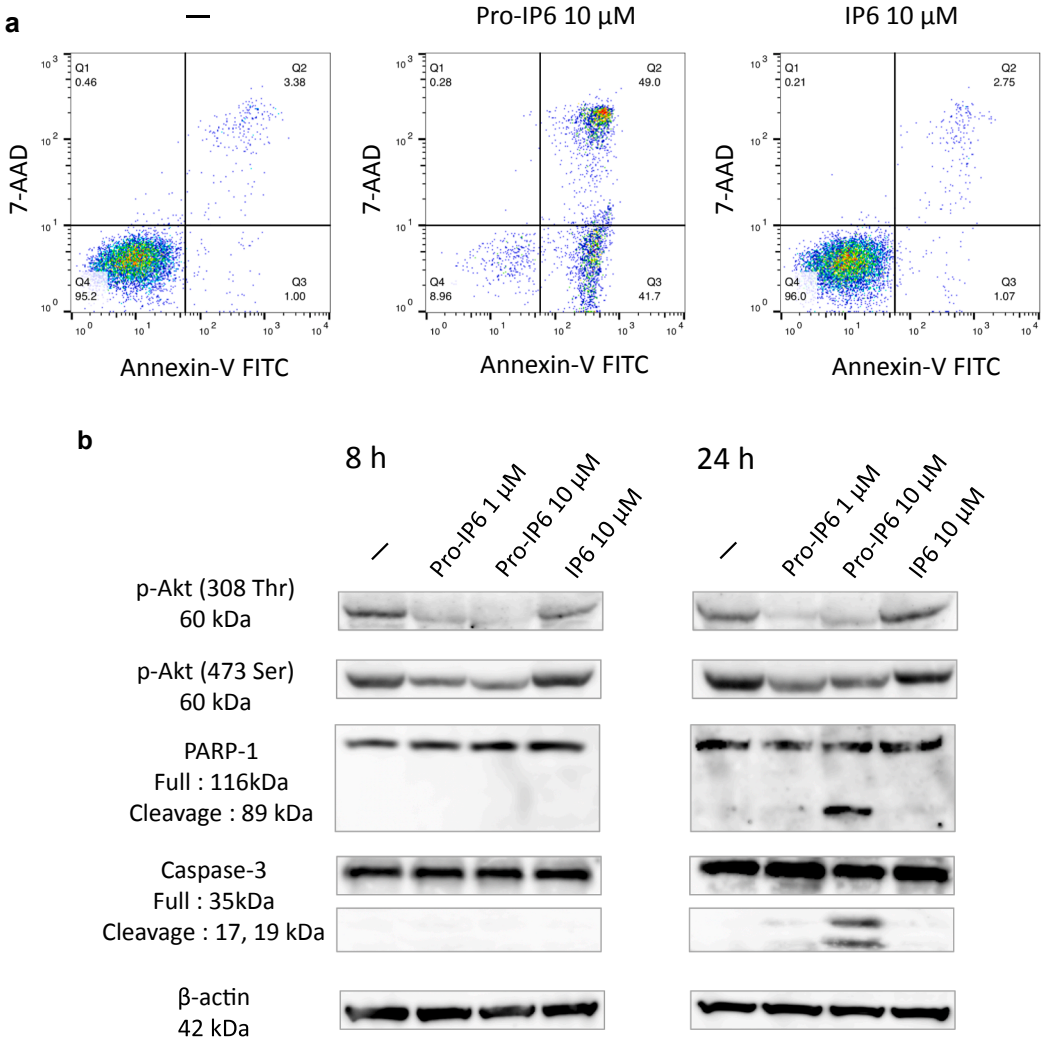

Fig. 3. Apoptotic effect and Akt activation of Pro-IP6 (1 or 10 μM) and IP6 (10 μM) in Jurkat cells. (a) FACS analysis. The cells were incubated with DMSO (control), IP6 in water, or Pro-IP6 in DMSO, and incubated for 24 h. Then FACS analysis was performed using FITC-conjugated annexin V and 7-AAD. (b) Immunoblot analysis. The cells were incubated with DMSO (control), IP6 in water, or Pro-IP6 in DMSO, and incubated for 8 or 24 h. Then immunoblotting was performed using anti-pAkt (Thr308), anti-pAkt (Ser473), anti-PARP-1, anti-Caspase-3, or anti-β-actin antibody.

^{13}C NMR (150 MHz, CD_3CN) δ 12.5, 16.9, 28.7, 34.6,81.9, 82.0, 82.1,82.2, 170.7, 170.8, 171.0.

HRMS (FAB+) m/s called for $C_{66}H_{114}O_{48}P_6Na$: 1883.4803. Found:1883.4766.

4.2. Biological assay

4.2.1. Cell culture and MTT assay

Human cervical cancer cell line HeLa was maintained in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum (FBS). Human leukemic T cell line Jurkat, M8166, MT-2 and K562 were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS. Normal human peripheral blood mononuclear cells (PBMC, Precision for Medicine, Bethesda, MD, USA) were purchased, and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS. Cell viability was measured by MTT assay as previously described [23].

4.2.2. Analysis of IP6 inside of a cell

HeLa cells were seeded (5×10^5 cells/1 mL/well), and cultured for 1d. IP6 water solution or Pro-IP6 DMSO solution (1%) was then added to the cell culture medium. As control, 1% DMSO was used. After incubation for 30 min, cells were washed with 1xPBS (1 mL x1), detached from dish by scraper, and moved to tubes. The cells were washed with 1xPBS (1 mL x3), suspended with MeOH containing 1% NP-40 (350 μL), and lysed by sonication. The lysate was passed through reverse phase-weakly basic anion exchange resin Oasis WAX 1 cc (Waters, Milford, MA, USA) pre-treated with MeOH (1 mL) and water (1 mL). The absorbate was washed with 50% MeOH water solution (1 mL), then eluted with 50% MeOH solution containing 2 M HCl (200 μL x2). The eluent was concentrated *in vacuo*, and dissolved in MeOH (50 μL). To the solution, approx. 10% trimethylsilyldiazomethane hexane solution (200 μL, Tokyo Chemical Industry, Tokyo, Japan) was added, and the mixture was stirred under argon atmosphere at 50 °C for 1 h. Then water (100 μL) was added, and hexane phase was removed. The residual solution was analyzed by HPLC LC-20AD (Shimadzu, Kyoto, Japan) with Mastro C18 column (2.1 mm × 100 mm, 3 μm, Shimadzu) using 0.1% formic acid water solution:acetonitrile = 85:15–65:35 (Flow rate: 0.3 mL/min; Retention time: 4.7 min) and MS spectometer amaZon speed (Bruker). This procedure to analyze IP6 inside of a cell was established by modification of a method developed by Shimadzu-Techno Research Inc. (Kyoto, Japan).

4.2.3. FACS and immunoblot analyses

To culture medium of Jurkat cells, IP6 water solution or Pro-IP6 DMSO solution (1%) was added. As control, 1% DMSO was used. The

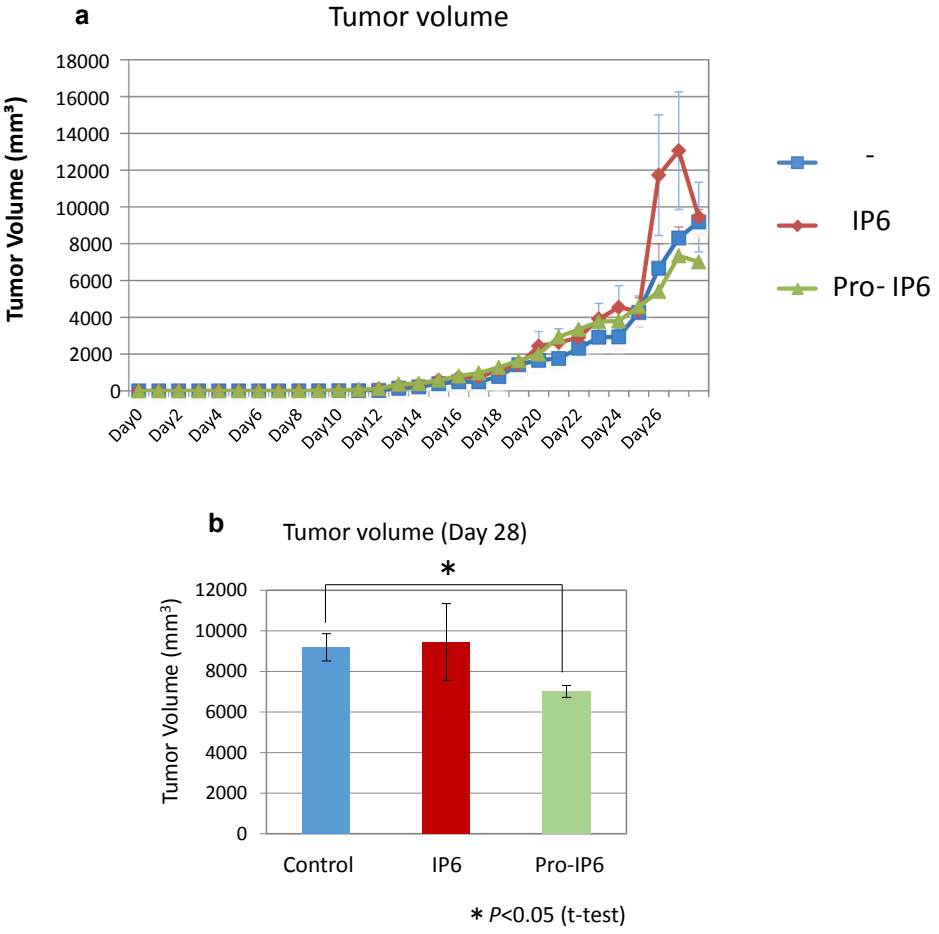

Fig. 4. In vivo anti-ATL activity using mice transplanted with ATL S1T cells. After transplantation into NSG mice, IP6 (7.1 mg/kg) or Pro-IP6 (20 mg/kg) was administered intraperitoneally to each mouse every day for 28 days (control: 3 mice; IP6: 3 mice; Pro-IP6: 4 mice). The tumor volume was continuously measured. On the 28th day, mice were sacrificed, and the tumor size was measured after complete removal. (a) Change in the tumor volume over time. (b) The tumor volume after complete removal on the 28th day.

cells were incubated for 8 h or 24 h. For FACS, the cells were washed with 1xPBS, incubated with Annexin V-FITC (PromoCell, Heidelberg, Germany) and 7-AAD (Becton Dickinson, Franklin Lakes, NJ, USA) for 15 min, and analyzed by FACS Calibur (Becton Dickinson). The data analysis was performed using FlowJo software (Becton Dickinson). For immunoblot, cells were lysed with Laemmli sample buffer, and analyzed as previously described [24]. As an antibody, anti-phospho-Akt (Thr308) (Cell signaling technology, Danvers, MA, USA), anti-phospho-Akt (Ser473) (Cell signaling technology), anti-PARP-1 (Sigma-Aldrich), anti-Caspase-3 (Cell signaling technology) or anti-β-actin (Sigma-Aldrich) was used.

4.2.4. Mice

NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) female mice, 4 weeks of age, were obtained from Charles River Japan (Tokyo, Japan). The mice were maintained as previously described [25]. Mice were acclimated for 1 week before experiments. This study was carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments, Science Council of Japan (http://www.scj.go.jp/en/animal/index.html). All animal procedures and their care were approved by the Animal Care and Use Committee of Rakuno Gakuen University in accordance with the Guide for the Care and Use of Laboratory Animals.

4.2.5. Administration of chemicals to NSG mice injected with S1T cells

S1T cells (5×10^6) [26] were washed for three times with PBS and injected subcutaneously in the postauricular region of 5-week-old NSG mice. Ten mice were randomly divided into three groups of 3, 3 and 4 mice, and used as control group, IP6 administration group, Pro-IP6 administration group, respectively. Pro-IP6 is insoluble in water, thus this compound was dissolved in olive oil. For IP6 administration group and Pro-IP6 administration group, each drug was administered at 11 μmol/kg, and the control group was administered vehicle intraperitoneally once daily. Mice were monitored daily for tumor size. Tumor volume was calculated according to the formula: $a^2 \times b \times 0.5$, where a and b are the smallest and largest diameters, respectively [25]. Up to 25 days, the tumor volumes of each group was about 4000 mm(2) but there was a sudden increase in tumor from the 26th day, considering the humanitarian end point, all mice were euthanized at 28 days after inoculation.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103240.

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